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Transgenic tomato (*Lycopersicon esculentum*) overexpressing *cAPX* exhibits enhanced tolerance to UV-B and heat stress

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Abstract

Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and hydroxyl radicals, are by-products of biological redox reactions. ROS can denature enzymes and damage important cellular components. Plants develop antioxidant enzymes, such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) to scavenge ROS and detoxify them. The effect of increased cytosolic ascorbate peroxidase (cAPX) on heat and UV-B stress tolerance was studied using transformed tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) plants. This research demonstrates, in either laboratory or field tests, the potential to enhance tolerance to heat, UV-B, and sunscald stress by gene transfer. Overexpression of cAPX in transgenic tomato enhanced resistance to heat (40 °C) and UV-B stress compared to wild-type plants. When leaf disks were placed at 40 °C for 13 hours, the electrolyte leakage of disks from wild-type were 93%, whereas two tested transgenic lines (A9, A16) exhibited 24% and 52% leakage respectively. When fruits of wild-type and transgenic plants were exposed to UV-B (2.5mW cm^{-2}) for five days, the extent of browning was 95%, and 33%, and 37%, respectively. In field tests, the detached fruits from field-grown transgenic plants showed more resistance to exposure to direct sunlight than fruits from wild-type plants. APX activity in leaves of *cAPX* transgenic plants was several folds higher than in leaves of wild-type plants when exposed to heat, UV-B, and drought stresses.

Key words: *Lycopersicon esculentum*, overexpression, ascorbate peroxidase (APX), heat, UV-B, oxidative stress, sunscald.

Introduction

Reactive oxygen species (ROS) including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), can have deleterious effects such as lipid peroxidation, DNA mutation and protein denaturation in cells (Bowler *et al.*, 1992; Scandalios, 1993; Apel and Hirt, 2004). ROS may be produced in response to abiotic and biotic stresses, and are associated with a number of physiological disorders in plants (Allen, 1995). These stresses, to varying degrees, affect crop growth, resulting in huge losses of plants and plant products annually throughout the world (Scandalios, 1993). Plants have evolved efficient non-enzymatic and enzymatic systems to cope with ROS. Non-enzymatic systems, such as ascorbate, glutathione, α-tocopherol, and carotenoids, can react directly with ROS (Allen, 1995). Enzymatic systems like SOD scavenge the superoxide anion. APX removes H_2O_2. Glutathione reductase (GR) also can remove H_2O_2 via the ascorbate-glutathione cycle (Noctor and Foyer, 1998; Scandalios, 1993). Scavenging of ROS is important for maintenance of normal plant growth (Allen, 1995).

Heat and ultraviolet B (UV-B, 280-320 nm) stimulate the production and accumulation of toxic ROS, which results in lipid peroxidation and membrane injury (A-H-Mackerness, 2000; Davidson *et al.*, 1996; He *et al.*, 2002; Jiang and Huang, 2001; Sairam *et al.*, 2000). An interaction of high temperatures and light intensity (UV-B) can induce sunscald in fruits of many horticulture crops (Rabinowitch *et al.*, 1983; Renquist *et al.*, 1989). Sunscald of fruits is manifested as either tissue browning or desiccation (Renquist *et al.*, 1989). Tolerance of tomato fruits to sunscald damage by controlled heat treatment was accompanied by an increase in superoxide dismutase (SOD) activity (Rabinowitch *et al.*, 1982; Rabinowitch and Sklan, 1980). Rabinowitch and Sklan (1980) reported that SOD activity levels were high in immature green fruits and declined to a minimum in the mature-green and breaker (early ripening) stages, which are known to be most susceptible to sunscald. During tomato ripening, oxidative processes such as lipid peroxidation, protein oxidation, and hydrogen peroxide content, increase at the breaker stage. In contrast, antioxidant enzyme activities of SOD and APX decreased at the breaker stage of ripening (Jimenez *et al.*, 2002).

Tolerance to heat and UV-B correlates with an increased capacity of the plants to scavenge or detoxify activated oxygen species (Chaitanya *et al.*, 2002; Davidson *et al.*, 1996; Mazza *et al.*, 1999; Sairam *et al.*, 2000), suggesting that increased antioxidant enzyme activity might protect plant tissues against sunscald. Wisniewski *et al.* (2002) reported that transgenic apple plants that overexpressed cAPX had improved resistance to heat stress. Leaf disks from wild-type (WT) apple plants exhibited 100% electrolyte leakage, whereas transgenic lines exhibited 40% to 75% leakage. Transgenic apple plants also showed tolerance to UV-B and freezing stress. Chen and Pan (1998) reported that

overexpression of Cu/Zn-SOD can enhance the tolerance to heat stress or UV-B radiation in *Arabidopsis thaliana*.

The overexpression of enzymes involved in scavenging ROS in plants by gene transfer technology may be able to increase tolerance of plants to oxidative stresses and improve plant performance under these conditions (Sen Gupta *et al.*, 1993a, b; Kubo *et al.*, 1995; McKersie *et al.*, 1999; Wang *et al.*, 1999; Wang *et al.*, 2005; Yoshimura *et al.*, 2000).

The objective of this study was to determine the tolerance of tomato plants and fruits to heat and UV-B stresses when *cAPX* gene is introduced into the plants.

Materials and methods

Plant material and growth conditions: Tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) was previously transformed by *Agrobacterium tumefaciens* with a binary vector containing pea *cAPX* cDNA (Mittler and Zilinskas, 1991). Independently-transformed *cAPX* lines of T_2 generation and wild-type (WT) plants were used in the experiments. Cuttings from regenerated transgenic T_2 plants were rooted in rooting medium complimented with antibiotic (MS + 50 mg L^{-1} kanamycin + 0.2 mg L^{-1} NAA + 400 mg L^{-1} cefotaxime + 7 g L^{-1} agar) and transplanted in peat moss soil (Lakeland Peat Moss, Inc., Edmonton, Alberta, Canada). They were grown in a greenhouse with natural lighting supplemented with sodium vapor lamps (1000W, Philips, Inc., Eindhoven, Netherlands) to provide a 16-h photoperiod at approximately 23/21°C (±2°C, day/night temperature).

Heat, UV-B, sunscald, and drought stress tests

Heat stress tests: Leaf discs from WT and T_2 plants were heat stressed and the disruption of membrane integrity was estimated by electrolyte leakage. Two leaf discs (0.62 cm in diameter) were punched out with a cork borer from the youngest fully expanded leaves of T_2 transgenic and WT plants of the same age. The leaf discs were immerged in a test tube containing 10 mL of deionized, distilled water. The base of the tube was submerged in a water bath at 40°C and removed after 0.5, 2, 3, 5, 7, 9, 11 and 13 h for testing. Following heat treatment, electrolyte leakage was measured using a conductivity meter (ElectroMark Analyzer, Markson Science, Inc., Del Mar, CA). Determinination of percent electrolyte leakage was done based on the method of Wisniewski *et al.* (1997). Means for all values are an average of two subsamples in each plant with three replications. The significance of differences between means were determined using Duncan's multiple range test at $P < 0.05$ level (NCSS-PASS software, NCSS Inc., Williamsport, PA). Arcsine square root transformation was performed before data analysis. Nontransformed means are presented.

Discs (0.62 cm) from fourth or fifth leaves of WT and T_2 transgenic *cAPX* lines were placed in an eppendorf tube (1.5 mL) and were incubated in a water bath (42°C) for 4 h. The samples were then frozen in liquid nitrogen and stored at –80°C for further enzyme activity gel analysis.

UV-B stress tests: Uniform mature green tomato fruits, randomly detached from WT and T_2 plants grown in the field (Lewis-Brown Farm at Corvallis, Oregon), were exposed to UV-B radiation provided by UV-B fluorescent lamps (Blak-Ray lamp, UVP, Inc., San Gabriel, CA, USA) in a room with no other source of light. The fruits were placed 20-cm away from the light source (2.5 mW cm^{-2}) and exposed 10 h per day for 3, 4, or 5 days. The extent of injury was based on colors from a white to yellow color of the epidermis, followed by browning when the injury was more severe. UV-B fruit injury was assessed by estimating the percent of exposure area that exhibited browning. The means of injury values are an average of three replicates. The significance of differences were estimated by Duncan's multiple range test at $P < 0.05$ level.

For UV-B stress enzyme activity gel analysis, shoots with three to four leaves from greenhouse-grown WT and T_2 plants were placed in tubes containing 50 mL of distilled water. The shoots were placed 20 cm below the UV light source for 4 h. After treatment, leaf discs (0.62 cm) were excised and frozen in liquid nitrogen before being stored at –80 °C.

Sunscald tests: In a field test, mature green tomato fruits, randomly detached from WT and T_2 plants were exposed to field conditions for 15 d (from September 8 to 23, 2002, Lewis-Brown Farm, Corvallis, Oregon). Fruit injury was visually recorded as described by Rabinowitch *et al.* (1986). Sunscald injury was characterized by the bleaching (with a brown or yellow halo around the bleached area) and necrosis of the pericarp. The injured area eventually became sunken and dry. The percent of injured fruit area was estimated visually. Mean values are an average of five replicates. The significance of differences between means were determined by Duncan's multiple range test at $P < 0.05$ level.

Drought stress test: For drought stress test, 15 d-old rooted WT and transgenic plants were grown in plastic pots (15 cm diameter; 14.5 cm height) on peat moss (Lakeland Peat Moss, Inc., Edmonton, Alberta, Canada). After four weeks in a greenhouse, the plants were subjected to drought stress by withholding water for 7 d. Leaf discs (1.02 cm in diameter) were excised from the fourth or fifth leaf of the treated plants, frozen in liquid nitrogen, and stored at –80°C for later use in enzyme gel activity experiments.

APX enzyme gel activity assay: About 100 mg stored leaf tissue was ground to fine powder in the liquid nitrogen and homogenized in 200 µl of grinding buffer (100 mM $NaPO_4$, pH7.0; 5mM ascorbate; 1 mM EDTA, pH 8.0; 10% glycerol; and 0.001% bromophenol blue), and centrifuged at 13,000g for 6 min at 4°C. The supernatant was collected and protein concentration was determined using a Protein Assay System (Bio-Rad; Hercules, CA). Approximately 70 µg of total protein was loaded into each lane of a non-denaturing, 10% polyacrylamide gel and electrophoretically (PAGE) separated for 5 h at 4ºC in a 1X Tris-glycine buffer (24 mM Tris, 192 mM glycine), with subsequent staining for APX activity as described by Mittler and Zilinska (1993).

Results

Heat stress tests: The electrolyte leakage of the leaf discs are presented in Fig. 1. The amount of leakage increased with time of exposure. The differences in heat stress resistance between the WT and cAPX- expressing plants (A9 and A16) were statistically significant after 2 h treatment (P <0.05). After 13 h treatment, the

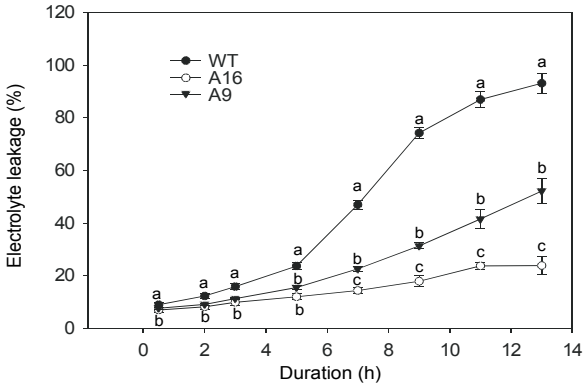

Fig. 1. Electrolyte leakage of leaf discs of wild-type (WT), and transgenic cAPX (Lines A9 and A16) tomato plants following heat stress (40 °C) of varying duration (0.5, 3, 5, 7, 9, 11, and 13 h). Values are means ±SE (n=3). Different letters indicate significant differences (P<0.05) between means within each sampling time (Duncan's multiple range test).

electrolyte leakage in WT plants was 93%, whereas that in transgenic tomato lines A9 and A16 was 24 and 52%, respectively.

UV-B stress tests: The first visible sign of damage was seen on day 3, with transgenic A9, A16 and WT fruits showing about 20, 34 and 61% injury, respectively. After 5 d, WT and transgenic A9, A16 fruits showed 95%, 33% and 37% injury, respectively, as evidenced by a brown discoloration of the exocarp tissue (Fig. 2). Compared to WT fruits, transgenic fruits suffered significantly less UV-B browning injury (P <0.05).

Sunscald tests: Exposure of detached fruits to natural sunlight under field conditions resulted in sunscald injury to all fruits after 15 d. However, the fruits of the transgenic tomatoes were less injured (Fig. 3). Sunscald injury in WT fruit averaged about 21%. In contrast the cAPX- transgenic tomatoes (A3, A9, A13, A16, A24 and A30) exhibited 1% to 10% sunscald injury. Significant differences (P <0.05) in fruit sunscald injury between transgenic APX lines and WT fruit were observed (Fig. 3).

APX enzyme activity: The APX enzyme activity gel densitometric assay revealed higher APX activity in transgenic plant lines (A9, A16) than in WT plants after UV-B (9- to 10-fold), heat (3- to 3.2-fold), and drought stress (5- to 6-fold) treatment (Fig. 4).

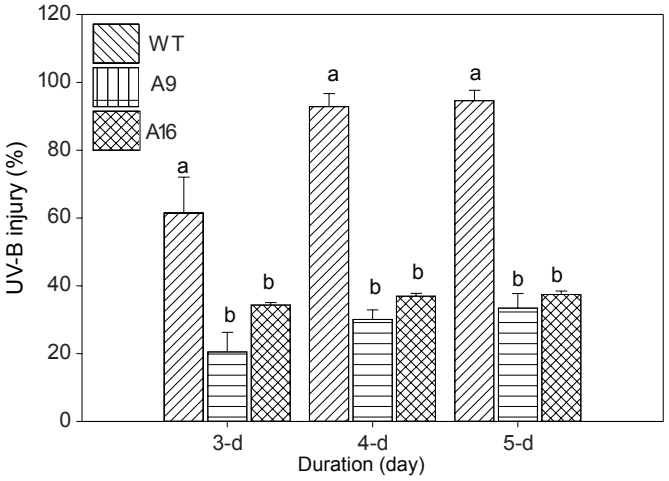

Fig. 2. Percent injury (exposed area exhibiting browning) in fruit of wild-type (WT) and transgenic cAPX (A9 and A16) tomato plants after UV-B (2.5 mW/cm2) treatment for 3, 4, and 5 days. Values are means ± SE (n=3). Different letters indicate significant differences (P<0.05) between means within a sample time (Duncan's multiple range tests).

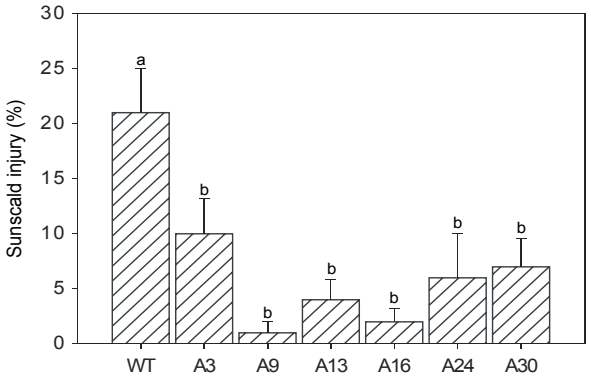

Fig. 3. Percent sunscald injury to detached fruit from wild-type (WT) and transgenic cAPX (A3-A30) tomato plants under field conditions for 15 d. Values are means ± SE (n=5). Different letters indicate significant differences (P<0.05) by Duncan's multiple range tests.

Discussion

The overexpression of the cAPX enzymes in tomato plants increased the resistance of their leaf and fruit tissues to heat and UV-B stresses, supporting the results reported by previous work in apple (Wisniewski *et al.*, 2002).

Electrolyte leakage in stressed transgenic plants was much less than in WT plants (Fig. 1) providing strong evidence that overexpression of cAPX resulted in enhanced protection of membrane lipid peroxidation caused by ROS during heat stress (Huang *et al.*, 2001). After UV-B stress, WT fruits showed more browning damage than transgenic fruits (Fig. 2), indicating that overexpression of antioxidants might play an important role in detoxifying heavy loads of ROS during UV-B stress to provide protection (Balakumar *et al.*, 1997; Mazza *et al.*, 1999).

Several transgenic fruit lines showed significantly less sunscald damage than WT fruits (P <0.05) (Fig. 3), suggesting that overexpression of antioxidant genes might increase tolerance to sunscald. APX enzyme activity was higher in *cAPX*-expressing plants after heat, UV-B, and drought stress (Fig. 4), which suggests that the overexpression of APX may play a role in protecting plant against ROS during various physiological stress (Allen, 1995).

We also tested SOD enzyme activity by gel assay and found that after heat and UV-B stresses, the SOD bands were very faint, indicating that SOD enzyme activity was very low (data not shown). The reason for this low enzyme activity is not known but may be related to the loss of SOD activity following long exposure to the stress treatment. It is also possible that the effect of SOD is indirect, because the product of SOD activity

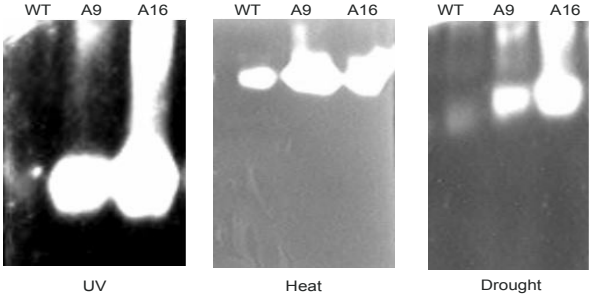

Fig. 4. APX enzyme activity gel assay. UV-B (2.5 mW cm^{-2}) for 4 h; heat (42 °C) for 4 h; and drought stress (withholding water for 7 d). About 70 µg protein was loaded in each lane of the native gel.

is hydrogen peroxide, which has been implicated as an elicitor of genes related to stress tolerance (Sen Gupta *et al.*, 1993b; McKersie *et al.*, 1996; Prasad *et al.*, 1994).

Overexpression of APX in transgenic tomato plants provides better protection against heat, UV-B and sunscald. This elevated enzyme activity might play an important role in increasing stress tolerance against fruit sunscald. Given these results, along with those of other studies showing heat and UV-B effects on SOD or APX in transgenic plants (Chen and Pan, 1998; Wisniewski *et al.*, 2002), we believe that a similar approach might be applicable to other important fruits that are sensitive to sunscald, such as apple, to improve their tolerance against this stress.

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The effect of anti-hail nets on fruit protection, radiation, temperature, quality and profitability of 'Mondial Gala' apples

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Abstract

The effects of crystal (transparent) and black nets on the protection of fruits from hail, the interception of light, temperature, humidity and fruit quality were evaluated over four seasons (from 2000 to 2003) at the IRTA-Experimental Station, Lleida (NE-Spain) on 'Mondial Gala' apples (*Malus x domestica* Borkh.). Nets demonstrated their efficiency for fruit protection against hail; decreased maximum orchard temperatures and increased minimum temperatures and relative humidity. Based on PAR values, on sunny days, the black net intercepted 25% more incident radiation than the control and the crystal net intercepted 12% more. The use of black net resulted in a significant reduction of colour intensity and days taken for maturity, and provided lower average yields for fruit harvested at the first picking. The crystal net was associated with intermediate values between black net and control, or similar values to those of the control. Both nets reduced fruit temperature and the incidence of sunburn improving global skin quality. The black net increased the vigour of the trees. Fruit firmness was not affected by the use of nets. Soluble solid content decreased when black net was used, while maturity was delayed in some seasons. There were no consistent effect with respect to titratable acidity and fruit cracking. The annual cost of the anti-hail nets was 1874 to 1612 € ha^{-1}, respectively, for crystal and black nets, depending mainly on the durability of the net. The annual cost of insurance was 760 € ha^{-1} and was determinated by site, cultivar, yield and price insured, and was lower than that of covering by nets. The gross profit corresponding to the crystal net (8896 € ha^{-1}) was lower to the control/insurance (9223 € ha^{-1}) and greater to the black net (7842 € ha^{-1}) because of the reduction in fruit colour. With 'Mondial Gala' apples, the use of both colour nets was not economically beneficial compared to the control.

Key words: Apple, *Malus x domestica* Borkh., 'Mondial Gala', net, hail protection, insurance, radiation, temperature, humidity, vigour, fruit colour, quality, cost, benefit.

Introduction

Apple production is important in Spain, which had a total surface area of 44674 ha in 2002. The Ebro Valley is the main area of production, and Catalonia - with 15292 ha - is the most important region (MAPA, 2003). The main cultivars are 'Golden' and 'Gala', which together account for around 60% of the total area dedicated to apple production. An increase in the damage to crops caused by hail storms has been observed over the last 15 years, particularly in the months of April and May. This has encouraged fruit growers in the Lleida area to establish a network of silver iodure burners covering almost the whole area. Even so, in some years and under certain conditions, this remedy does not produce completely satisfactory results.

In addition to using silver iodure burners, spanish fruitgrowers have also insured their crops with the public company Agroseguro, which receives subsidies from both national and regional governments. One of the most common options is to insure crops against hail damage with lower cost than anti-hail nets, but in the case of hail, the loss of production presents a problem for fruit industry companies that wish to maintain customers and also results in an increase in total costs (associated with unused grading machines, cold store facilities, *etc.*). Furthermore, in the case of some new cultivars that have been developed according to the "club" formula, insurance companies establish a maximum price payable (0.31 € kg^{-1} in the case of 'Pink Lady'), whereas their planting/production costs and added value are much higher than those of standard cultivars. In the main apple producing countries of the EU (Italy, France, Austria, *etc.*) and America (Argentina, Chile, Mexico, *etc.*), the nets have been increasingly employed to protect fruit against hail damage in recent decades mainly because the huge increase of the insurance cost. This technique now constitutes the only effective way to protect fruit against this threat. The efficiency of nets in fruit protection and their effects on the interception of light, fruit colour and quality, sunburn, installation systems, costs, and modifications of orchard climate have all been widely documented by authors from Italy, France, Austria, Argentina, Chile, Mexico and South Africa, *etc.* (Andrews and Johnson, 1996; Bru, 1996; Coreau *et al.*, 1997; Reigne, 1997; Vercammen *et al*., 1998; Vercammen, 1999; Crété, 2000; Yuri *et al*., 2000; Peano *et al*., 2001; Dussi *et al*., 2005; Gindaba and Wand, 2005, 2006; Vittone *et al*., 2006). However, little information is available relating to the effects and efficiency of this technique with respect to deciduous fruit crops grown in Spain, although some preliminary results have been reported in NE-Spain by Iglesias and Alegre (2004).

This paper is a synthesis of data collected during the 2000-2003 growing seasons at the IRTA-Experimental Station of Lleida with 'Mondial Gala' apples. The objective of the trial was to evaluate the effects of two different coloured nets on the protection of fruit against hail, fruit colour and quality and the effect on orchard climate (temperature and humidity) and light interception. Also

an economical analysis of using the two colours of nets has been done.

Materials and methods

Study site, plant material, climatic conditions and net characteristics: The study was conducted in the 2000, 2001, 2002 and 2003 seasons in a plantation of 'Mondial Gala' apple trees on M9 Pajam 2 rootstock, planted at the IRTA-Estació Experimental de Lleida (Mollerussa, NE Spain), in 1994. The rows were oriented from NE to SW. Trees were trained with a modified central-leader system, spaced at 4 x 1.4 m and grown on Tipic Xerofluvent, coarse-silty, mixed (calcareous), mesic soil, with an average depth of 0.85 m. Irrigation was same for all the treatments and applied based on evapotranspiration (Etc) minus the effective rainfall; was provided daily by means of a drip system, which consisted of two 4 L h^{-1} drippers per tree. This particular area of Spain is subjected to periods of high summer temperatures (>30ºC) and very low rainfall (413, 296, 430 and 528 mm, respectively, for the 2000, 2001, 2002 and 2003 seasons). In the pre-harvest and harvest period (from 1st July to 15th August), important differences between seasons were recorded with respect to temperature and rainfall. Lower temperatures were registered in 2000 and 2002, temperatures were normal in 2001 and extremely dry and atypically hot conditions occurred in 2003 (Fig. 1).

Nets were installed in May 1998. The orchard was covered with nets from the end of April (after blooming) to October. The net characteristics were:

Black net: Black polyethylene net, diameter 0.28 mm, Beniagro GVM 2.5 x 3 (cell size 3 x 7.4 mm). Non-overlapping system with a slight inclination towards the centre of the interrows. Estimated lifespan: 15 years.

Crystal net: Transparent polyethylene net, diameter 0.28 mm, Beniagro GVM 2.5 x 3 (cell size 3 x 7.4 mm), with the same system as the black net. Estimated lifespan: 8 years.

Five-metre high poles, with 14/16 cm diameter, located at 12.5 m intervals, were used to support the nets. Estimated lifespan: 24 years.

Experimental design: A completely randomised block design was used, with four blocks assigned to each of three treatments: black net, crystal net and control. This design was used to control the effect due to the possible variations in tree vigour due to the effect of nets. Each treatment and block consisted of five rows of 15 trees. Two trees per treatment were selected from the central row of each block based on uniform crop load and vigour.

Light interception by nets: The effect of nets on the interception of light was measured annually as a percentage of total above-canopy Photosynthetically Active Radiation (PAR), using a Ceptometer mod. Sun Scan SS1-UM-1.05 (Delta-T Devices Ltd Cambridge, UK) with a 64 sensor photodiode linearly sorted in a 100 cm length sword. Sun Scan response was almost entirely within the PAR wavelength band of 400-700 nm. The sword ceptometer was placed in the middle of two rows and also parallel to them for each treatment, positioned 1.10 m above ground level. Five readings per treatment were taken at 2 hour intervals, between 9.00 and 21.00 (7.00 and 19.00 solar time) several times in each season (July-August), after shoot growth had stopped, on both sunny and cloudy days. Light distribution in relation to tree height was also measured at 7 heights between 0.5 and 3.5 m above the ground on 10th August 2000 at 14.00 and 18.00 (12.00 and 16.00 solar time) and compared with incident radiation recorded at a height of 4.5 m for each treatment.

Orchard temperature, fruit temperature and relative humidity: The effect of nets on orchard temperature and relative humidity was recorded using Hobo®Pro RH/Temp/2x External mod H08-007-02 automatic sensors (Equipos de Instrumentación y Control S.L., Madrid, Spain), installed within the canopy, and located 1.2 m above the ground level at the centre of the block of each treatment.

Fruit temperature was measured three times in sunny days

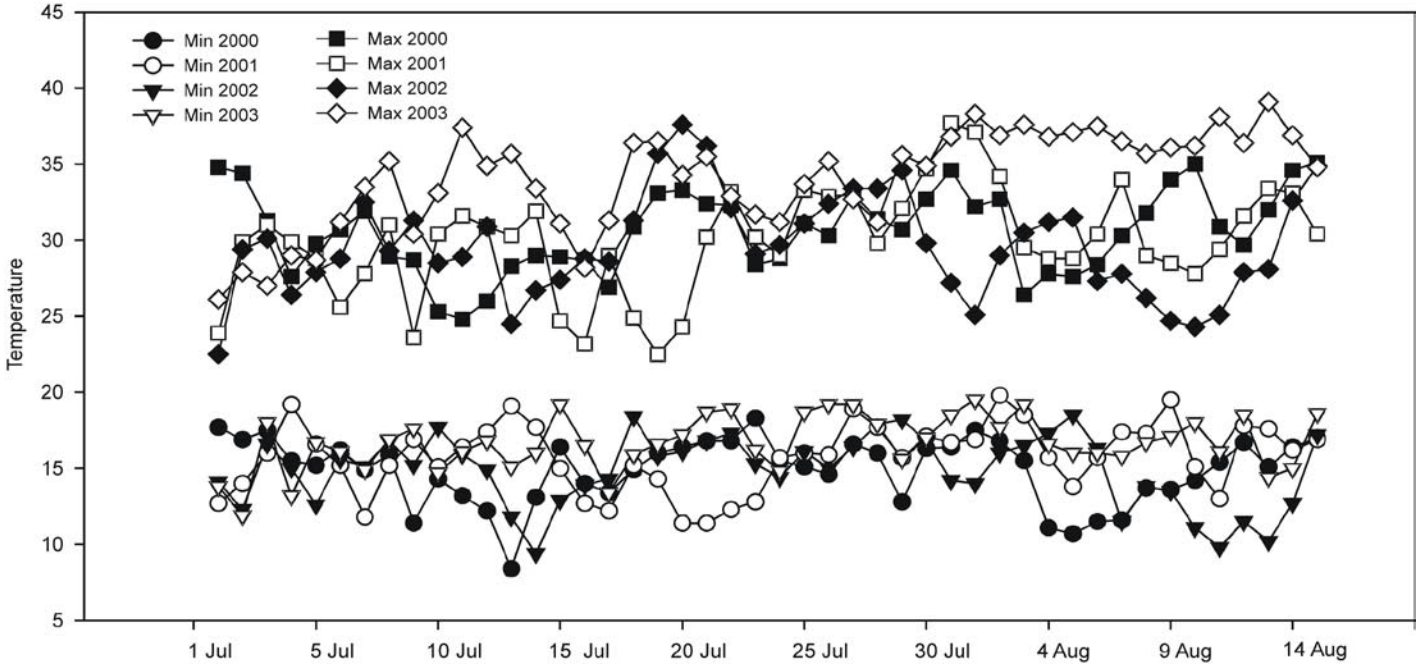

Fig. 1. Daily maximum and minimum temperatures over the period 1st July – 15th August for the 2000, 2001, 2002 and 2003 seasons

and three more times in cloudy days of July and August 2000. These measurements were made using a Crison Model 637 (Crison Instruments, Barcelona, Spain) digital thermometer. The temperature sensor was introduced 3 mm under the apple skin tangentially to the surface of the exposed side of the fruit, as proposed by Ryall and Pentzer (1982). At each measurement date, fruit temperature was recorded for 10 fruits per treatment (5 fruits/tree x 2 trees) picked at around 1.6-2.0 m above ground level on the side directly exposed to sunlight during the period of maximum daily temperature, from 15:00 to 15:30 (13:00 to 13:30 solar time).

Fruit colour and size measurements: Apple colour was measured with a Minolta Chroma Meter CR-200 portable tristimulus colorimeter (Minolta Corp, Osaka, Japan) and fruit chromaticity was recorded according to Commission Internationale d'Eclairage L*, a* and b* colour space coordinates (Hunter, 1975). Hue angle was calculated as described by McGuire (1992) and expressed in degrees.

In each season, from 2000 to 2003, 20 fruits were selected and marked in the inner (around the central axis) and outer (periphery area) canopies on each of two trees per treatment-block on 25th June; 10 fruits were selected from each of two tree sides; five from the top (1.2-2.0m above ground level) and five from the bottom (0.6-1.2 cm). Fruit colour measurements were recorded *in situ* on each one of the four sampling dates (five sampling dates in 2002 and 2003), from 5th July through to commercial harvest on 10th August (Fig. 4). Colour values were measured at both the reddest (exposed side) and greenest (shaded side) points on the fruit equator. Harvest date was 138 days after full bloom, when flesh firmness was <17.5 lb and soluble solid content was >13.0%.

Commercial value of the crop depends on both, fruit size and fruit colour. At commercial harvest, fruit size distribution based on 5 mm-interval fruit diameter categories and average of red colour fruit-surface coverage were determined for the whole crop for two trees per treatment and block on which fruit colour was recorded by using an electronic grading machine (SAMMO s.r.l., Model S2010, Italy). Four complementary trees per replication were marked and their fruits were picked in two harvests. The criteria established for the first harvest were: fruit colour >40% of fruit surface and fruit size diameter >70 mm. Professional pickers were used and instructed to harvest only those fruits which had a sufficiently red colour to comply with the EU extra colour grade (1/2 of their surface with a good red colour).

Fruit quality parameters: The same 20 fruits marked per tree (40 fruits per block-treatment) were also used at commercial harvest for fruit quality determinations. Flesh firmness was determined with a table penetrometer (Penefel, Copa technologies, France) with an 11 mm diameter tip and expressed in lb. Two readings were taken from opposite peeled sides of 40 fruits per block-treatment on which colour was recorded. Soluble solid concentration (SSC) were determined by measuring the refractive index of a blended composite of wedges taken from 40 unpeeled apples per block/treatment, using an Atago-Palette 100 digital refractometer (Atago Co., Tokyo, Japan) and expressed as %. Titratable acidity (TA) was determined for the same composite by titrating to a final pH value of 8.2 with 0.1 N NaOH and expressed in g of malic acid L^{-1}. The effect of nets on fruit maturity was based on starch conversion and determinated using the Ctifl-Eurofru code with values ranging from 1 (immature) to 10 (mature).

Complementary determinations: The average number of fruits damaged by hail was visually recorded for the whole production when fruits were graded at commercial harvest. The effect of nets on the incidence of sunburn was evaluated with respect to the same sample used to determine fruit quality parameters. Tree vigour was determinated on the basis of measurements of trunk-cross-sectional-areas (TCSA) taken 20 cm above the graft union, measured at the start of the experiment and annually in winter time.

Data analysis: Analysis of variance was preformed separately each year for PAR values, orchard and fruit temperature, chromaticity values, yields, fruit size, fruit colour and quality, according to a complete randomized block model with each block being a replication unit, using the Statistical Analysis System software (SAS Institute Cary, N.C., 1997): statistical significance was tested at $P = 0.05$. In the case of chromaticity values, data from each block represented the mean for two fruit sides (E: exposed side, S: shaded side) for 20 fruits per tree and two trees per block and treatment. Plots were arranged in a randomized complete block design. When the analysis was statistically significant (F-test), mean separation was carried out by Tukey's test at P=0.05, using the mean square error for each sampling date and parameter evaluated. Differences between treatments and seasons were evaluated by analysis of variance and were tested using the General Linear Model procedure (PROC GLM of SAS) as a randomized complete block design to determine the statistical significance of single and double-way interactions. Season and treatment were considered as fixed effects, while blocks, trees, and fruits were designated as random effects.

Results and discussion

Effects of nets on the interception of radiation, orchard temperature and humidity: The values for radiation and light interception were similar for all four seasons (2000-2003). PAR values recorded under the nets on a sunny day (19th July) and on a cloudy day (17th July) in 2000, are presented in Fig. 2. Greater average values for interception were recorded between 13:00 and 15:00 (11:00 and 13:00 solar time), the period when the incidence of sunlight is most vertical. On sunny days, both nets intercepted more light radiation than the control without a net: average light interception values were 25 and 12% for black and crystal nets, respectively. Light interception by black nets was twice as great as for crystal nets on sunny days, whereas the difference was smaller on cloudy days. Clouds caused a reduction in total incident radiation of around 50% (Fig. 2). In the period from the 1st July to 17th August of the four seasons studied (2000-2003) only a mean of 5 days were cloudy, the rest were sunny as usual in warm areas.

Absolute values of PAR and the effects of nets on light interception were similar to those reported by Peano *et al*. (2001), Vercammen (1999) and Créte *et al*. (2001). Dussi *et al*. (2005) recorded respective decreases in PAR of 28 and 45%, using 15 and 55% density shade nets in Argentina (Alto Valle).

The solar radiation registered on sunny days resulted in high PAR values (maximum 1600-2000 µmol m^{-2} s^{-1}), which are common in southern Europe arid conditions. On cloudy days (complete clouds cover), the maximum values ranged from 800-900 µmol m^{-2} s^{-1}. In both cases, the registered values were twice as great as those for northern Europe (Belgium, Germany, *etc.*) and for this reason radiation disponibility is not a limitation even when black nets is used (maximum PAR values around 1200 µmol m^{-2} s^{-1}).

Measurements of light intercepted at different tree heights (from 0.50 to 3.50 m) in the middle row of each treatment were taken, before harvest, at two different times on 28th July 2000 at 14:00 and 18:00 (12:00 and 16:00 solar time) provided similar values. The recorded differences between treatments were significant and very similar at both measurement times. At 14:00, the average of light intercepted, did not depend on tree height because the incidence of sunlight was vertical. At 18:00, the interception of sunlight increased with increased tree height due to the shading of the lower parts of the trees (data not shown). This explains the increased exposure to light of the upper parts of the trees and their consequent better fruit colouration, because sunlight is the most important factor regulating anthocyanin synthesis and colour as reported by Saure (1990) and Lancaster (1992) in apple skin and Dussi and Huysamer (1995) in pear skin.

Maximum, mean and minimum daily temperatures for the period 1st July- 17th July 2001 (period representative of summer conditions in warm regions) showed that the use of nets exerted a limited influence on orchard temperature (Fig. 3). Maximum temperatures tended to be lower under the nets (3 ºC), due to the interception of radiation or "shade effect" which is greater than the gain of temperature caused by the use of nets due to their role in the interception of air circulation or "greenhouse effect". Bigger differences were recorded on bright and sunny days (1st, 2nd July,..) and lesser ones on cloudy days (8th July, 18th July). Minimum temperatures tended to be lower in the control

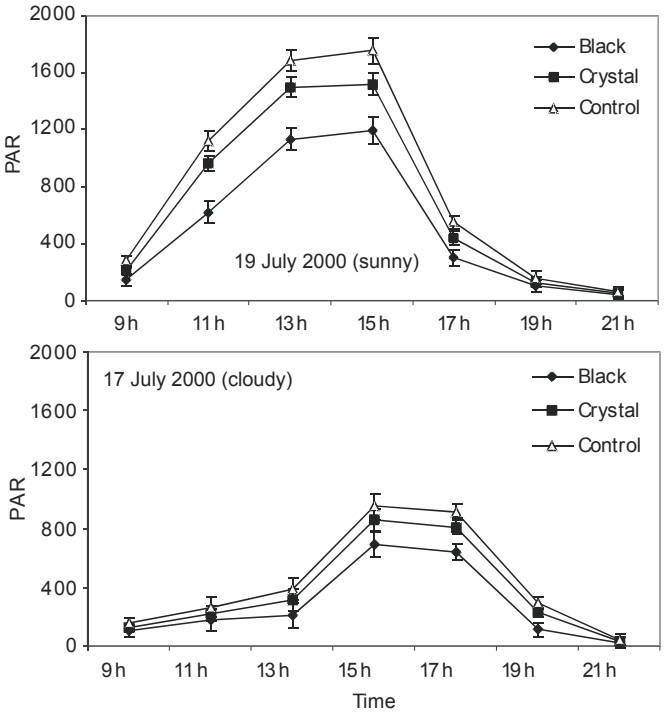

Fig. 2. Diurnal curve of photosynthetically active radiation (PAR±SE) (µmol m^{-2} s^{-1}) as affected by different coloured nets or no net on a sunny day (19th July) and a cloudy day (17th July) 2000.

by 1 ºC (Fig. 3) than in the nets because of the greenhouse effect and the low radiation at this time of the day. Similar results were reported by Vercammen (1999) and Crété *et al*. (2001), indicating that the influence of nets upon maximum orchard temperatures and their role in increasing minimum temperatures was not clearly demonstrated. Reigne (1997) found a moderate increase in maximum temperatures associated with the use of nets and Vaysse (1997) reported a moderate decrease (<1 ºC). Peano *et al*. (2001) did not find any clear temperature effect associated with the use of nets and only noted that maximum

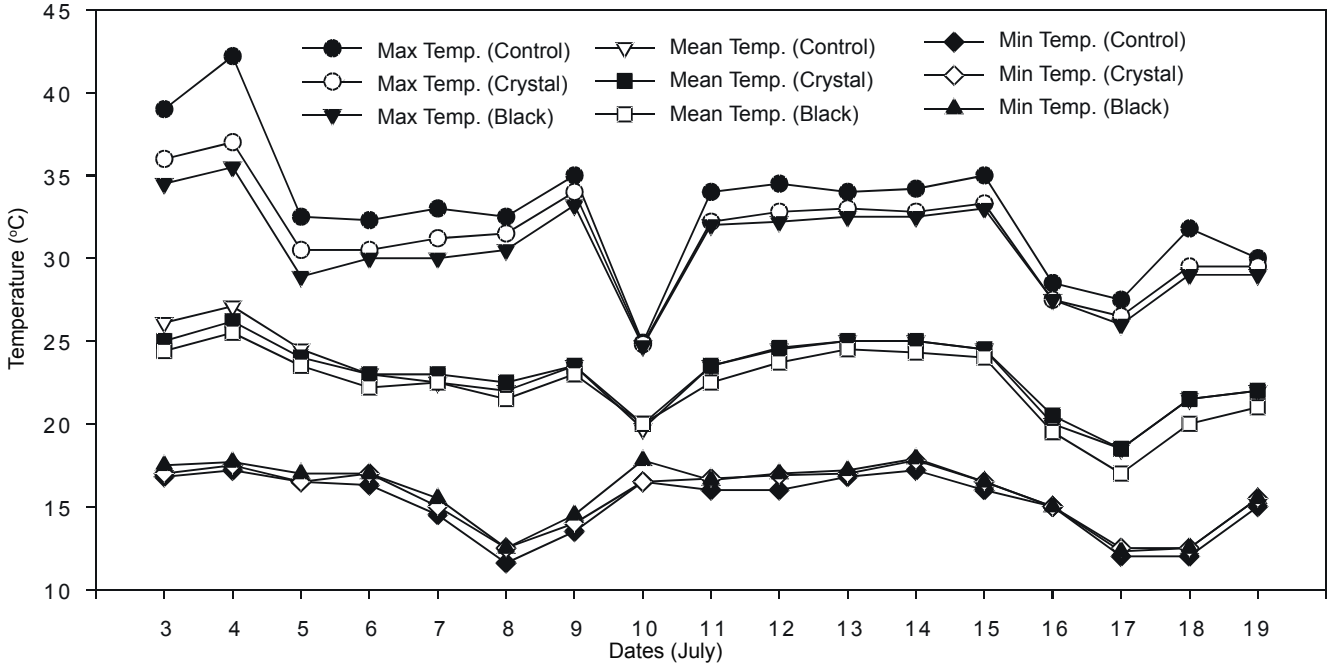

Fig. 3. Maximum (upper), mean (centre), minimum (lower) daily and mean temperatures per treatment (right) affected by the use of two different coloured nets during the period 3rd -19th July 2001

were slightly increased. It would be interesting to quantify the interception of air circulation by the nets in order to know the greenhouse effect.

Orchard humidity increased by the use of both nets but no significant differences were observed with respect to maximum relative humidity (data not shown). These results were in line with those reported by Crété *et al*. (2001), indicating a 2-6% increase in humidity associated with the use of nets. These authors also reported a decrease in evaporation by11% in July associated with the use of nets and a significant reduction in wind speed, which also resulted in a decrease in skin bruising.

Fruit temperature: Temperature measurements registered during the maximum daily orchard temperatures, in summer 2000, showed the positive effects of nets on both, reductions in fruit temperature (Table 1) and in the incidence of sunburn (Table 3). This reduction in temperature was related to radiation intercepted by the nets (Fig. 2). The black net was associated with a greater temperature reduction (around 4 ºC) than the crystal net (2.5 ºC) when compared to the control. On cloudy days, without direct sun radiation over the canopy, fruit temperatures were lower and differences among treatments were minor (1-2 ºC). Also the differences between air and fruit temperatures were smaller compared to sunny days. It is also interesting to note that the higher fruit temperatures associated with sunny days in summer (47.1 ºC), were around 12 ºC higher than air temperatures due to direct exposure to sunlight (Table 1). For this reason the use of shade nets, evaporative cooling and kaolin-based particle film technology were the three techniques commonly used to reduce radiation and fruit temperature in apple and pear, as reported by Dussi *et al*. (1997, 2005), Gindaba and Wand (2006) and Iglesias *et al*. (2002, 2005).

Table 1. Internal fruit temperature[y] and orchard air temperature (°C) of `Mondial Gala' apples affected by the use of two different coloured nets under different conditions at 15:00 (13.00 solar time) on six different dates in 2000

Treatment/ conditions	Date			Mean	Nets vs control
	25th July	6th Aug.	10th Aug.		
Sunny					
Black	41.6*	39.1	40.1	40.2 c	-4.1
Crystal	42.6	41.4	41.9	41.9 b	-2.4
Control	47.1	43.1	42.9	44.3 a	
Air temperature	34.1	31.8	32.6	32.8	
Cloudy	21th July	3th Aug.	14th Aug.		
Black	36.3	33.3	36.4	35.3 b	-1.9
Crystal	36.9	34.9	36.9	36.2 ab	-1.0
Control	38.1	35.6	37.8	37.2 a	
Air temperature	35.1	33.8	35.6	34.8	

(*):Each value is the mean of 10 measurements on the exposed side of the fruit.
[y] Different letters in the same column represent significant difference at $P < 0.05$ by Tukey range test if the F-test was significant in the ANOVA.

Fruit colour: The evolution of fruit colour, based on Hue values (with higher values indicating less colour), over the 4 weeks before commercial harvest and Hue values at commercial harvest, revealed significant differences between treatments both at harvest and also during the 2-3 weeks before harvest, for all seasons except 2000 (Fig. 4), as a result of optimal temperatures for colour development. This was because this is the period of maximum anthocyanin biosynthesis in 'Gala' apples (Iglesias, 1996; Iglesias *et al*., 2005). When four seasons were compared, Hue values prior to harvest and at commercial harvest showed significant differences from season to season, and the interaction *season x treatment* was significant. The crystal net produced either similar values to the control (2001 and 2002) or values between the black net and the control (2003). The higher values associated with use of the black net paralleled a reduction in fruit colour with respect to the control and also evidenced a delay and reduction in fruit colour development. This could be explained by a lower availability of carbohydrates necessary for anthocyanin biosynthesis due to the reduction of light disponibility. It is well known that anthocyanin biosynthesis in apple is directly affected by light (Lancaster, 1992; Arakawa, 1988) and temperature (Faragher, 1983; Arakawa, 1991). The reduction of radiation is responsible for down-regulation of photosynthetic capacity of leaves and consequently a lower light saturated photosynthetic rate compared to the control (Gindaba and Wand, 2006). Takos *et al*. (2006), more recently, reported that in red skin apple cultivars several flavonoid genes required for anthocyanin synthesis were coordinately transcribed in reponse to light exposure.

When fruit colour on both sides of the tree (east-west) and for different heights were compared, lower values were recorded on the west sides and in the upper parts of trees (data not shown). This was related to the greater exposure to light on the higher parts of the canopy and into the sunset side of the tree, as reported by Jackson *et al*. (1977) and Dussi *et al*. (2005). The interactions *treatment x tree side* and *treatment x canopy height* were not significant, and the black net reduced fruit colour for the whole canopy. In 2003, temperatures were unusually high during the summer period and this resulted in poor colour development compared with 2000 and 2002, even in the case of optimum exposure to light on the exposed sides of fruits without nets. Under these conditions the effect of both high temperatures and significant reductions in exposure to light associated with the use of nets explained the dramatic reduction in fruit colour with respect to other seasons. Under these critical conditions, even the slight reduction in exposure to light occasioned by the crystal net resulted in a reduction in fruit colour with respect to the control in 2003, but this did not occur in 2000, 2001 and 2002 (Fig. 4), when temperatures were more favourable for colour development.

Average of fruit colour are important in order to determine its commercial value, and a minimum of 50% of fruit surface should be coloured to comply with the EU Extra colour grade requirements and more than 80% for Extra Fancy requirements. Average values for fruit colour distribution for the total yield of each treatment at commercial harvest are presented in Fig. 5. Significant differences were observed between seasons and the interaction *season x treatment* was significant with respect to fruit colour and Hue values. The greatest values for fruits with <40% of fruit colour were recorded in 2003. Intermediate values were obtained in 2001 and the lowest values in 2000 and 2002. When the averages of fruits >80% of fruit colour were compared, the greatest values were obtained for 2000 and 2002 seasons. These results were similar to those discussed above for Hue values (Fig. 4), and showed the negative effect of the black net in reducing average values for fruits with >80% and 40-80% of fruit colour.

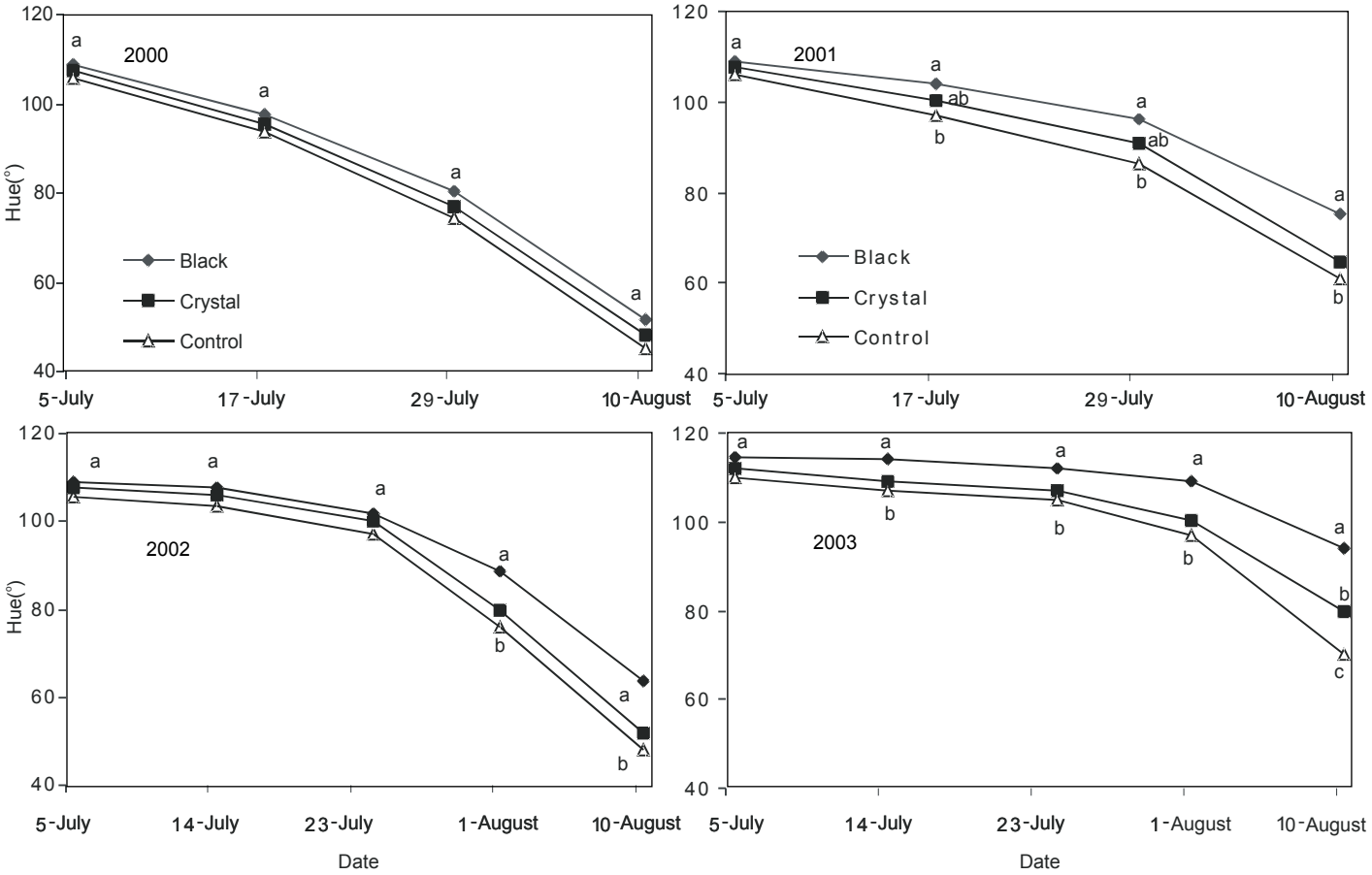

Fig. 4. The effect of two different coloured nets on fruit colour evolution (Hue) for 'Mondial Gala' apples in the preharvest period of the 2000, 2001, 2002 and 2003 seasons. Treatments with the same letter for the same season and data are not statistically different according to the Tukey Test (P<0.05). (Season x treatment interaction for hue values at harvest was significant at P<0.01). Each point represents the mean of two fruit sides of 20 fruits per tree and 8 trees per treatment (2 trees.block-1 x 4 blocks).

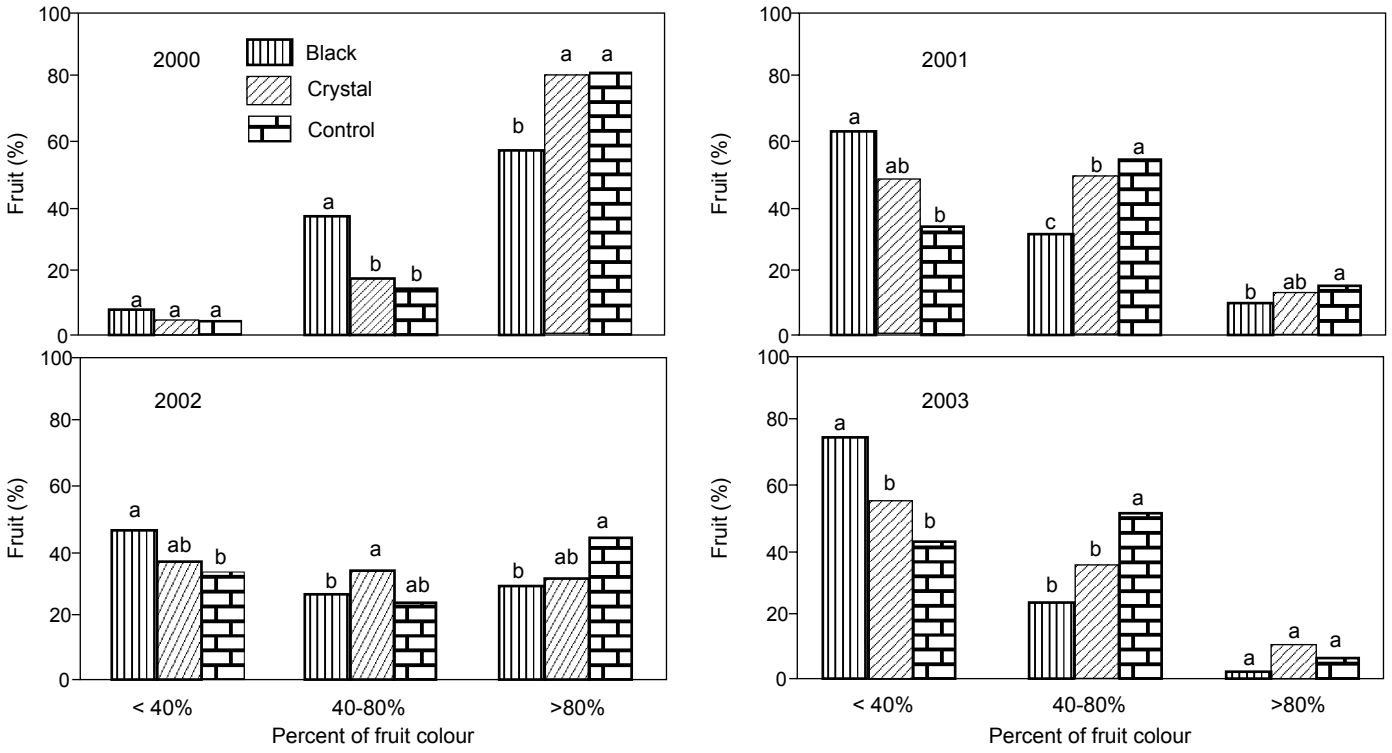

Fig. 5. The effect of two different coloured nets on the average of fruit colour for all the yield of 'Mondial Gala' apples at commercial harvest in the 2 ferent according to the Tukey Test (P<0.05). (Season x treatment interaction for % of fruits for each different degree of colour was significant at P<0.01).

Crystal net provided similar values to the control, except in 2003 when reduced the averages of 40-80% and increased those of <40%, indicating a reduction of fruit colour (Fig. 5).

The total yield harvested at the first picking date (>70 mm size and >50% of coloured surface) revealed lower values associated with the use of the black net and similar values between the crystal net and the control in 2000, 2001 and 2002, and intermediate values for the control and use of the black net in 2003. Even in seasons favourable to fruit development, such as 2000, the black net reduced fruit colouration. Mean values for all seasons only indicated significant differences between the control and black net (Fig. 6). Important differences were observed between seasons, with higher average values being obtained in 2000 and lower values in 2003. For this reason, the interaction *season x treatment* was also significant with regard to average values associated with fruit harvested at the first picking.

All previously presented results relating to fruit colour confirm those reported by other authors working with bicoloured cultivars such as 'Royal Gala', 'Jonagold' and 'Elstar' (Reigne, 1997; Vercammen, 1999; Créte, 2000; Peano *et al*., 2001). They also illustrate the negative effect of the black net on fruit colour; in the case of the crystal net, similar values were obtained to the control for seasons with normal or optimum temperatures, and values between those obtained using the black net and the control were obtained in warm seasons, such as 2003. Dussi *et al*. (2005) working with the cultivar 'Fuji', in Argentina, and Gindaba and Wand (2006) working with 'Royal Gala' in South Africa, also reported a significant reduction in fruit colour under anti-hail nets. Red colour development was influenced by the season due to the direct effect of temperatures on anthocyanin biosynthesis in 'Mondial Gala' apples as reported by Iglesias *et al*. (2002).

Tree vigour and yield: No difference between treatments were observed at the end of the first season (2000) after the installation of the nets on tree vigour. The annual increase in TCSA showed greater values for trees under the black net in 2002 and 2003 and intermediate values for crystal net in 2001 (Table 2). Furthermore, annual shoot growth was significantly higher in all seasons when using the black net in comparison to the crystal net and the control, as reported by Peano *et al*. (2002). Shading by nets (specially black net) has effects similar to over-tree evaporative cooling; decreasing plant temperature and water stress, and reducing the incidence of sunburn. This has been widely documented by several authors (Parchomchuk and Meheriuk, 1996; Recasens *et al*., 1998; Gindaba and Wand, 2005, 2006; Iglesias *et al*., 2005). As discussed above, fruit colour was significantly reduced by net shading because of decreased direct sunlight on the fruits and consequently a reduction of anthocyanin bioshynthesis rate.

Table 2. The effect of two different coloured nets on tree vigor[z], measured as the trunk-cross-sectional-area (TCSA), of 'Mondial Gala' apples over 1999 to 2003 seasons

Treatment	TCSA (cm^2)**				
	1999*	2000	2001	2002	2003
Black	33.9a	43.2a	48.6a	55.6a	63.8a
Crystal	35.0a	41.9a	45.3ab	51.3b	57.9b
Control	35.2a	40.3a	44.1b	50.2b	56.1b
Significance (*P*)	0.353	0.080	0.031	0.009	0.001

[z] Different letters in the same column represent significant difference at $P \leq 0.05$ by Tukey range test if the F-test was significant in the ANOVA. (*): Initial TCSA, before installation of nets. **: TCSA mesured in November

The trial was located in a warm region with sunny days and high radiation levels (Fig. 2) over the most part of summer. In these conditions, light disponibility compared to cold regions of the North European countries, is not a limitation, even under black nets. For this reason the increase of vigour could be explained by the positive effect of shading (black net) which resulted in a significant reduction of both, the radiation reaching trees and consequently the level of evapotranspiration. This resulted in a reduction in plant water stress, an increase in photosynthesis and increased availability of carbohydrates: all of these circumstances are conducive to an increase in vigour, as observed in commercial orchards. The reduction in stress is also favoured by a reduction in maximum temperatures and increase in orchard humidity associated with the use of nets. In these conditions we suggest that this reduction is more important than the reduction in photosynthesis caused by the interception of radiation by the nets. Gindaba and Wand (2005) reported a down-regulation of photosynthetic capacity due to anti-hail net which resulted in reduced fruit size on 'Royal Gala' and 'Cripps Pink' apples. Furthermore, the greater humidity found under the nets leads to a decrease in plant water stress. This confirms the data presented by Créte *et al.* (2001), which indicated that it is possible to reduce irrigation needs by about 15% with respect to the control because of an 10% evaporation decrease.

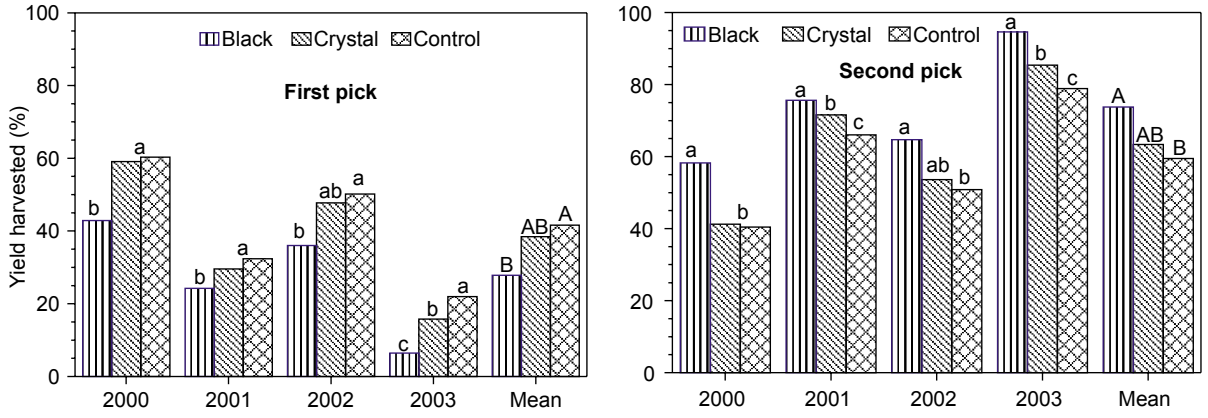

Fig. 6. Percentage of yield harvested by picking for 'Mondial Gala' apples for the period 2000-2003, as affected by the use of different coloured nets. Columns with the same letter for the same season and picking were not statistically different according to the Tukey Test ($P<0.05$). (Season x treatment interaction for % of yield harvested by picking was significant at $P<0.01$).

Table 3. Effect of two different coloured nets on mean yields, fruit size, quality parameters, starch index, incidence of cracking and sunburn and hail damage, for 'Mondial Gala' apples at commercial harvest in the 2000, 2001, 2002 and 2003 seasons.

Season	Treatment	Yield	Weight	Size mm (% yield)			Firmness	SSCs	T.A.	Starch	Crack.	Sunburn	Hail
		(kg tree^{-1})	(g fruit^{-1})	<70	70-80	>80	(lb)	(ºBrix)	(g L^{-1})	index	(%)	(%)	affe. (%)
2000	Black	31.7	185.7 b	19.2	67.7	13.1 b	15.4	13.3	3.9	7.2 b	2.1	1.2 b	0.0
	Crystal	25.1	190.5 ab	11.7	67.2	21.1 a	14.9	13.1	3.3	7.1 b	3.7	1.8 b	0.0
	Control	30.6	198.0 a	14.1	70.7	15.2 b	15.6	13.2	3.0	8.6 a	3.9	4.9 a	0.0
2001	Black	38.1	144.0	36.9	52.4	10.6	16.5	11.8 b	2.8	8.2	4.2	2.3 b	0.0
	Crystal	39.9	155.0	23.8	64.2	11.9	16.3	12.8 a	2.5	8.1	6.1	4.3 b	0.0
	Control	37.9	141.0	37.0	49.1	12.9	16.9	12.7 a	2.8	7.4	6.4	7.5 a	0.0
2002	Black	28.1	168.5	27.1	52.9	19.9 a	16.7	10.6 b	5.4	7.4	0.0	0.0	0.0
	Crystal	26.4	161.7	31.2	52.9	15.9 ab	16.6	12.6 a	6.1	7.1	0.0	0.0	0.0
	Control	28.2	154.2	37.9	48.4	13.6 b	16.9	12.0 a	5.8	7.0	0.0	0.3	0.0
2003	Black	29.4	153.0	29.4	61.9	8.6	14.3	11.2 b	2.8 a	7.1 b	12.6 b	0.3 c	0.0 b
	Crystal	28.9	154.0	27.8	62.1	10.1	14.2	12.0 a	2.6 ab	7.4 b	15.8ab	3.8 b	0.0 b
	Control	29.4	152.0	24.7	61.8	13.3	14.7	12.4 a	2.5 b	8.8 a	20.5 a	12.0a	10.6 a

Columns with the same letter for the same season were not statically different according to the Tukey Test (P<0.05).

Mean yields (kg tree^{-1}) recorded during the period 2000-2003 were not significantly affected by the use of nets (Table 3). These results support those presented by Reigne (1997), but Peano *et al*. (2002) reported a decrease by the effect of anti-hail nets due to modifications in the plant physilogy.

The effect of nets on fruit size, fruit maturity and quality parameters: Fruit size distribution, fruit weight and fruit firmness were not significantly affected by the use of nets (Table 3). Similar observation have been reported by Borin and Saoncella (2000) and Crété (2000). Soluble solid content decreased when the black net was used, but values were similar for the crystal net and the control. These results agree with those reported by Coreau *et al*. (1997), Crété *et al*. (2001), Peano *et al*. (2001) on 'Gala' and 'Fuji' apples, and confirm that shading reduces the soluble solid content of fruits, delaying their ripening. In opposite, a better exposition of the leaves to light has been related with a better fruit sugar content (Jackson *et al*., 1977; Dussi *et al*., 2005). Nets had no effect either on titratable acidity or on fruit firmness, but maturity, expressed by starch conversion, was delayed by about a week in 2000 and 2003, but not in the other seasons. Similar results were reported by several other workers (Borin and Saoncella, 2000; Peano *et al*., 2001). However, Ghindaba and Wand (2006) did not find any differences in fruit firmness, soluble solid content and starch conversion when using a shade net with 'Royal Gala' apples.

According to Reigne (1997), the use of nets did not have any influence upon the incidence of pests (*Cydia*, *Aphis*, etc) or diseases (*Venturia*, *Podosphaera*, *etc.*). Demaria *et al*. (2006), reported a significant decrease of moth pest population and a reduction of fruit-damages with anti-hail nets.

The effect of nets on fruit sunburn and cracking: The use of nets had a positive effect on the reduction in number of fruits affected by sunburn, in spite of 'Mondial Gala' is not a very sensitive cultivar to this disorder compared with 'Fuji' or 'Granny Smith' (Carbó *et al*., 2004). Crystal net produced intermediate results between black net and the control (Table 3). The reduction of sunburn associated with the use of nets, especially the black net, and the increase in overall skin quality (but decrease of fruit colour) has been widely reported for the cultivars 'Golden', 'Gala', 'Granny Smith', 'Fuji' and 'Pink Lady' (Andrews and Johnson, 1996; Yuri *et al*., 1996; Crété, 2000; Peano *et al*., 2001; Dussi *et al*., 2005; Gindaba and Wand, 2005, 2006). This can be explained by both, the reduction in direct incident radiation on the fruit (Fig. 2), and by the reduction in fruit temperature under the net (Table 3). This finding is in agreement with most of the research indicating high temperatures and intense solar radiation as the main causes of sunburn in apples (Yuri *et al*., 1998). For this reason, shading by nets has been used in several parts of the world with high temperatures and intense solar radiation to reduce sunburn and increase skin quality in apples (Andrews and Johnson, 1996; Yuri *et al*., 1996; Dussi *et al*., 2005; Gindaba and Wand, 2005; 2006), specially on non coloured apples such as 'Golden' or 'Granny Smith'. As reported in warm areas, the reduction of fruit colour observed on bicoloured or red cultivars should be considered and the use of white or grey nets is recommended (Peano *et al*., 2001), in spite the minor protection against sunburn.

Fruit cracking was not affected by the nets, except in 2003 when greater values of fruit cracking were recorded, probably due to the unusually high summer temperatures, which also reduced fruit colour. In this season nets significantly reduced the averages of fruit cracking compared to the control (Table 3).

Efficiency of nets against hail protection: The only hail storm recorded took place in May 2003. The results obtained showed the efficiency of the nets in protecting fruit compared to the control (Table 3). The stability offered by this system in windy storms has also been reported by several researchers from different countries, working with the same system (Bru, 1996; Vercammen, 1999).

Economic evaluation

Installation costs: Beniagro-type GVM 2.5 x 3, crystal and black coloured nets were used in the trial. The total cost of net installation (nets, poles, poles anchorages, rented machinery, net installation, labours, *etc.*) was 14358 € ha^{-1}. The cost of the support structure for a planted orchard (poles + support cables) was 6625 € ha^{-1}. In the case of a new plantation, the same poles used to support the nets can support the trees and this makes it possible to share the cost of supporting trees, which is estimated at around 2000 € ha^{-1} (the cost of poles and their positioning).

Annual cost of nets and insurance: These costs have been calculated for the orchard where the trial was carried out in the region of Lleida (Mollerussa). Annual costs of using nets (nets, installation, structure, rented machinery, labour, fold and unfold the nets each year, *etc.*) were 1874 and 1612 € ha^{-1}, for crystal and black nets, respectively (Table 4). We consider a lifespan for the support structure (poles, support cables) of 24 years, and lifespans of 8 and 15 years, respectively, for the crystal and black nets. The annual cost of using nets was compared with the annual cost of insurance (only protection against hail) for the cultivar 'Mondial Gala', considering a mean yield of 50 tons ha^{-1} at the Mollerussa site. The final annual cost of insurance was 760 € ha^{-1} (Table 4) and was subsidised by ENESA (48%) and DARP-Generalitat de Catalunya (15%). The cost (€ kg^{-1}) of insurance was lower than that of covering the orchard with nets and directly depended on the yield insured: the cost increased as the yield increased. The greater cost of the crystal net was due to its lower life expectancy (8 years) than the black net (15 years). In contrast with the case of insurance, the cost associated with the use of nets (€ kg^{-1}) was inversely related to the yields obtained.

The effect of nets on gross income: As explained earlier, the use of nets influenced some parameters of fruit quality as fruit colour, damage by sunburn and cracking, and consequently fruit price. Furthermore, as the insurance (control), the installation of nets resulted in annual recurring cost. Both factors are considered to evaluate the effect of the nets on the gross income without considering the other costs of production -labour, inputs, irrigation, *etc.*-, because these are the same for the three treatments of 1 ha of 'Mondial Gala' apples. Considering the same yield for the three treatments and based on the mean data corresponding to the 2000-2003 period, shown in Fig. 5 (fruit colour distribution), Fig. 6 (yields harvest by the two harvests) and Table 3 (fruit size, damage by cracking and sunburn), the total income from fruit sale, the cost of protection against hail, and the benefit are presented in Table 4. Compared to the control (insurance without using net), the benefits (total gross profit) of using crystal net was lower than using black net. Crystal net reduced sunburn, without affecting negatively fruit colour, but the annual cost of its installation is more than two times compared to the insurance and greater than black net. The decrease of benefit when using black net, even after reduction of cracking and sunburn, is due to the decreased fruit color: average of yields on the first picking and increase of non marketing yield.

The results discussed above are from 'Mondial Gala', with a limited insurance price. In the case of new developed cultivars as 'Pink Lady', 'Kanzi', *etc.*, the fruitgrower price is greater (0.50-0.60 € kg^{-1}), but the price to insure is limited (0.31 € kg^{-1}) and can't cover the value of the fruit. In this case the benefit of using crystal net (black net reduces colour) increase the benefit compared to the control which is nowadays used in the main areas of fruit production in Spain.

On the basis of the results presented, we can conclude that the system evaluated was very effective at protecting fruit against hail damage. A study of annual benefits shows similar benefits when the control and the crystal net are compared and the disadvantage of using black nets due to the reduction of fruit colour on 'Mondial Gala' apples. Net cost was higher for the crystal net than for the black one due to its shorter lifespan (8 and 15 years, respectively). This relative cost decreased when yields increased; just the opposite to what happened in the case of insurance. The use of nets offers greatest return in case of cultivars with high market values, due to the limits of maximum insurable value.

Table 4. Differences on gross profit due to the use of two coloured nets and control (without nets), over a 1 ha orchard surface of `Mondial Gala' apples at Lleida (Spain)

Concept per ha	Treatment		
	Control	Black net	Crystal net
Total yield (kg ha^{-1})*	52000	52000	52000
% of total yield < 70 mm *a	26.9	28.1	23.6
% of total yield affected by sunburn*	6.2	1.0	2.5
% of total yield affected by cracking*	7.7	4.7	6.4
Commercial yield (kg ha^{-1})	**30784**	**34424**	**35100**
Yield harvested at 1st picking ** (%) (diameter > 70mm and > 50% fruit	41	28	37
Yield decrease at 1st picking with respect to the control (%)	_	-13	-4
Harvest at 1st picking* (kg)	**12621**	**9639**	**12987**
Price 1st picking (€ kg^{-1}) ***	0.34	0.34	0.34
Income 1st picking (€)	42913	3277.2	4415.6
Yield at 2nd picking**(%)	59	72	63
Harvest at 2nd picking (kg)	18163	24785	22113
Yield <40% fruit colour *(%)b	29	46	31
Price yield no market (€ kg^{-1})***	0.06	0.06	0.06
Income yield no market (a+b)	**2597**	**2965**	**2692**
Price 2nd picking (€ kg^{-1})***	0.24	024	0.24
Income 2nd picking (€)	**3094.9**	**3212.2**	**3661.9**
Total fruit income (€ ha^{-1})	**9982.9**	**9454.1**	**10769.5**
Difference to control (€ ha^{-1})	**-**	**-528.8**	**786.6**
Insurance cost (€ ha^{-1} $year^{-1}$)	**760.0**	**-**	**-**
Total nets cost (€ ha^{-1} $year^{-1}$)	**-**	**1611.6**	**1873.8**
Difference to control (€ ha^{-1})	**-**	**851.6**	**1113.8**
Total gross profit' (€ ha^{-1})	**9222.9**	**7842.5**	**8895.71**

(*): data from Table 3; (**): data from Figure 6; (***): mean fruit grower prices from 2000 to 2003. (y) : without considering production costs (labour, inputs, irrigation, *etc.*).

For all of the factors studied, the black net always had a greater impact than the crystal net, resulting in a reduction of fruit colour, soluble solid content and sunburn, and causing a subsequent delay in maturity. It would therefore adivisible to use crystal nets in warm areas with poorly or medium coloured cultivars.

It would also be interesting to study the effect of using nets on plant hydric status and water needs and their influence upon tree vigour. These are considerations of potential interest due to the effects of nets on radiation, humidity, evapotranspiration and temperature.

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Induction of phenolic compounds biosynthesis with light irradiation in the flesh of red and yellow apples

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Abstract

Effect of light irradiation on the accumulation of phenolic compounds was investigated in the flesh slices of three apple (*Malus domestica* Borkh.) cultivars. 'Fuji' and 'Jonathan' with red skin and 'Orin' a yellow-green one were used in this study. The irradiation was carried out at 10, 17, 24 and 30℃ for 96 hours, using a mixture of white plus ultraviolet fluorescents. Phenolic acids, anthocyanin and flavonols were the phenolics that increased rapidly by irradiation whereas flavanols, procyanidins and dihydrochalcones did not change in either mature or in ripe fruits of all the three cultivars. There was a positive correlation between anthocyanins, phenolic acids and flavonols in examined cultivars both at the mature and ripe stages. Optimum temperature for the synthesis of phenolic acids, anthocyanins and flavonols was 24℃ regardless to the maturity stage and variety. Total phenolic content of 'Fuji' increased through ripening but it decreased in 'Jonathan' and 'Orin'. Therefore, the irradiation to the flesh might be a very useful method for the study of the regulation mechanism of the phenolic compounds accumulation.

Key words: Apple, light irradiation on flesh, ultraviolet-B, flavonoids, phenolic acids, high performance liquid chromatography

Introduction

Apple fruit is known to contain large amounts of phenolics such as chlorogenic acid, flavanols, flavonols, procyanidins and anthocyanins (Awad and de Jager, 2002; Awad *et al.*, 2000). The studies using animals and *in vitro* work have been conducted to define mechanisms by which apples may help in preventing chronic disease caused by lipid and DNA oxidation (Boyer and Liu, 2004). The antioxidative potential of apple, however, is known to depend on the concentration and composition of phenolics (Kondo *et al.*, 2002), which is influenced by many physiological and environmental factors (Lancaster, 1992). Moreover, distribution of these metabolites differs within a fruit (Awad *et al.,* 2000).

The ability for anthocyanin accumulation in apple skin varies within varieties. Red-skinned apples accumulate anthocyanin, while yellow-green ones do not and most of the commercial cultivars with red skin accumulate anthocyanin in the skin but not in the flesh. Although the biosynthesis mechanisms of anthocyanin and other phenolics have been widely studied in the skin, limited information about the flesh is available. Stimulative effect of irradiation on the anthocyanin accumulation, influenced by bagging, temperature and maturity has been reported, recently. These studies show that UV-B stimulates anthocyanin synthesis in the apple skin (Arakawa *et al.,* 1985; Arakawa, 1988; Arakawa, 1991; Wang *et al.,* 2000).

Because of lower content of phenolics, specifically flavonols, in the flesh compared to the skin, pulp may have lower bioactivity than the peel. Many researches have been conducted on the regulation of phenolics accumulation in the skin, and little is known about the flesh. The aim of this study was to investigate the regulation mechanism of anthocyanin and other phenolic compounds accumulation in the flesh, with special attention given to light and temperature as two key regulatory factors. Mature and ripe fruits of three cultivars with varied genetic background were studied.

Materials and methods

Fruits of 'Jonathan' and 'Fuji' (red cultivars) and 'Orin' (yellow-green) were harvested from 15-year-old trees grafted onto *Malus prunifolia* Borkh. var. *ringo* Asami rootstocks. The trees were grown at the experimental orchard of the Faculty of Agriculture and Life Science, Hirosaki University. Sampling dates for mature and ripe stages of each cultivar were: 'Jonathan'; 123 and 145 days after full bloom (DAFB), 'Fuji'; 134 and 165 DAFB and 'Orin'; 131 and 170 DAFB, respectively. Three fruits of each cultivar were equatorially cut and immediately dipped in a solution of Polyclar SB 100 (20 g L^{-1}), in order to protect the tissues from browning, and then sterilized with 5 percent ethanol for 1 minute. Three sectors of these samples were put into petri dishes and covered with clear polyethylene sheet to maintain the moisture of the tissues during irradiation. The other three halves were wrapped in aluminum foil, to maintain dark conditions during irradiation, as controls. The irradiation was carried out at 10, 17, 24 and 30℃ using a mixture of a white fluorescent lamp (FL20-S W, Toshiba, Tokyo; 4 W m^{-2}) and a UV-B fluorescent (FL20-S. E., Toshiba Tokyo; 1.3 W m^{-2}) with a filter eliminating radiation below 290 nm. The irradiation was carried out for 96 hours, continuously, and some water was daily sprayed on the samples during the irradiation period. The irradiated surface (almost one millimeter in thickness) was then separated and frozen in liquid nitrogen then kept at –80℃ until analysis. Phenolic extraction was carried out with 15 percent acetic acid in methanol added to 1 g of finely powdered tissue and then kept in 4℃ for 2 hours. These samples were centrifuged at 10,000 g for 15 minutes at 0℃(Himac CR 15, HITACHI, Japan). The

supernatant of samples was filtered through a 0.45µm disposable syringe filter and used for flavonoids quantification by high performance liquid chromatography (HPLC) coupled to a diode array detector with wavelength set at 280, 350 and 530 nm. The column was a 1.5 mm I.D.×250 mm (Grand C18-UG 120-5 SE, MASIS, Inc., Aomori, Japan) with a 1.5 mm I.D.×35 mm guard column. Elution solvents were (a) 1.5 percent phosphoric acid in water of which the flow was 90 µL min^{-1} and (b) 1.5 percent phosphoric acid, 20 percent formic acid and 25 percent acetonitrile in water with 10 µL min^{-1}. Samples (5 µL) were injected on to the column which was maintained at 30℃.

According to standard analysis, identified substances were categorized to six groups; namely, phenolic acids (chlorogenic acid), anthocyanins (cyanidin 3-galactoside), procyanidins (procyanidin B2), flavanols (catechin and epicatechin), flavonols (quercetin glycosides), and dihydrochalcones (phloridzin). Mature and ripe fruit flesh of three cultivars were used for biochemical analysis.

Results

Accumulation of phenolic acids, anthocyanin and flavonols in response to UV-B irradiation: Irradiated flesh of mature and ripe fruits of all three cultivars accumulated phenolic acids, flavonols and anthocyanin (Figs. 1 and 2). 'Fuji' and 'Orin' accumulated the highest flavonols and phenolic acids at 24 and 30℃; whereas for 'Jonathan' 24℃ was optimum. However, the concentration of anthocyanin was lower than phenolic acids and flavonols in three varieties. Chlorogenic acid of 'Orin' was the predominant phenolic compound after irradiation.

Total phenolic content of 'Fuji' increased from mature stage to ripening but in 'Jonathan' and 'Orin' it decreased. Interestingly, total phenolic content of Fuji (before and after irradiation), both at mature and ripe stages, was higher than that of 'Jonathan' followed by 'Orin'. 'Jonathan' accumulated the highest anthocyanin, resulted in redder color, followed by 'Fuji' and 'Orin'. Anthocyanin biosynthesis in the pith of receptacle area was higher than the cortex of receptacle.

Lack of response of flavanols, procyanidins and dihydrochalcones to UV-B irradiation: Irradiation did not stimulate the accumulation of flavanols, procyanidins and dihydrochalcones both in mature and ripe stages in all three cultivars (Figs. 1 and 2). In ripe 'Orin' the flavanols increased slightly at 24℃, but it was not significant. Flavanols increased with fruit ripening regardless to irradiation temperature. Flavanol content of 'Jonathan' was higher than that of 'Fuji' and 'Orin'.

Correlation between the accumulation of anthocyanin and other phenolics: There was not same pattern of correlation between anthocyanin biosynthesis and other phenolics in the examined cultivars (Table 1). In 'Fuji' and 'Jonathan', phenolic acids and flavonols showed a positive correlation with anthocyanin accumulation, which was not affected by ripening. In 'Orin', on the other hand, the correlation coefficient values between anthocyanin versus phenolic acids and flavonols decreased from mature stage to full ripening.

Discussion

Our results showed that the flesh of all three cultivars used in this study had the potential for accumulating anthocyanin as well as many other polyphenolics, although temperature and maturing stage affected it. The flesh of 'Jonathan' accumulated the highest

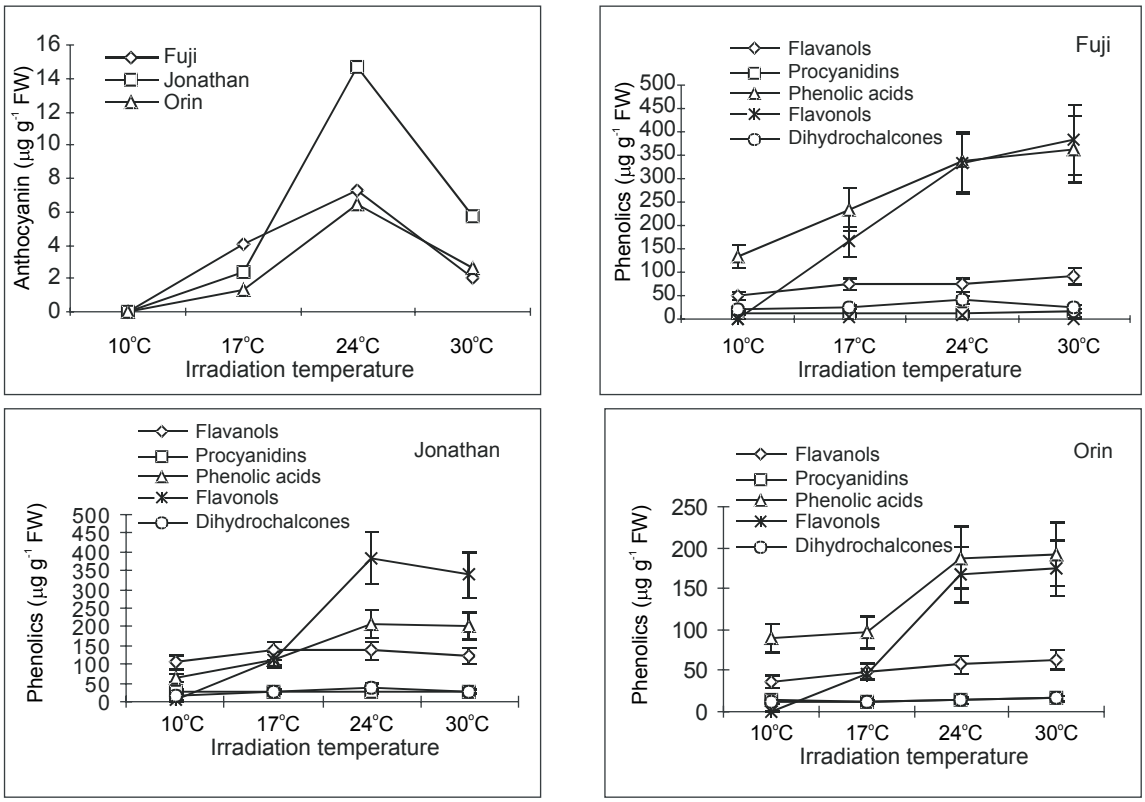

Fig. 1. Effect of UV-B irradiation on the accumulation of anthocyanin and other phenolic compounds in three apple cultivars at mature stage. Phenolic content of the three cultivars (µg g^{-1} FW), irradiated at 10, 17, 24 and 30℃. Vertical bars represent SE .

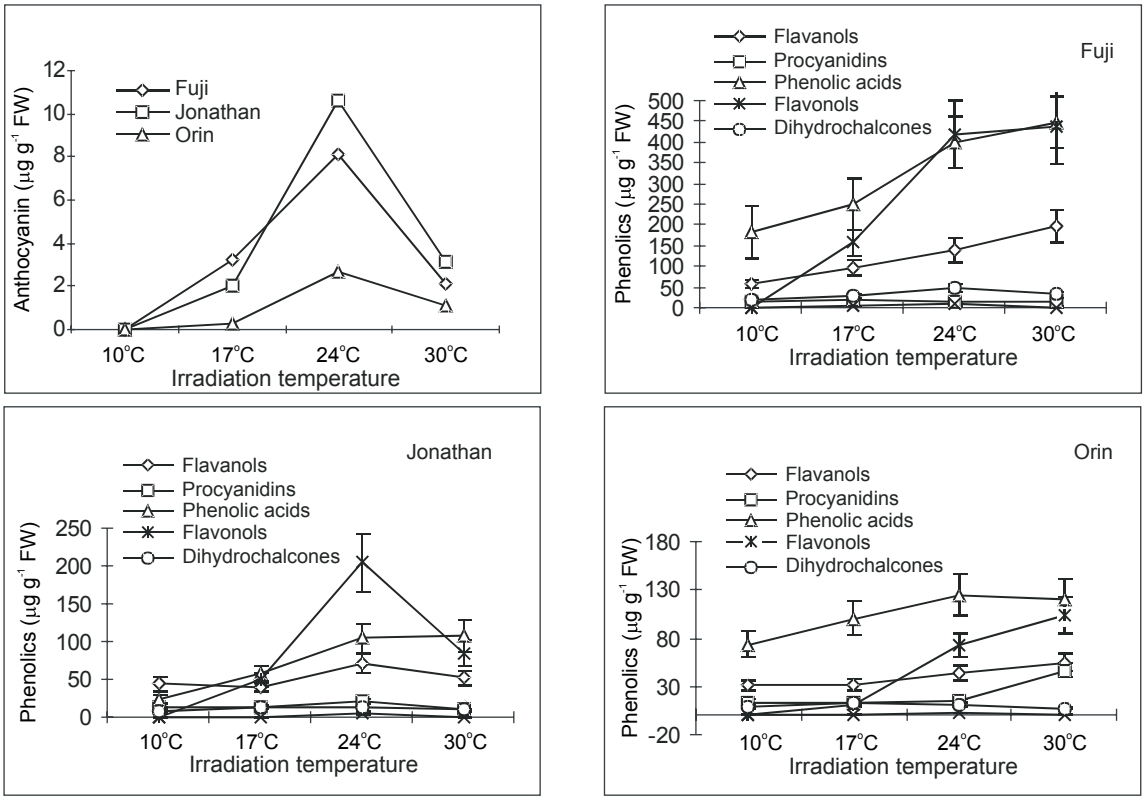

Fig. 2. Effect of UV-B irradiation on the accumulation of anthocyanin and other phenolic compounds in three apple cultivars at ripe stage. Phenolic content of the three cultivars (µg g^{-1} FW), irradiated at 10, 17, 24 and 30℃. Vertical bars represent SE .

anthocyanin followed by 'Fuji' and 'Orin', respectively, which is same as the order of the anthocyanin synthesis ability of the skin of these cultivars suggesting that the light threshold of the flesh is same as that of the skin.

While apple flesh contains low level of flavonols as reported by Awad *et al.* (2000), it increased with light irradiation showing that the synthesis of this group of flavonoids is a light-dependent process. A positive correlation between anthocyanins, phenolic acids and flavonols, but not the other phenolics, show that the expression of the genes controlling the synthesis. The quantity and quality of phenolics changed with irradiation, depending on the irradiation temperatures. Optimum temperature for the synthesis of phenolic acids, anthocyanins and flavonols was 24℃, regardless to the maturity stage and variety. The response of flesh to light irradiation is different from that of skin. Our previous study showed that temperature had different effects on phenolic accumulation in skin depending on the maturity stage; mature and ripe fruits accumulated the highest phenolics at 17 and 24℃, respectively (Bakhshi and Arakawa, in press).

Activation of the expression of the flavonoid biosynthetic genes by enhanced light has recently been reported in other studies (Lancaster, 1992; Jaakola *et al.*, 2004). All the three cultivars examined in this study accumulated low amounts of phenolics at 10℃ which coincides with our former results on the skin. Although low temperatures have been suggested to induce Phenylalanine Ammonia-lyase (PAL) activity (Lancaster, 1992), it seems that low temperature could reduce the UV-B enhancement of quercetins and chlorogenic acid.

Phenolic acids, procyanidins, catechins and dihydrochalcones were the phenolics identified in the flesh among which phenolic acids increased significantly with irradiation. This might be related to the induction of PAL activity by UV-B, which mediates the synthesis of coumaroyl CoA. Comaroyl CoA then gives rise to phenolic acids and flavonoids. Anthocyanin biosynthesis in the irradiated flesh of 'Orin' that does not naturally accumulate red color might be related to the simultaneous effect of UV-B and wounding on the induction of PAL activity (Lancaster, 1992). However, apple sectors kept in the dark during irradiation did not show any change in their phenolic content showing that only wounding is not enough for phenolic accumulation (data not

Table 1. Correlation coefficient value (r) of anthocyanin versus other identified phenolic compounds in the light (white plus UV-B) irradiated flesh of 'Fuji', 'Jonathan' and 'Orin' cultivars

	Mature			Ripe		
Phenolic classes	'Fuji'	'Jonathan'	'Orin'	'Fuji'	'Jonathan'	'Orin'
Flavanols	0.39 ns	0.51 ns	0.55 ns	0.47 ns	0.56 ns	0.38 ns
Procyanidins	0.19 ns	0.22 ns	0.28 ns	0.36 ns	0.58 ns	0.21 ns
Phenolic acids	0.61 *	0.77 **	0.9 **	0.64 *	0.68 *	0.65 *
Flavonols	0.84 **	0.92 **	0.88 **	0.81 **	0.93 **	0.79 **
Dihydrochalcones	0.21 ns	0.34 ns	0.16 ns	0.36 ns	0.28 ns	0.12 ns

*, ** Significant at *P*=5% and 1%, respectively; ns- not significant.

shown). Other than anthocyanin and quercetin, phenolic acids increased with irradiation. This coincides with the result of Awad *et al.* (2000) who reported that the level of chlorogenic acid in the shaded side of skin (receiving low light) was lower than that of the sun exposed side.

In the last two decades, interest in using natural ingredients in foods has increased dramatically. The emphasis given to natural antioxidants results from concerns over the toxicity of some synthetic antioxidants and from the research findings that point to a relationship between active dietary ingredients, such as natural antioxidants, and their protection of cells from free radical-induced oxidative stress in the human body (Valenzuela *et al.*, 2003).

Our results show that apple fruit is a potentially good source of phenolic compounds as natural antioxidants. The present study revealed that light and temperature have strong effect on the phenolics accumulation in the apple flesh as well as the skin. This finding offers the exciting possibility of improving the polyphenolic content by optimizing the environmental factors. Light irradiation on the flesh of apple also seems to be a good method for the study on the expression of the genes controlling the accumulation of phenolic compounds.

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a* values to follow lycopene concentration during ripening and storage of tomato (cv. Caruso)

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Abstract

The ripening of tomato fruit is a highly regulated process during which colour, flavour, aroma and texture change in a coordinated manner. This research work aims to correlate the colour changes measured objectively with the lycopene concentration in tomatoes during ripening at room temperature (21 and 26°C). These results were compared with colour and lycopene content of pink and light red tomatoes stored at 14°C, temperature used to prevent ripening and therefore extend the shelf life of the fruits. The duration of heat treatment at 100ºC was previously optimized in order to release the maximum lycopene from chromoplasts during extraction. An a* value of 20 for the peel corresponds to an increase of lycopene content of *Caruso* tomato from 9 to 43 mg/100 g TSS, at room temperature. The shelf life of pink and light red tomatoes can be extended to two weeks at 14ºC without loss of lycopene content, presenting the same content as green tomatoes ripened at room temperature for one week.

Keywords: Tomato, *Lycopersicon esculentum* Mill., lycopene, colour, ripening, storage

Introduction

The composition of tomato (*Lycopersicon esculentum* Mill.) in carotenoids differs qualitatively and quantitatively with factors such as cultivar or varietal differences, stage of maturity, climatic or geographic effects, part of the plant, conditions during production, postharvest handling, processing and storage (Delia and Rodriguez-Amaya, 2003).

Carotenoids are responsible for the pleasing yellow, orange or red colour of many foods. The role of some of these compounds as provitamin A precursors has been known for years. Other beneficial effects to human health which have been more recently attributed to carotenoids are of enhancement of the immune response and reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases, cataract and mascular degeneration, these actions not being restricted to the provitamin A (Olson, 1999). Since the human body is unable to synthesize carotenoids from endogenously produced biochemicals, the body is totally dependent on dietary sourced (exogenous) carotenoids.

The principal carotenoids in tomato are beta-carotene, beta-cryptoxanthin, lycopene, lutein and zeaxanthin. Lycopene is inactive vitamin A but is a more efficient antioxidant than beta-carotene (Di Mascio *et al.*, 1989). It has been linked with reduction of the risk of cancer, especially lung, stomach and prostate cancer (Giovannucci, 1999). In nature, carotenoids are protected by the cellular structure of the plant tissues. On the other hand, this natural protection lowers bioavailability.

The intactness of the cellular matrix determines the bioavailability of different nutrients (Van het Hof *et al.*, 2000). Heat treatment can have a deleterious effect on the micronutrient content of vegetables but at the same time the bioavailability of some nutrients can increase. There exist a lot of different opinions whether processed tomatoes contain a higher lycopene concentration or not. Food processing may improve lycopene bioavailability by breaking down cell walls, which weakens the bonding forces between lycopene and tissue matrix, thus making lycopene more accessible (Delia and Rodriguez-Amaya, 2003; Dewanto *et al.*, 2002; Gärtner *et al.*, 1997; Stahl and Sies, 1992; Thompson *et al.*, 2000). Other authors also established heating at 100°C to release the maximum lycopene from the chromoplasts (Sharma and Le Maguer, 1996). In other studies, heating of tomato products under different processing conditions may cause degradation of lycopene (Cole and Karpur, 1957; Klaui and Bauernfeind, 1981; Miki and Akatsu, 1970; Noble, 1975).

Data on the effects of processing on the carotenoid content of foods are therefore conflicting. The way of calculating the lycopene concentration is most important. When heating tomato pulp the lycopene content in pulp increases. However, this increase is directly related to the increase in tomato total soluble solids (TSS) concentration during heating. In fact, lycopene content in pulp may decrease (Sharma and le Maguer, 1996) when expressed per mass of TSS. In several of published papers (Gärtner *et al.*, 1997; Stahl and Sies, 1992; Thompson *et al.*, 2000; Khachik *et al.*, 1992; Nguyen and Schwartz, 1999; Nicoli *et al.*, 1999), the authors did not take into account the increase of tomato TSS. In consequence, it seemed that the lycopene level increased.

Tomato ripening is a regulated process during which colour, flavour, aroma and texture change in a coordinated manner. Tomato fruit colour is an important quality attribute, and it is the initial aspect evaluated by the consumers. Fruit colour is

related to the lycopene and chlorophyll content changes during the ripening process (Bramley, 1997).

Tomato fruits are chilling sensitive and the recommended storage temperature varies with the maturity stage (Hardenburg *et al.*, 1986). Temperature management is critical to maintain quality. Ripe tomato fruits can be stored at 10°C, without visible symptoms of chilling injury, although flavour and aroma may be negatively affected (Maul *et al.*, 2000).

This research work aimed to correlate the colour changes measured objectively with the lycopene concentration in tomatoes during ripening at room temperature (21 and 26°C). An objective of the work was also to compare these results with colour and lycopene content of pink and light red tomatoes stored at 14°C, temperature used to extend the shelf life of the fruits (Hardenburg *et al.*, 1986).

Materials and methods

Plant material: Tomato (cv. Caruso) at different stages of ripening (according to the experiment) were purchased at a local hypermarket (Matosinhos, Portugal).

Heat treatment: Red tomatoes (stage 5, light red, and stage 6, red, USDA, 1975) were cut into pieces and the seeds were removed. The tomato was blended in a plastic beaker covered with aluminium foil, to prevent the destruction of the lycopene by light. The blending was continued till a rough, smooth tomato pulp was formed. Afterwards the rough tomato pulp was further homogenized for 10 min with a homogenizer (Ultra-Turrax T25, Janke & Kunkel, IKA-Labortechnik, Germany). The homogenate was divided into two fractions. One fraction was kept to analyze the lycopene content in the fresh tomato and the other fraction was heated to 100°C with a magnetic stirrer at maximum speed. After starting to boil, the tomato pulp was left simmering for different periods of time (5 to 15 min) at 100°C. Both heated and non-heated samples were collected and 5 g from each sample were weighed in glass beakers covered with aluminium foil. Lycopene content was determined in these samples.

Ripening experiment: Tomatoes at the stage of breakers (stage 2, USDA, 1975) were exposed to light at room temperature (21 and 26°C) and left to ripen during 10 days. This colour stage was chosen because consumers may purchase tomatoes at this stage and leave them to ripen at room temperature.

Tomatoes were analysed for colour and lycopene content each day. Three replicates of three tomatoes were used for each day.

Storage experiment: Pink and light red tomatoes (stages 4, pink, and 5, light red, USDA, 1975) were stored at 14°C (this temperature is recommended to stop the ripening process and store tomatoes of stage 4 and stage 5) and a relative humidity 90-95% for two weeks (Hardenburg *et al.*, 1986). These colour stages were chosen because consumers usually buy tomatoes at these ripening stages. The colour and lycopene content were analysed during that time. Three replicates of three tomatoes were used for each colour stage and for each day.

Colour determination: The colours of peel and pulp were measured by using a hand-held tristimulus reflectance colorimeter (Minolta CR-300, Minolta Corp., Ramsey, New Jersey, USA). Colour was expressed as the CIE-L* a* b* uniform colour space. L* indicates lightness, a* chromaticity on a green (-) to red (+) axis, b* the chromaticity on a blue (-) to yellow (+) axis. Numerical values of a* and b* were converted into Hue angle ($H° = \tan^{-1} b^*/a^*$) and chroma ($Chroma = (a^{*2} + b^{*2})^{1/2}$) (Francis, 1980). Ten measurements were performed on each tomato.

Tomato colour was also classified according to the USDA colour chart (USDA, 1975).

Lycopene determination: The method used for the determination of lycopene concentration was adapted from Goula *et al.* (2006). A sample of 5 g of homogenized tomato pulp (fresh or processed at 100°C) was mixed with acetone and petroleum ether and stirred in a glass beaker. The mixture was transferred to a Kitazato and connected to a vacuum pump. The extract was washed three times with distilled water. The acetone was then removed helping to prevent the formation of stable emulsions.

The absorbance was measured at 505 nm using a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer (Shimadzu Corp., Tokyo, Japan) after calibration with petroleum ether. Lycopene determination was performed in triplicate. The extinction coefficient used in the calculations was 2820 for a 1% (w/v) solution (Pearson, 1976) and the lycopene content expressed in mg/ 100 g TSS.

Total soluble solids determination: The amount of total soluble solids was determined at room temperature by using refractometry (hand-held Atago refractometer model ATC1).

Statistical analysis: The descriptive analysis consisted of the calculation of mean and standard deviation for cardinal variables and of frequencies for ordinal variables. Kolmogorov-Smirnov test was used to verify the normality of the distribution of cardinal variables. The *Mann Whitney* test was used to compare medium orders of two independent samples and the *Kruskal-Wallis* to compare medium orders of three or more independent samples (non parametric tests).

The Spearman correlation coefficient was calculated to quantify the intensity between pairs of variables (non parametric correlation) (Finney, 1980). P level of critical significance was used to reject null hypothesis (rejected when $P<0.05$).

Results and discussion

Heat treatment: The effect of different heating times was investigated. In the beginning, it became clear that the heating time optimum was between the 5 and 10 min heating. The maximum lycopene content seems to correspond to a heating time between 5 and 7 min (data not shown). For further experiments 6 min heating time was used in order to maximize the release of lycopene from tomato tissues.

Ripening experiment: At colour stage 1, tomatoes are still green, meaning, there is no red pigment, so there is no lycopene present. At colour stage 2 the level of lycopene is still very low. Only when tomato changed from colour stage 4 to stage 5, the lycopene level increases rapidly because during this change the tomato turns from pink to light red. Differences between lycopene concentration of level 4 and 5 were statistically significant ($P<0.02$) at both temperatures. Levels of around 40 mg/ 100 g

TSS were achieved for tomatoes at colour stages 5 and 6 (Table 1). Stage 5 was achieved after 7 days of storage while stage 6 was achieved after 10 days. Lycopene concentration did not increase from stage 5 to stage 6 (Table 1).

Table 1. Lycopene concentration in heated tomato samples during ripening at 21and at 26ºC

Ripening temperature (°C)	Colour stage (USDA, 1975)	Lycopene (mg/ 100 g TSS)	Standard deviation
21	3	5.8	0.00
	4	9.0	2.38
	5	42.9	9.73
	6	40.6	22.79
26	2	6.4	0.00
	3	4.3	0.82
	4	15.4	10.05
	5	43.9	11.24
	6	28.9	0.00

A strong positive curvilinear relationship was found between the a* values of tomato peel and pulp and lycopene concentration at both temperatures (21 and 26ºC). Low lycopene contents correspond to low a* values. For a* values around 15 (pulp) and 20 (peel) the lycopene concentration increased abruptly, corresponding to the change from stage colour 4 to stage colour 5 (Figs. 1 and 2). For high values of a* (colour stage 5 and 6), the peel presented higher values than the pulp. This means that the red colour is more intense in the peel, probably because the peel contains higher lycopene concentration than the pulp. No correlation was found for the other colour parameters (L*, b*, hue and chroma) (data not presented).

Storage experiment: After two weeks of storage at 14°C pink and light red tomatoes presented no symptoms of chilling injury. No pitting or decay (Wills *et al.*, 1998) were observed which indicates that this temperature was adequate for storage of tomatoes at these maturity stages.

After two weeks, tomatoes at these stages of colour exhibited lycopene contents (Table 2) similar to the green tomatoes (stage 2) ripened to the same stages of colour (ripening experiment) after 6 and 7 days of storage, respectively. The same tendency was observed for the peel and pulp (Figs. 3 and 4).

Table 2. Lycopene concentration of heated tomato samples after two week storage at 14ºC

Colour stage (USDA, 1975)	Lycopene (mg/ 100 g TSS)	Standard deviation
4	14.6	3.80
5	41.7	12.35

Processing tomatoes is a value-added step in terms of lycopene bioavailability. By heating processed tomatoes (blended and simmered) at 100°C for 6 min, the bioavailability of lycopene seems to increase, due to a breakdown of the cell walls of the tomato pulp.

The a* parameter (CIE-L*a*b*) is a good parameter for quantifying the lycopene level in tomatoes. The evolution from colour stage 4 to colour stage 5 at room temperature (21 and 26°C), involves dramatic increase in the lycopene content from 9 mg $100g^{-1}$ TSS to 43 mg $100g^{-1}$ TSS. This happens for a* values around 15 for the pulp and 20 for the peel. The latter is a good

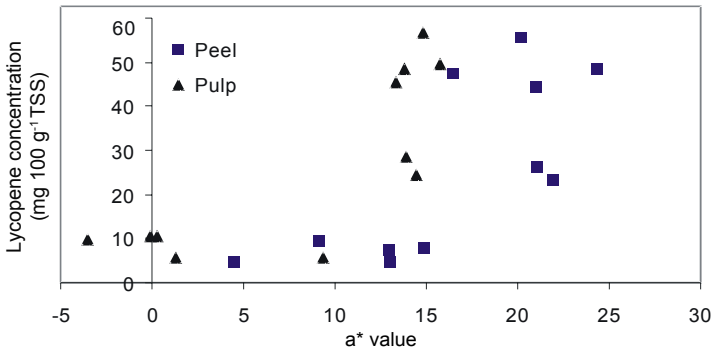

Fig. 1. Lycopene concentration of heated tomato samples in function of a* value during ripening at 21ºC.

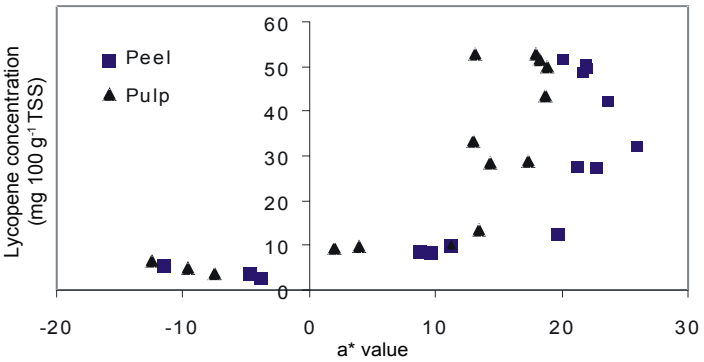

Fig. 2. Lycopene concentration of heated tomato samples in function of a* value during ripening at 26ºC.

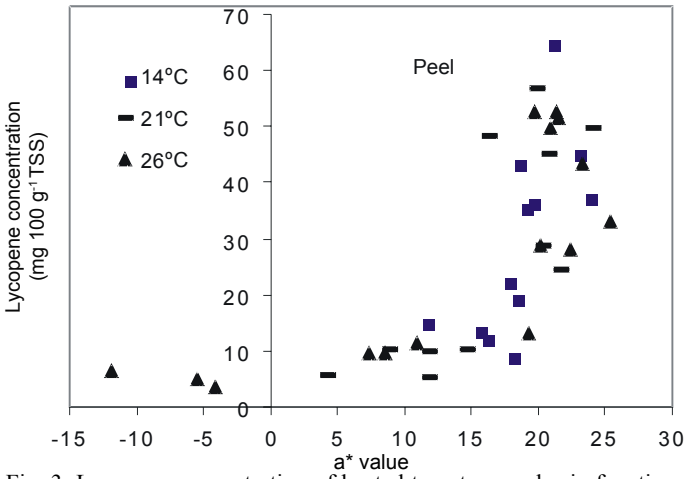

Fig. 3. Lycopene concentration of heated tomato samples in function of a* value of the peel during ripening at 21ºC and 26ºC and during storage at 14ºC.

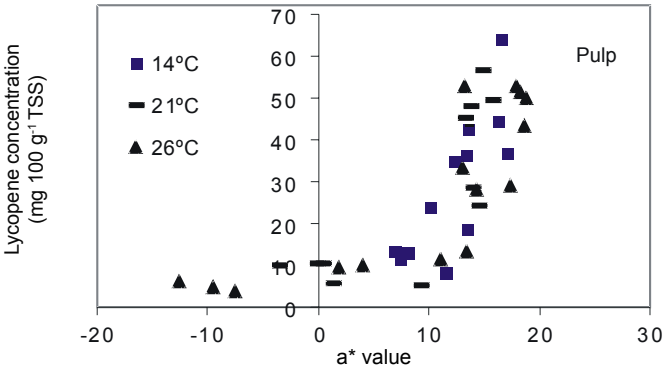

Fig. 4. Lycopene concentration of heated tomato samples in function of a* value of the pulp during ripening at 21ºC and 26ºC and during storage at 14 ºC.

indicator for the consumption of tomato (cv. Caruso) since it corresponds to high lycopene content and colour measurement of the peel by CIE- L*a*b* which is not a destructive method. The storage temperature (21 or 26ºC) does not influence the lycopene content for a specific stage of ripening.

Pink and light red tomatoes (colour stages 4 and 5), usually purchased by consumers, can be successfully stored for two weeks at 14°C, presenting lycopene contents similar to green tomatoes (colour stage 2) after being exposed to room temperature for one week, turning to light red (colour stage 5) tomatoes.

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Effects of abusive temperatures on the postharvest quality of lettuce leaves: ascorbic acid loss and microbial growth

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Abstract

Changes in lettuce leaf quality (ascorbic acid contents and microbial populations) at two abusive temperatures (8 and 15ºC), simulating the commercial storage conditions for fresh vegetables were analyzed. A storage temperature of 8°C was chosen to simulate abusive refrigerated storage and a storage temperature of 15°C was chosen to simulate room temperature. Quality indicators evaluation in samples, stored at abusive temperatures were compared with sample from optimal storage temperature (0°C). First order kinetics is assumed for ascorbic acid degradation. Ascorbic acid degradation rate in lettuce leaves stored at abusive temperatures was from 2.7 to 2.9 times faster than at 0°C. The growth curve of total microbial counts was fitted with the Gompertz and Logistic models. These models allowed us to predict the vegetable microbiological shelf life. Temperature is the controlling factor for lettuce shelf life and quality; microbial quality was retained 1.6 and 4 times longer at 0ºC with respect to 8 and 15ºC, respectively.

Key words: Abusive temperature storage, nutritional quality, microbiological quality, shelf life, growth models, lettuce, *Lactuca sativa*, ascorbic acid retention

Introduction

Vegetables are important components in a balanced human diet. They are low in fat, low in calorie with high carbohydrate and fiber contents. They provide significant levels of some micronutrients. Fresh vegetables have a short shelf life when they are exposed to conditions that destroy their quality attributes. Fresh vegetable quality is affected by various factors, including post-harvest handling and storage conditions (temperature, relative humidity, light and atmosphere composition). Minimum safe low temperatures and high relative humidity control are the most important tools for extending the shelf life of most fresh vegetables. Temperature is the single most important variable and its improper manipulation causes evident changes in the sensory characteristics of fresh vegetables that condition consumers acceptability. Abusive storage temperatures also lead to reduce levels of some nutrients, mainly ascorbic acid, and favor microorganisms proliferation to levels that may exceed tolerable levels (Francis *et al.*, 1999). Fresh fruits and vegetables probably receive the greatest temperature abuse at the retail level. Temperature abuse is a function of time and temperature during holding and the relative perishability of a particular commodity (Paull, 1999).

Fresh vegetables are a major source of ascorbic acid, a nutrient that besides its vitamin action, is valuable for its antioxidant effects, its stimulation of the immune system and other health benefits that are being actively investigated and reported (Giannakourou and Taoukis, 2003). While the assessment of ascorbic acid content does not represent a complete nutritional evaluation, this compound is very labile and therefore it gives a sensitive index of relative nutritional quality (Perrin and Gaye, 1986).

Another basic phenomenon that confronts the extension of fresh vegetables shelf life is the microorganisms proliferation. Disease-causing microorganisms' growth is a food safety concern, especially with high pH vegetables. Visible growth and off-odors caused by microorganisms are also objectionable. Various treatments and strategies have been evaluated to aid in the management of post-processing microbial populations, *e.g.* use of sanitizing solutions (chlorine water, hydrogen peroxide, sodium dichloroisocyanurate, etc.), modified atmosphere control and irradiation with a gamma source (Zagory, 1999). However, most of the research evaluating the efficiency of sanitizer for eliminating microorganisms to date has not been promising (Brackett, 1999).

The first few hours after harvest can be crucial for the fresh vegetables shelf life. Although temperatures close to 0 °C are recommended, fresh vegetables may be prepared, shipped and stored at 5 °C and, sometimes as high as 10 °C (Watada *et al.*, 1996). Handling at this elevated level can hasten deterioration because Q_{10} of biological reactions range from 3 to 4 and possibly as high as 7 within this temperature region (Wiley, 1997). The paramount marketing tool for fresh vegetables is strict avoidance of temperature abuse during processing, distribution, and merchandising to achieve the required or near maximum shelf life for each product (Schlimme, 1995). Although research are needed to determine the kinetic parameters that help to model the changes in quality attributes of fresh products (Giannakourou and Taoukis, 2003), these studies were done in similar conditions found in commercial practices. The significance of laboratory studies and

to a lesser extent simulated shipping studies may not be relevant to commercial practices in many cases (Paull, 1999).

The purpose of the present work was to analyze changes in lettuce leaf quality kept at two abusive temperatures (8 and 15 ºC) simulating the commercial sales practice of fresh vegetables. A storage temperature of 8 °C was chosen to simulate abusive refrigerated storage and a storage temperature of 15 °C was chosen to simulate room temperature. Quality indicators evaluation in sample stored at abusive temperatures was compared to the optimal storage temperature (0 °C). Quality changes of lettuce leaves were analyzed through nutritional indicators (ascorbic acid retention), microbiological (total microbial counts) and other physicochemical parameters such as soluble solids contents, water loss and weight loss. In the present work, only tap water immersion was used to eliminate superficial microflora; in this way, only temperature effects on microbial evolution was evaluated. In addition, Gompertz and Logistic models were applied to predict the vegetable microbiological shelf life.

Materials and methods

Sample preparation: Heads of Romaine lettuce (*Lactuca sativa*, type Cos, variety Logifolia) were harvested at optimal maturity when they reached a marketable size (approximately 20-24 leaves per head). They were immediately transported to the laboratory. To reduce natural variability among samples, lettuce leaves were sorted for integrity, color and size uniformity, and lack of defects. Outer leaves were discarded and only photosynthetic leaves (green leaves) were included in the samples. Lettuce leaves were dipped in tap water for 4 min at room temperature (20 °C) in a ratio of one part of lettuce for 10 parts of water. Lettuce leaves were then centrifuged for 30 sec at 200 rpm in a salad spinner to remove excess water. Leaves were piled up in 120 g stacks and placed in polyethylene bags (25 x 20 cm, useful volume: 1.8 l) with an O_2 permeability of 520-4000 cm^3m^{-2} day^{-1}, CO_2 permeability of 3900-10000 $cm^3m^{-2}day^{-1}$ and water vapor of 4-10 g $m^{-2}day^{-1}$. Samples were placed in boxes with overall dimensions of 0.4 x 0.3 x 0.3 m, made of heavy-duty, 0.60 cm thick, transparent acrylic, with 97-99% relative humidity, and stored at 0, 8 and 15°C for approximately 200 h. In samples stored at 15ºC ascorbic acid and microbiological determinations were stopped earlier because, from a sensorial point of view, they were deteriorated beyond any commercial value.

Physicochemical determinations: Samples were weighed to determine weight loss during storage. Moisture was determined by the weight loss of 10 g samples after 24 h at 80°C (Bastrash *et al.*, 1993). 30 g of lettuce leaf juice was obtained with a home juice maker and centrifuged at 2000 rpm for 5 min. Juice samples were diluted (1:1) with distilled water. Soluble solids were determined on the diluted samples with an Abbe refractometer (Kim *et al.*, 1993). At each sampling period, two samples from each experimental condition were taken for the analysis. Analyses were performed on each sample in duplicate.

Determination of ascorbic acid: Ascorbic acid contents were determined according to Moreira *et al.* (2003). Lettuce leaves were ground with a tissue homogenizer (Multiquick, MR 5550 CA, Braun) for 1 min. Twenty grams of lettuce was homogenized with 40 mL of oxalic acid solution 0.5 g / 100 (w/w). The mixture was filtered through Whatman # 42 filter paper. Aliquots (10 mL) of the filtrate were titrated with 2,6-dichloroindephenol. To prepare solutions, copper-free water was used. Since copper may be present in the lettuce samples, titration was performed rapidly to minimize interfering effects. Ascorbic acid contents are reported as mg / 100g sample on a wet basis. Ascorbic acid determination was performed on three different experimental runs at each storage temperature. For each experimental run two samples were taken and analysis was performed in triplicate.

Microbial population: Ground lettuce (10 g) was macerated in a buffer solution (PO_4K_3, pH = 7.2). Total microbial counts were done on plate count agar (PCA) after incubation for 48 h at 35°C (ICMSF, 1983, Mossel and Moreno García, 1985). Microbial counts in fresh lettuce leaves were performed on three independent experimental runs for each storage condition analyzed. At each sampling time, two sample lots were analyzed by duplicate.

Primary growth models application: Experimental values were fitted with the Gompertz model (Zwietering *et al.*, 1990) and the Logistic model (Ricker, 1979). The Gompertz model is:

$$\log N = A + C \cdot e^{(-e^{(-B\cdot(t-M))})}$$

Where, log N is the decimal logarithm of microorganisms counts at time t; A is the asymptotic value when time decreases indefinitely (approximately equivalent to the decimal logarithm of the initial microorganisms counts); C represents the increase in the logarithm of microorganisms counts when time increases indefinitely (number of log cycles); B is the maximum rate of growth related to time M and M is the time required to reach the maximum rate of growth. The logistic model is: (Ricker, 1979)

$$\log N = A + \frac{C}{1 + e^{(D - F*t)}}$$

Where, Log N, A and C has the same meaning as in the Gompertz model. D is a dimensionless parameter and F is the rate of growth relative to the mean time of the exponential phase.

Statistical analysis: Linear correlations significance was tested according to Volk (1980). Differences among slopes for the evolution of ascorbic acid at different storage temperatures were tested as indicated by Volk (1980). Whenever differences are reported to be significant, a 95% confidence level was used.

Estimation of the parameters in both Gompertz and the Logistic models was done with the function *lsqcurvefit* from Mathlab 7.0.

Results and discussion

Physicochemical parameters: Lettuce leaves weight losses during storage are presented in Fig. 1. The weight loss increased with the storage temperatures and, after 170 h of storage, they amounted to 0.6, 1.0 and 1.6 g / 100 g (w/w) in samples held at 0, 8 and 15°C, respectively. Weight loss of fresh vegetables can be attributed to: (1) evaporation of surface water remaining on the product after washing; (2) dehydration, that is water loss due to the difference in water vapor pressure between the surrounding atmosphere and the foodstuff; and (3) respiration, which consist in the breakdown of carbohydrates to produce carbon dioxide and water. Since the samples were kept in polyethylene bags with very low permeability to water vapor and with relative humidity

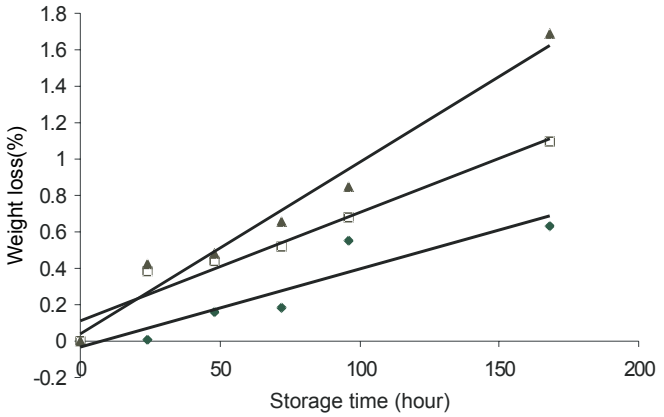

Fig. 1. Lettuce leaf weight loss, as percentage of fresh weight, during storage at 0ºC (♦) (y= 0.0043x – 0.0343, R= 0.94), 8ºC (□) (y= 0.0059x + 0.1156, R= 0.97) and 15ºC (▲) (y= 0.0094x + 0.0432, R= 0.98).

above 98%, weight loss would be due to respiration.

Initial water contents in lettuce leaves were 94.56 ± 0.53 g / 100 g (w/w). After 170 h of storage the water contents of samples stored at 0°C were 94.84 ± 0.21 g / 100 g (w/w). In samples stored at 8°C they were 95.14 ± 0.29 g / 100 g and in samples stored at 15°C they were 95.83 ± 0.6 g / 100 g (w/w). These increases in water contents would correspond to water produced by respiration. While carbon dioxide is lost to the atmosphere, water is retained by the food and weight loss is independent of moisture loss (Sastry and Buffington, 1982). Polyethylene bags provide high relative humidity, so dehydratation typically is not a problem. However, moisture may condense on the inner surface of the bag, detracting visual appearance and providing conditions for microbial growth.

Initial soluble solid contents in lettuce leaves were 3.9 ± 0.24°Brix. After 170 h of storage, the soluble solids contents of samples held at 0 and 8°C were not significantly different from the initial value (P<0.01). However, in samples stored at 15°C the final soluble solids contents were significantly lower (P<0.01) with a value of 3.06 ± 0.32°Brix. This decrease in soluble solids could be attributed to sugar consumption through respiration.

Ascorbic acid contents: The initial ascorbic acid contents of lettuce leaves ranged between 6.0 and 16.6 mg/100 g of sample with a mean value of 10.9 ± 2.9 mg / 100 g (n=40). Moreira *et al.* (2005) and Roura *et al.* (2003) reported similar initial mean values for ascorbic acid contents in lettuce leaves (8.3 ± 1.0 and 9.4 ± 1.6 mg / 100 g of sample, respectively). The wide range of values for the initial ascorbic acid contents in lettuce leaves would reflect the influence of various factors such as climate conditions, cultural practices, maturity at harvest, harvesting method and post-harvest handling conditions (Lee and Kader, 2000). Of course, the inherent variability in biological systems would also contribute to this variability.

First order kinetics is assumed for ascorbic acid degradation. Therefore, the natural logarithm of ascorbic acid contents ratio to the initial ascorbic acid contents against time should fall on a straight line with the slope representing the apparent reaction constant. Ascorbic acid contents in lettuce leaves kept at the different storage temperature are presented in this way in Fig. 2. The slopes of the straight tendency lines for samples stored at 8 and 15°C were not significantly different between themselves

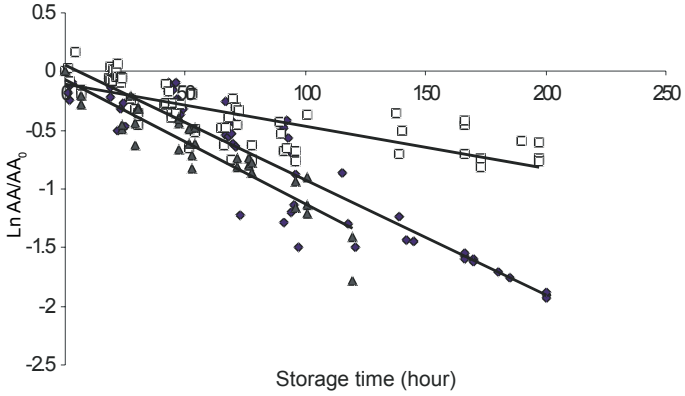

Fig. 2. Ascorbic acid degradation (ln AA/AA_0) in lettuce leaves stored at 0ºC (♦) (y= -0.0036x – 0.1084, R= 0.76, N= 70), 8ºC (□) (y= -0.0098x + 0.0538, R= 0.93, N= 56) and 15ºC (▲) (y= -0.0106x - 0.065, R= 0.92, N=32). AA is the ascorbic acid content (mg AA/100 g sample) at any time of storage and AA_0 (mg AA_0/100 g sample) at time zero. Ascorbic acid determination was performed on three different experimental runs at each storage temperature. For each experimental run two samples were taken and analysis were performed in triplicate.

but were significantly different (P<0.01) from the slope of the tendency line for samples stored at 0°C. These results indicate that ascorbic acid degradation rate in lettuce leaves stored at abusive temperatures was from 2.7 to 2.9 times faster than at 0°C. After 200 h of storage at 0°C ascorbic acid retention in lettuce leaves was about 46 g / 100 g. In samples kept at 8°C, the retention was about 14 g / 100 g. Samples held at 15°C were only kept for 100 h, a time at which they were completely spoiled. At that time, the ascorbic acid retention was already down to 22 g / 100 g. Tulio *et al.* (2002) analyzing the effects of storage temperature on the postharvest quality of jute leaves reported that the ascorbic acid content declined in all storage temperature conditions as storage period was increased. However, the reduction in the ascorbic acid contents was gradual at low storage temperatures but rapid at high storage temperatures.

Leafy vegetables lose ascorbic acid in postharvest operations and it has been attributed to both temperature and water loss (Kader, 1999). Changes in water contents in the samples kept in polyethylene bags were minimal and would not suffice to explain the losses in ascorbic acid. Ascorbic acid losses could be attributed to temperature effects and tissue structural changes due to biological deterioration factors (senescence and microbial activity).

Microbial growth: Initial total microbial counts were in the range of 4.0 to 4.4 x 10^6 CFU/ g. Similar results had been found in a previous work (Roura *et al.*, 2003).

Total microbial counts on lettuce leaves during storage are presented in Fig. 3 as log CFU/ g versus time. Experimental results are widely scattered. This may be because determinations were done on three different experimental runs on samples of an actual foodstuff, with the concurring variability associated to biological material.

The growth curve of total microbial counts fitted with the Gompertz and Logistic models including the r^2 values are shown in Fig. 3. Parameter A in both models was fixed at the log of the mean initial counts on lettuce leaves (6.82 CFU/ g). Both models produced very similar patterns for total microorganisms growth in samples stored at 0°C (Fig. 3A), with an incubation

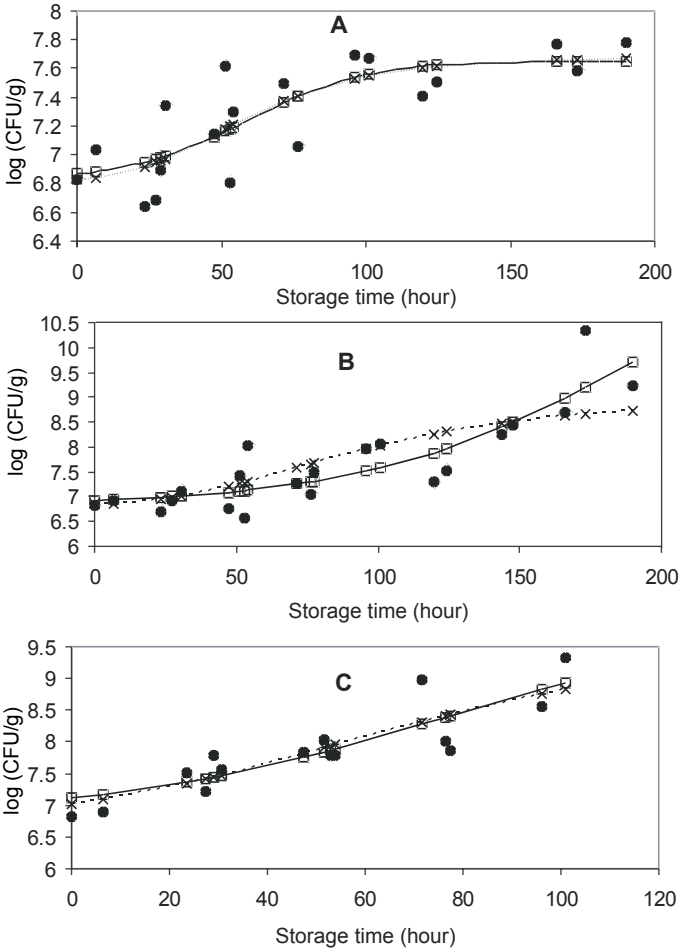

Fig. 3. Growth curve of total microbial during storage at 0ºC (**A**), 8ºC (**B**) and 15ºC (**C**) fitted with the Gompertz (--x--, R= 0.87) and Logistic models (-□-, R= 0.85).

phase lasting about 20-25 h, a logarithmic growth phase and a stabilization phase with logarithmic of microbial populations around 7.65 CFU/ g. For samples stored at 8°C, however, the best fit for the logistic model indicates that the growth of total bacteria is still in the exponential phase after 200 h of storage (Fig. 3B). On the other hand, the best fit for the Gompertz model, indicates that the stabilization phase would have been reached by 175 h of storage. The logarithm of microbial populations reached at 8ºC was around 8.7 CFU/ g, that is, about one log cycle above the stabilization value for samples stored at 0°C. Finally, for samples stored at 15°C, best fits for both models indicated that the total microbial growth was in the exponential phase after 100 h of storage (Fig. 3C). It should be pointed out that samples were stored at 15°C for only 100 h because, by that time, they had deteriorated beyond any commercial value.

French regulations imposed 8ºC as a maximum storage temperature for minimally processed vegetables. This limit was lowered to 4ºC, but vegetables are often stored or distributed at higher temperatures. This legislation allows a maximum of 5.0 x 10^7 CFU/ g for the safe consumption of minimally processed vegetables (Francis *et al.,* 1999). Accepting this value as a threshold that must not be surpassed, from a bacteriological point of view, the keeping time of fresh lettuce would be around 8 days for lettuce kept at 0°C; 5 days for lettuce kept at 8°C and 2 days for lettuce kept at 15°C.

The main aspects of this study were the establishment of vitamin C losses and microbial counts for Romaine lettuce leaves kept at two abusive temperatures and the comparative estimation of their shelf life with respect to the ideal storage temperature (0ºC). For ascorbic acid degradation and microbial evaluation, the profiles obtained were supported through a high number of experimental data (a mean of 52 data per fitted curve) during 100-200 hours after harvest, in order to be able to predict the quality parameters in a reliable way.

Exposure to undesirable temperatures resulted in faster ascorbic acid degradation rates detrimental to nutritional quality. The rate of degradation of ascorbic acid in lettuce leaves stored at abusive temperatures would be from 2.7 to 2.9 times faster than at 0°C.

If fruits or vegetables are handled at abusive temperatures, sanitizer agents applied to reduce initial microbial counts will do little to extend shelf life or limit microorganism growth. Mishandling of these products, exposing them to abusive temperatures, would make microbial levels go right back up to pre-wash levels or higher. In the present work, only tap water immersion was used to eliminate superficial microflora; in this way, only temperature effects on microbial counts were evaluated.

Model can be a powerful tool for microbial analysis, quickly providing an initial estimate of a microorganism's behavior. In this work, two primary models (Gompertz and Logistic), were compared for their ability to describe the total microbial counts as a function of storage temperatures applied to lettuce. These models allowed us to predict the vegetable microbiological shelf life. Temperature is the controlling factor for lettuce shelf life and quality; microbial quality was retained 1.6 and 4 times longer at 0ºC with respect to 8 and 15ºC, respectively.

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Shelf-life and quality of apple fruits in response to postharvest application of UV-C radiation

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Abstract

In this study, UV-C irradiation (1.435×10^{-4} w cm^{-2}) was used to maintain fruit quality of 'Red Delicious' and 'Golden Delicious' apple cultivars during storage. Apple fruits were irradiated in three different treatments (0, 5 and 15 min), and were stored in a cold storage at 1±1ºC with 85-95% RH for 6 months. At the end of storage, irradiated fruits for 15 min had lower pH and total soluble solids/titratable acids ratio and higher titratable acids and firmness than irradiated fruits for 5 min and control fruits. A significant difference was observed among total soluble solids of irradiated 'Red Delicious' fruits for 15 min, irradiated fruits for 5 min and control fruits at the end of storage. 'Red Delicious' apples had lower total soluble solids and total soluble solids/titratable acids ratio and higher firmness than 'Golden Delicious' apples after 6 months. Our results showed that UV-C irradiation can be used to reduce loss of fruit quality during long period storage of apples.

Key words: UV-C irradiation, apple, 'Golden Delicious', 'Red Delicious', fruit quality.

Introduction

Apple (*Malus domestica* Borkh.) fruits are commonly stored for long periods at low temperatures and under controlled atmosphere. During this period, usually quality and nutritional value of fruit decreases. A number of techniques like prestorage heat treatment (Klein and Lurie, 1992), treatment with chemical compounds (Leverentz *et al.*, 2003) and modified atmosphere (Hertog *et al.*, 2001) have been used. However, some chemical compounds may pose serious health hazard and environmental risks. Additionally, consumers prefer agricultural products without chemicals residues and, hence, alternative methods to control postharvest disease and to extend product shelf-life are required (Marquenie *et al.*, 2003).

UV-C is generally harmful but can produce beneficial effect on horticultural crops at low doses, a phenomenon known as hormesis (the stimulation of plant response by low levels of inhibitors or stress) (Lukey, 1982). UV-C radiation has been shown to be a promising postharvest treatment, because it delays the ripening process and does not affect quality during storage (Maharaj *et al.*, 1999).

Ripening of fruit involves complex physiological changes. The ripening process is generally accompanied by increased pigmentation, sugar, pH, ethylene and decreased firmness, starch and ascorbic acid (Wills *et al.*, 1981). Purohit *et al.* (2003) concluded that Ber (*Zizyphus mauritiana* Lamk.) fruits irradiated for 6 hours with UV-C radiation had lower TSS and TSS/TA ratio and higher TA than other treatments and control fruits. Maharaj *et al.* (1999) and Vicente *et al.* (2004, 2005) also reported increase in fruit firmness due to UV-C irradiation.

This experiment was conducted to examine the effect of UV-C treatment on shelf life and the physico-chemical changes in 'Golden Delicious' and 'Red Delicious' apples.

Materials and methods

'Golden Delicious' and 'Red Delicious' apple fruits were harvested manually on optimal date for commercial harvesting (150 days after full bloom for 'Red Delicious' and 170 days after full bloom for 'Golden Delicious' apples) (Naseri, 2004) from 15-years old standard trees from an orchard in Urmia, Iran, during 2004-2005 growing year. Fruits of uniform shape, size and free from fungal infection were selected.

UV-C radiation was provided by fluorescent germicidal lamp (30 w, 90 cm) with a peak emission at 254 nm. Irradiation was carried out under ambient condition for 0, 5 and 15 min. The intensity of radiation was 1.435×10^{-4} w cm^{-2}. Fruits were placed at approximately 25 cm from the lamp and rotated so that their blossom and stem ends faced the lamp to ensure uniform irradiation. After irradiation, fruits were stored at 1±1 ˚C and 85-95% RH for 6 months. After sampling from cold stored fruits, they were stored for 7 days at 25ºC.

Fruit physical and chemical parameters were measured periodically after treatment and 45 days storage at 1±1 ˚C plus 7 days at 25 ˚C in 12-apples samples per treatment (3 apples per replication) per cultivar. Total soluble solids (TSS) were determined with a hand-held refractometer. Titratable acids (TA) was determined by titration with 0.1 N NaOH and expressed as % malic acid (A.O.A.C, 1980). Fruit firmness was determined on opposite sides of the fruit after peel removal using a penetrometer with 8 mm diameter tip. pH was measured with pH meter. TSS/TA ratio was also calculated.

Completely randomized factorial design with four replications was used. Every replicate had 24 fruits. An analysis of variance was used to analyze difference between means. The Duncan's multiple range test was applied for mean separation at P=0.05.

Results and discussion

Quality measurements revealed that during storage, in irradiated fruits, pH, total soluble solids and TSS/TA ratio increased and firmness and titratable acids decreased slowly than control fruits (Fig. 1). After 6 months storage at 1±1 ˚C plus 7 days at 25 ˚C, irradiated fruits for 15 min had significantly low pH and TSS/TA ratio and high titratable acids and firmness than other treatment and control fruits. Total soluble solids were not affected by UV-C radiation (Table 1). In 'Red Delicious' apples, irradiated fruits for 5 and 15 min had low TSS than control fruits. But in 'Golden Delicious' apples, there weren't significant differences between treatments. Differences for pH between irradiated 'Red Delicious' and 'Golden Delicious' apples were non-significant (Fig. 2).

Table 1. Effect of UV-C radiation on firmness, titratable acids (TA), pH and TSS/TA ratio after 6 months at 1±1 ºC plus 7 days at 25 ºC

Irradiation time (min)	Firmness (N)	TA (%)	pH	TSS/ TA ratio
0 (control)	25.4[c]	0.10[c]	4.57[a]	156.3[a]
5	28.0[b]	0.15[b]	4.22[c]	98.2[b]
15	29.9[a]	0.17[a]	4.44[b]	86.5[c]

Similar letters in columns have no significant differences at $P \leq 0.05$.

After 6 months storage at 1±1 ˚C plus 7 days at 25 ˚C, 'Golden Delicious' fruits had high TSS and TSS/TA ratio and low firmness than 'Red Delicious' fruits. pH and TA were not affected by cultivar (Table 2).

Table 2. Effect of cultivar on firmness, total soluble solids (TSS) and TSS/TA ratio after 6 months at 1±1 ºC plus 7 days at 25 ºC

Cultivar	Firmness (N)	TSS (%)	pH
'Red Delicious'	31.85[a]	14.70[b]	107.9[b]
'Golden Delicious'	23.92[b]	15.08[a]	119.4[a]

Similar letters in columns have no significant differences at $P \leq 0.05$.

Like other plant development processes, fruit ripening is significantly influenced by growth regulators of which ethylene plays a key role, however, polyamines have also been implicated to control ripening (Pandey *et al.*, 2000; Shamaa and Alderson, 2005**)**. In climacteric fruits, burst of ethylene production is first detectable sign of ripening that occurs during ripening and precedes the respiratory climacteric. During ripening, changes in color and texture are under the control of ethylene whereas flavor development in fruit is not much influenced (Shamaa and Alderson, 2005**)**.

Polyamines are a group of nitrogen-containing compounds that accumulate in plants in response to environmental stress (Evans and Malmberg, 1989; Barka *et al.*, 2000). Polyamines and ethylene may compete for the intermediate S-adenosyl methionine (SAM) which produces the propylamine moiety for their biosyntheses (Pandey *et al.*, 2000; Shamaa and Alderson, 2005**)**. Maharaj *et al.* (1999) showed that optimal doses of UV-C produced higher levels of free and conjugated polyamines particularly putrescine, compared with the control in mature green tomato fruits. Level of putrescine seems to increase in plants subjected to stress and these includes UV irradiation treatments (Shamaa and Alderson, 2005**)**.

The increase in TSS may be related to the moisture loss and hydrolysis of polysaccharides. UV radiation checks the moisture loss, thereby, increasing TSS retardation (Lu *et al.*, 1993).

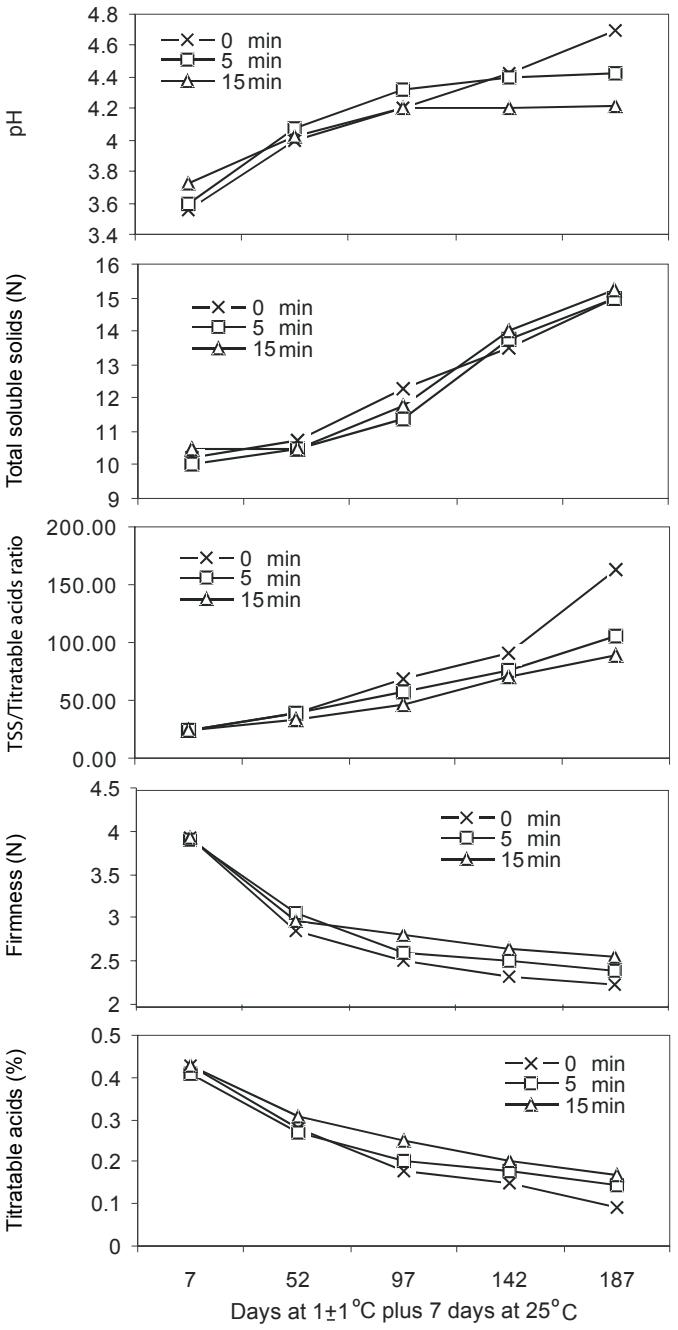

Fig. 1. Changes of pH, total soluble solids, firmness, titratable acids and TSS/TA ratio during storage at 1±1 ºC plus 7 days at 25 ºC.

Fig. 2. Interaction effect of UV-C radiation and cultivar on total soluble solids and pH after 6 months at 1±1 ºC plus 7 days at 25 ºC ($P \leq 0.05$).

Therefore, lower rate of increase in TSS of UV-C treated fruits could be attributed to reduced moisture losses and reduced hydrolysis of polysaccharides.

The rate of respiration and ethylene production reduces after UV-C treatment (Maharaj *et al.*, 1999; Vicente *et al.*, 2004). Maharaj *et al.* (1999) suggested that since UV radiation caused a shift in ethylene production rates, the climacteric respiration pattern was also shifted. The shift in ethylene production peak is associated with UV treated fruit suggests that SAM was probably a limiting factor in UV treated fruit. UV could have also caused irreversible membrane deterioration, which could have affected the activity of ACC oxidase (loosely bound to membrane), and thus explain the reduced ethylene production in such fruit (Maharaj *et al.*, 1999).

The higher TA in irradiated fruits could be due to lower respiration rate in the fruits and lower usage of these compounds in respiration process. The lower pH in irradiated fruits is related to high TA in these fruits. The low TSS/TA ratio resulted in higher retention of TA in irradiated fruits.

Maharaj *et al.* (1999) proposed that polyamines play role like conjugation of calcium to pectic acid and other polysaccharides and, therefore, maintain cell wall from hydrolytic enzymes. Additionally, Knee and Bartley (1981) proposed that loss in cohesion of cell walls may result from S-adenosyl methionine methylation of the free carboxylic groups in the pectin material and disruption of calcium cross linkage of adjacent polyuronides. Since SAM is probably routed to polyamines synthesis, it wasn't readily available for disruption of calcium bridges that could lead to increased firmness of the UV treated fruit (Maharaj *et al.*, 1999). Furthermore, Kramer *et al.* (1989) have shown that polyamines inhibit softening by reducing the activity of cell wall degrading enzymes such as polygalacturonase. Barka *et al.* (2000) reported that UV-C treatments could reduce the activity of cell wall degrading enzymes, *i.e.* polygalacturonase, pectin methyl esterase, xylanase, β-D-galactosidase and protease, and delay softening by affecting the cell wall disassembly rate. Reduction in polygalacturonase activity in UV-C treated fruits of tomato was reported by Stevens *et al.* (2004). These could explain the higher level of firmness found in UV-C treated apples.

Present investigation revealed that irradiated 'Red Delicious' fruits for 15 min had higher values of total soluble solids at the end of storage. 'Red Delicious' apples after 6 months had lower total soluble solids and total soluble solids/titratable acids ratio and higher firmness than 'Golden Delicious' apples. The results revealed that UV-C irradiation can be used to reduce losses of fruit quality during long storage period of apple.

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Effects of sugars and aminooxyacetic acid on the longevity of pollinated *Dendrobium* (Heang Beauty) flowers

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Abstract

The vase life of detached pollinated *Dendrobium* (Heang Beauty) orchids are affected by loss of energy source and the production of pollination-induced ethylene. The physiological changes that follow these two events are discoloration, thinning of petals and hyponasty. In order to circumvent this problem, individual detached pollinated *Dendrobium* (Heang Beauty) flowers were treated with solutions containing different concentrations of sucrose or glucose, Aminooxyacetic acid (AOA) and a combination of sugars and AOA. Discolouration, petal thickness and hyponasty were observed and data was recorded daily. Weight loss of flowers and pH of all solutions were also measured daily. Results showed that the best treatment solution in extending the longevity of the flowers were solutions containing 4% sucrose + 0.5mM AOA. Flowers held in this treatment also showed a delay in discoloration, thinning of petals and hyponasty. The inclusion of AOA into solutions resulted in low pH and contributes to better water uptake and delayed turgor loss in flowers.

Key words: *Dendrobium,* pollination, ethylene, AOA, flowers, discoloration, sucrose, glucose

Introduction

The cut flower industry is a US$ 2 billion industry and is an important revenue for some of the major cut flower producers in the world. Orchids are among the highly demanded cut flowers and are produced by countries like Thailand, the Netherlands, Hawaii, Singapore and Italy. However, like any other cut flower, orchids face vase life-related problems such as excessive water loss, decline in respirable substrates and sensitivity to exogenous or endogenous ethylene that hastens senescence and wilting of the flowers (Hew, 1994).

Vase-life is a yardstick for the longevity of cut flowers and is an important target for improving flower characteristics, whether by chemical treatment or plant breeding (Yamada *et al.,* 2002). The influence and effect of sugars and ethylene are two major areas which have been vastly studied to tackle the problem of flower quality and vase life of cut flowers.

Several studies have focused on the effect and mechanism of ethylene inhibitors. Compounds such as aminooxyacetic acid (AOA), aminoethoxyvinylglycine (AVG) and silver thiosulphate (STS) effectively delay senescence of climacteric flowers by inhibiting the synthesis or action of 1-aminocyclopropane-1 carboxylate synthase (ACCS) (Yang, 1984). Petal senescence is generally accelerated by treatment with exogenous ethylene and delayed by anionic STS, which prevents ethylene action (Beyer, 1976). For example, gladiolus flowers were reported to last longer (Hwang *et al.,* 1995) when they were treated with silver STS.

Furthermore, the use of sugars in holding solution of cut flowers has been extensively studied and has yielded a great amount of success in a variety of cultivars. Inclusion of sugars in the vase solution of carnation flowers reduced their sensitivity to ethylene (Mayak and Dilley, 1976). Sucrose, that extended vase-life of rose (Kuiper *et al.*, 1995) and sweet pea (Ichimura and Hiraya, 1999), also extended vase-life of gladiolus (Marousky, 1971; Bravdo *et al.*, 1974). From these observations, it is clear that sugars like sucrose have a negative effect on the process of cell death leading to petal senescence.

This study aims to further establish the effectiveness of AOA and sugars in prolonging the vase life of pollinated *Dendrobium* (Heang Beauty).

Materials and method

Plant material: *Dendrobium* (Heang Beauty) orchids were taken from the glasshouse in the University of Malaya. Flowers were cut at the peduncle, and were hand-pollinated by removing the pollinia from the anther to the stigma on the same flower using forceps. The cut flowers were held in glass vials containing 22ml of distilled water (control) or solutions containing chemicals. It was important for the peduncle of the flowers to be immersed in the solutions to enable efficient uptake of water and nutrient. The weight of the flowers were between 1.24-1.27g. All flowers were held at room temperature (26 ± 4°C).

Ethylene measurements: Ethylene production of detached unpollinated and pollinated *Dendrobium* flowers was measured using a Hewlett-Packard Gas Chromatography every 12 hours. Flowers were weighed as individual flowers were held in 30 mL glass centrifuge tube throughout the experiment. Tubes were sealed for 2 hours before readings were taken. After each reading, the seals were removed until the next time point.

Preparation of chemicals: AOA (Sigma) was tested at concentration of 0.25 and 0.5mM. Glucose (Sigma) and sucrose (Sigma) were used at 4%. All solutions were prepared at the

beginning of the experiments and were not renewed.

pH readings: The pH of the solutions was determined daily with a pH meter (HANNA Instruments).

Water uptake and fresh weight: The difference between consecutive weighing of the vial plus solution (without the flower) was used to calculate the water uptake. Evaporative water loss from the surface of the solution was negligible. The weight of each flower was determined daily by subtracting the weight of the vial and solution from weight of vial, solution and flower.

Measurement of thickness: Thickness of each flower was measured daily by using a micrometer (Mitutoyo Micrometer).

Flower wilting: Vase life of flowers was considered terminated when the flowers reached full closure. The experiment was run against a flower treated with distilled water as control.

Colour measurement: The colour of petals was measured daily using a Minolta CR-200 Chromameter using the L.a.b Munsell Colour System. L* indicated value, a* and b* measures hue and chroma. Colour change was calculated using L*, a* and b* values and the formula: L* x a* / b*

Results

In this experiment, ethylene production of detached pollinated *Dendrobium* flowers were measured against unpollinated *Dendrobium* flowers to confirm the climacteric response in the flowers. As shown in Fig. 1, pollinated flowers produced ethylene at 60 hours (1.25 µg $g^{-1}h^{-1}$), 84 hours (1.4 µg $g^{-1}h^{-1}$) and 108 hours (1.3 µg $g^{-1}h^{-1}$) after pollination . Thereafter the ethylene production decreased and remained at 0 µg $g^{-1}h^{-1}$. Unpollinated flowers (control) did not produce any ethylene throughout the experiment.

Pollinated *Dendrobium* held in distilled water (control), had the shortest vase life (3 days) compared to pollinated flowers in the other treatments. Flowers treated with 0.5mM AOA, 4% glucose with 0.5 mM AOA and 4% sucrose with 0.5 mM AOA proved to be the better treatments in extending longevity (Table 1). The combination of 4% sucrose and 0.5mM AOA was the best treatment because of its ability to extend vase life up to 13 days.

Table 1. Vase life of pollinated *Dendrobium* (Heang Beauty) held in different treatments

Treatment	Full closure (day)
Distilled water	6 ±0.3
Glucose 4%	8 ±0.5
Sucrose 4%	7 ±1.0
0.25mM AOA	7 ±0.5
0.5mM AOA	11 ±0.3
Sucrose 4% + 0.5mM AOA	13 ±0.5
Glucose 4% + 0.5mM AOA	11 ±0.0

Generally, all the treatments showed weight increment in the first 3 days and began to decrease thereafter. The increment may be due to the initial water uptake from the solutions to fulfil the nutrient requirement of the cut flowers. However, at day 3, the water uptake was no longer effective to increase the fresh weight of the flowers and the weight began to drop. The fresh weight became lower when the flowers started to close (result not shown). Control showed a drop in weight of flowers at day 2 and the rapid loss of weight continued throughout the experiment. On the other hand, flowers treated with 0.5mM AOA, sucrose 4% + 0.5mM AOA and glucose 4% + 0.5mM AOA managed to delay the onset of weight loss to day 4 and day 5 with a more stable weight loss throughout the experiment (Fig. 2).

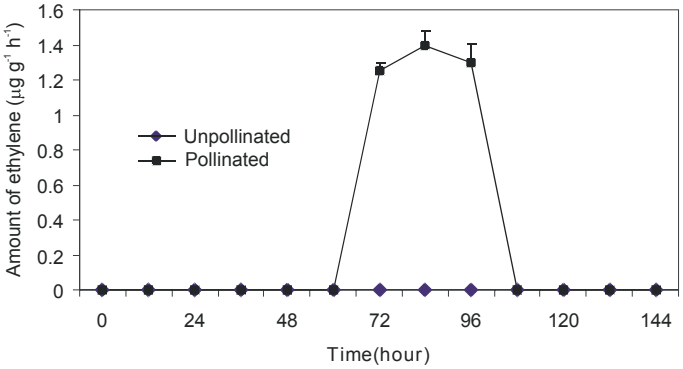

Fig. 1. Ethylene production of unpollinated and pollinated detached *Dendrobium* Heang Beauty.

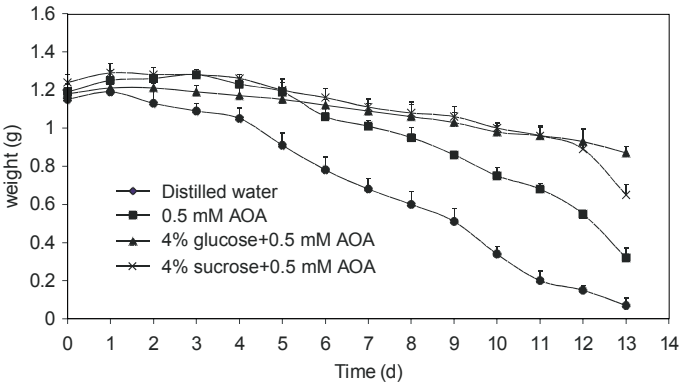

Fig. 2. Weight of detached pollinated *Dendrobium* Heang Beauty held in different treatment solutions.

Table 2 shows the total water uptake of *Dendrobium* flowers held in different treatments throughout the treatment. Flowers held in 4% sucrose +0.5mM AOA showed the highest amount of water uptake (4.46 mL), followed by flowers held in 0.5mM AOA (3.94 mL) and flowers held in 4% glucose+ 0.5mM AOA (3.83 mL). Total water uptake by the flowers held in the rest of the treatment solutions was not significantly different from the control.

Table 2. Total water uptake by pollinated *Dendrobium* (Heang Beauty) flowers held in different treatments

Treatments	Total water uptake (mL)
Distilled water	2.00
Glucose 4%	2.17
Sucrose 4%	2.19
0.25mMAOA	2.65
0.5mM AOA	3.94
Sucrose 4%+0.5mMAOA	4.46
Glucose 4%+0.5mMAOA	3.83

Fig. 3 shows the change in thickness of flower petals of pollinated *Dendrobium* throughout the experiment. In this observation, the positive results coincided with the longevity of flowers. This is evident as flowers treated with 0.5mM AOA, 4% glucose with 0.5 mM AOA and 4% sucrose with 0.5 mM AOA managed to show a delay in decrement of petal thickness which only occurred at day 6 (4% sucrose + 0.5mM AOA) and day 4 (0.5mM AOA, 4% glucose) compared to control which showed decrease in petal thickness on day 1.

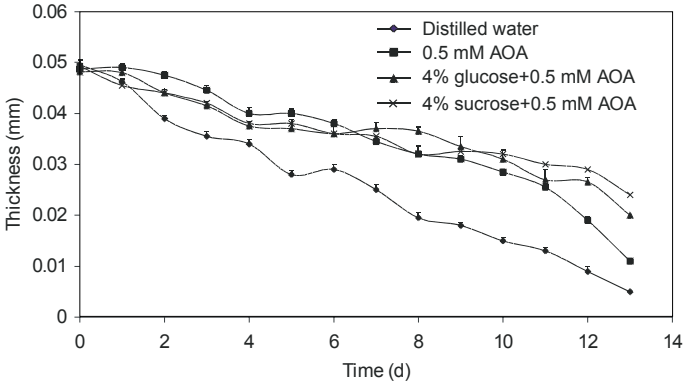

Fig. 3. Thickness of petals of pollinated *Dendrobium* (Heang Beauty) flowers held in different treatment solutions.

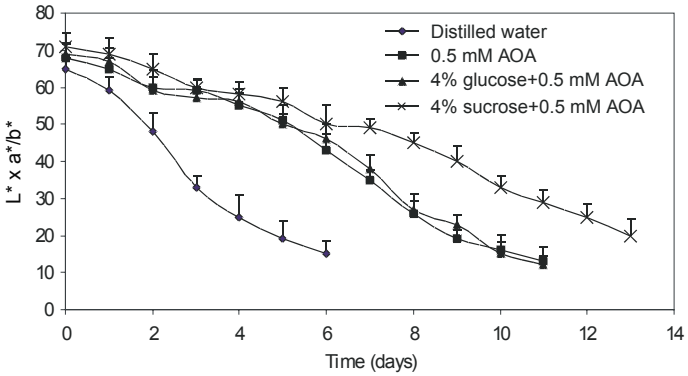

Fig. 4. Colour change of petals of pollinated *Dendrobium* (Heang Beauty) flowers held in different treatment solutions.

The decrease in L.a.b value is an indication of discoloration of petal, from white to eventually transparent. Flowers held in 0.5mM AOA, 4% glucose+0.5mM AOA and 4% sucrose+0.5mM AOA showed a lower rate of discolouration of petals compared to the control (Fig. 4).

Table 3 shows the change in pH values of the different treatments. It was found that the pH of solutions containing AOA (0.025 mM AOA, 0.5 mM AOA, sucrose 4% +0.5mM AOA and glucose + with 0.5mM AOA) were maintained around 3 throughout the experiment. Treatments with sugars however, showed higher pH values (5.0-6.0) throughout the experiment. The pH values of the solutions directly influence the water relations of the flowers.

Table 3. pH value of treatment solutions of at day 0 and day of termination of the experiment

Treatment	pH of solution at initial wilting	pH of solution at day of termination
Distilled Water	4.8±0.01	6.2±0.02
4% Glucose	4.2±0.02	5.3±0.01
4% Sucrose	4.4±0.02	5.8±0.02
0.25 mM AOA	3.6±0.03	3.8±0.03
0.5 mM AOA	3.1±0.01	3.5±0.01
4% Glucose + 0.5 mM AOA	3.3±0.04	3.4±0.04
4% Sucrose + 0.5 mM AOA	3.1±0.02	3.3±0.02

Discussion

It is a well known fact that the effect of ethylene on wilting can be inhibited by ethylene inhibitors. Woltering and van Doorn (1988), classified petal senescence of 93 species from 22 families into three types as follows:

(i) Type I, wilting apparently mediated by ethylene;

(ii) Type II, wilting apparently not mediated by ethylene;

(iii) Type III, abscission apparently mediated by ethylene.

In the cut flower species showing Types I and III of petal senescence, *i.e.* ethylene-sensitivity, vase-life could be improved by treatment with ethylene inhibitors or by genetic transformation with ethylene related genes (Reid and Wu, 1992; Chang *et al.,* 1993). The *Dendrobium* falls under the category of flowers where the senescence and abscission are mediated by ethylene. Furthermore this particular flower also shows pollination induced ethylene production which eventually acts as a signal for the flower to undergo senescence-like physiological changes.

Inhibitors of ethylene production and action have shown to slow down floral abscission (Sexton *et al.*, 1985). Aminooxyacetic acid (AOA) is an inhibitor of pyridoxal phosphate-requiring enzymes including 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, a key enzyme of ethylene synthesis (Abeles *et al.,* 1992). The results from this experiment further establishes the effectiveness of AOA as an ethylene inhibitor as the treatment with 0.5 mM AOA resulted in a significant extension of vase life compared to that of the control.

The post harvest life of flowers is strongly dependent on the carbohydrate status and the acceptable amounts of metabolic sugars are factors that affect the rate of senescence. Keeping flowers in vase solutions containing sucrose has been shown to extend their vase-life (Ho and Nichols, 1977). Supplying cut flowers with exogenous sugar maintain the pool of dry matter and respirable substrates, especially in petals, thus promoting respiration and extending longevity (Coorts, 1973; Rogers, 1973).

Aarts (1957) suggested that exogenous sucrose in some way maintains the structure and semi-permeability of the plasma membrane. It was demonstrated that sucrose antagonized the effect of abscisic acid in promoting the senescence of roses (Borochov *et al*., 1976). Furthermore, treatments of cut flowers with sucrose are found to be beneficial in delaying senescence processes (Yakimova *et al.,* 1996). Exogenous supply of sugars delays wilting in many flowers and this effect is due to maintenance in starch and sugar levels in cut flowers (Rattanawisalanon *et al*., 2003).

Previous studies have found that sucrose interact with other internal plant hormones in regulating the process of senescence. It was shown that sucrose enhances the effect of cytokinins in delaying senescence of flowers and reduces the effect of ethylene in promoting it (Mayak and Dilley, 1976). Sugar is found to be involved in enhancing the function of certain organelle such as mitochondria. Kaltaler and Steponkus (1976) found that in mitochondria isolated from cut flowers pretreated with sucrose, respiratory control values were maintained over longer periods. Hence, they concluded that the main effect of applied sugar in extending longevity is to maintain mitochondrial structure and functions. All of these findings show a close relationship with the results and effect of sugars observed in this experiment.

However, the main disadvantage of sugars in the vase solution is the promotion of bacterial growth when not accompanied by

an adequate antimicrobial agent; and it may therefore clog the xylem vessels and inhibit the uptake of both water and dissolved sugars (van Doorn, 1997). As is shown from the results, the effectiveness of sugar as a continuous energy supply for cut flowers was retarded due to the absence of an antimicrobial agent causing stem blockage. Hence, the turgidity and overall quality of the vase life was not significantly maintained throughout the experiment.

Rattanawasilanon *et al.* (2003) found that inclusion of AOA in the vase water, together with a sugar, had a positive effect on the time to flower senescence. In pollinated flowers, the effect of AOA on wilting may be due to a reduction of endogenous ethylene synthesis; however, such an effect is present only when AOA is combined with sugars (Rattanawasilanon *et al.,* 2003). Treatments with AOA or AOA + sucrose effectively retarded the longevity of cut spray-carnation flowers (Yakimova *et al.*, 1997). Our result is in agreement with both these findings as the combination of AOA and sucrose showed the longest vase life of pollinated *Dendrobium*.

AOA therefore, can act as an antibacterial agent, which inhibits bacterial growth (Rattanawasilanon *et al.,* 2003). The ability of AOA as an antimicrobial agent is attributed to the maintenance of low pH which results in a non-conducive environment for bacterial growth. Low pH seems to play the role of an antibacterial agent as bacterial growth is virtually halted at a pH of three or lower (Ketsa and Narkbua, 2001). Water uptake was also enhanced by acidic (pH 3 to 4) and warm water (43^{o}C) (Dole and Schnelle, 2002). Therefore, solutions with low pH throughout the experiment (0.5mM AOA. sucrose 4% + 0.5mM AOA) considerably delayed both abscission and petal wilting as water relations were improved and maintained.

In this experiment post pollination symptoms observed were discolouration of petals, change in petal thickness, weight loss and changes in pH values. Sucrose 4% with 0.5mM AOA was proved to be the best chemical treatment in delaying the petal senescence of *Dendrobium* (Heang Beauty). The treatment was able to maintain the highest water uptake by the flowers throughout the experiment. The lowest rate of thickness decrement of this treatment indicated their ability to slow down the pectin hydrolysis of petals throughout the experiment. It remains to be the centre of future studies to investigate the consequences of different ethylene inhibitors for their contribution in prolonging the vase life of cut flowers.

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Agronomic attributes of saffron yield at agroecosystems scale in Iran

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Abstract

In order to study effective factors in production of saffron, a series of studies was carried out during 2001 and 2002. In these studies, four selected location, Birjand, Qaen, Gonabad and Torbat-Haydarieh were spotted as the main saffron producing areas in Iran. Data was collected from 160 saffron farms, aged between 1 and 5 years. Results indicated that age of saffron farms, corm size, irrigation interval, and summer irrigation had positive linear relationship with yield. Age of saffron farms had the most pronounced effects on yield and was the most important component in all linear equations. Age of farms, irrigation intervals and corm size were major factors contributing to yield. The longest irrigation interval was observed for Gonabad (24 days) and the shortest was for Torbat-Haydarieh (12 days). Highest actual yield was for Torbat-Haydarieh which is an indication of better farm management in comparison with other areas. Maximum yield of 4 kg ha^{-1} was frequent but many farms produced over 7 kg ha^{-1} yield.

Key words: Saffron, irrigation interval, summer irrigation, corm size

Introduction

Saffron is an expensive spice and has been grown for a long time in many parts of the world including Spain, Italy, Greece and Iran. Today more than 95% of saffron in the world is produced in Iran and most of this production is from central and southern Khorasan (Kafi *et al.*, 2002). Saffron is an important cash crop for the small holding in Khorasan province and more than 85000 farmers are involved in its production (Kafi *et al.*, 2002). Saffron is a family based crop and most of farming practices particularly picking flowers are carried out by family members or community cooperation. Not only this crop is a cash crop for the farmers but also it has a strong tie with their social life.

Saffron is used locally and it has traditional medicinal uses (Koocheki, 2004). Recent reports on saffron as a cancer curing agent has brought more attention to this crop (Abdullaev, 2002). Saffron production does not require much water but timeliness of irrigation, particularly the first irrigation is very important for flower emergence and length of flowering period (Kafi *et al.*, 2002). Low temperature in autumn is a crucial factor for flower emergence (Molina *et al.*, 2004). There are many factors contributing to the yield of saffron. The most important factors are environmental conditions and farming practices such as age of farm, corm size, method of planting, irrigation application, irrigation interval and recent practice of summer irrigation which is not usual practice for saffron. The purpose of present study was quantitative evaluation of the magnitude of factors affecting crop yield by a comprehensive survey at farm level for two years.

Materials and methods

Saffron producing area of southern Khorasan, in which 95% saffron is produced, were investigated in four main counties namely Birjand, Qaen, Gonabad and Torbat–Haydarieh in two growing seasons 2001 and 2002. Data were collected from 160 farms with a very diverse criteria including the size (500 m^2 to 2 ha), age of saffron fields (1 to 5 year), farming practice (farmers skill) and farming background. A comprehensive survey was made during two years, associated with farming practices such as date of planting, time of first irrigation, time of first flowering period, the amount of manure used, irrigation frequency (including summer irrigation, if any), size of corm, planting method and yield, by personal reference to the farmers and direct monitoring. Climate data were collected from the nearest climate recording station. Statistical analyses were made on the relationship between yield and yield attributing factors and correlation coefficients were calculated accordingly.

Farming practices and phenological stages of plant particularly first time of flower appearance and length of flowering were correlated to the farming practices such as time of irrigation and corm size for the whole area. Analyses were made by Excel, SigmaStat and SPSS software.

Results and discussion

Date of planting: Table 1 indicates that the most frequent date of planting in the area was 1–10th of September, however in Torbat-haydarieh this practice was more frequent in 10-20th of August and in Gonabad 11- 20th of September. Date of planting varied with different geographical locations. In Spain corms are planted during mid May to early June (Behnia, 1991) and in Kashmir mid July to late August (Farooq and Koul, 1993)

Planting method: In general, two planting methods of hill and row are in practice (Table 2). In traditional systems, more tendency is towards hill planting where 1 to 15 corms are located in each hill (Mollafilabi, 2004). In general, 14 % of farms are planted through this method (Table 2). In row planting, corms are planted in rows which are 20cm apart from each other.

Age of farm: In Fig. 1, relationships between yield and age of

Table 1. Distribution frequency of planting date in different areas (%)

Counties	10-20 August	21-31 August	1-10 September	11-20 September	21-30 September	1-10 October	11-20 October
Brijand	12.5	20.0	25.0	10.0	10.0	10.0	12.5
Qaen	20.0	27.5	42.5	5.0	5.0	0	0
Gonabad	0.0	0.0	10.0	40.0	15.0	22.5	12.5
Torbat- haydarieh	37.5	22.5	22.5	7.5	5.0	0.0	0.0
Total area	17.5	17.5	25.0	15.6	8.7	8.1	6.2

Table 2. Frequency(%) and yield (kg ha^{-1}) of saffron under different planting methods in different areas

Counties		Planting method	
		Row	Hill
Brijand	Percentage	35.00	65.00
	Yield	2.70	4.06
Qaen	Percentage	27.50	72.50
	Yield	3.74	4.52
Gonabad	Percentage	30.00	70.00
	Yield	2.60	2.82
Torbat-Haydarieh	Percentage	12.50	87.50
	Yield	5.36	5.26
Total area	Percentage	26.25	73.76
	Yield	3.60	4.18

farms up to 5 years are presented. Yield in the first year was low and the maximum yield was obtained in 5th years, but usually saffron farms are kept up to 10 years (Behnia, 1991; Kafi *et al.*, 2002; Negbi, 1999). With increasing age of farm from 1 to 5, yield was increased (Fig. 1). This trend is most pronounced for Torbat–Haydarieh followed by Birjand. Higher yield in Torbat–Haydarieh and Birjand is associated with more suitable farming practices. Age of farms in Iran, which at present are more than 8 years, has been recommended to be reduced to 4 or 5 years, because yield can be improved by shortening the average age of saffron farms from 8 -10 to 4 or 5 years.

Size of corm: The correlation between size of corm and yield is shown in Fig. 2. With increasing size of the corm, yield also increased, and there was a good correlation between these two variables. Big corms cause earlier and vigorous flower emergence and therefore higher yield is obtained. This type of corm produces bigger daughter corms for next seasonal growth (DeMasstro and Ruta, 1993; McGimpsey *et al.*, 1997). In Table 3 proportion of corms with different size is shown. As it is seen in Torbat–Haydarieh small size corm was not used and the proportion of small size corm was less than 5 % for other counties. Medium size corm 7 to 10 g was used more than other types in different counties. There are references (Sadeghi, 1998), indicating that corms with less than 7 g have a low flowering potential and corms with 9 g are the most frequent with optimum flowering potential and corms with 15 g (not frequently used), yield more flower and a saffron yield of 7 kg ha^{-1} in first year (Kafi *et al.*, 2002).

Table 3. Distribution (%) of corm size in different counties

Counties	Corm size (g)		
	8	10	12
Brigand	5.0	60.0	35.0
Qaen	5.0	50.0	45.0
Gonabad	2.5	72.5	25.0
Torbat-Haydarieh	0.0	62.5	37.5
Total area	4.2	61.2	35.6

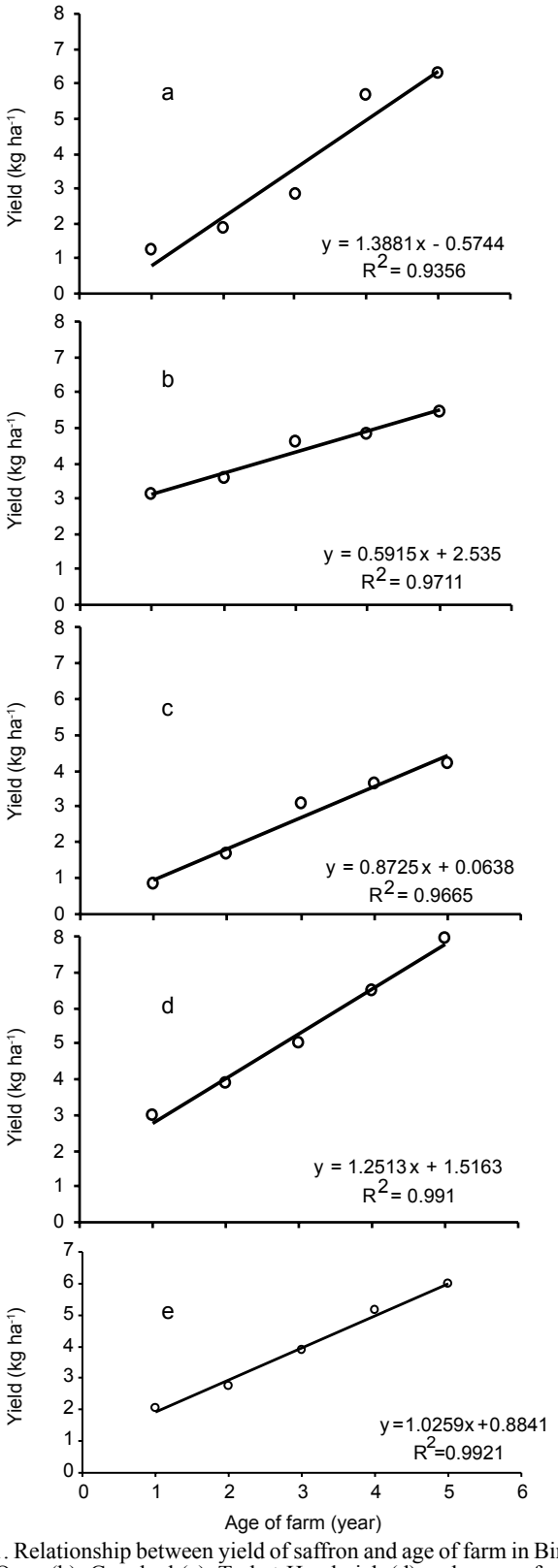

Fig 1. Relationship between yield of saffron and age of farm in Birjand (a), Qaen (b), Gonabad (c), Torbat-Heydarieh (d) and mean of whole area (e).

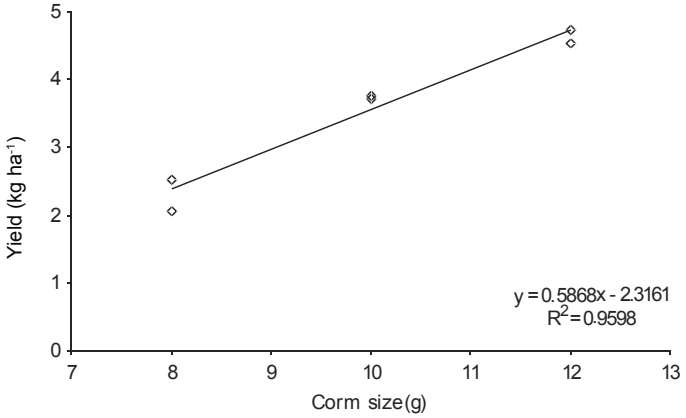

Fig. 2. Relationship between yield and corm size

Irrigation interval: There was a good correlation between irrigation intervals and yield of saffron (Fig. 3). By reducing irrigation interval, yield increased for different age groups and also for the average of all age groups. Higher yield obtained with lower irrigation interval has also been confirmed elsewhere (Mosaferi, 2001).

Three different irrigation intervals that were used for different counties are shown in Fig. 4. It is observed that irrigation with 24-days interval was most frequent in Birjand, Qaen and Gonabad and 12-days interval was most frequent in Torbat–Haydarieh, a reason for higher yield. There was no irrigation interval with 12 days in Gonabad.

Summer irrigation: Based on the physiological characteristics of saffron (growth start in early autumn with decreasing temperature of the area), first irrigation is required to stimulate flower emergence.

After flower emergence and harvesting, which normally last 30 days, vegetative growth starts and the leaves emerge. During winter days growth of leaves continue and by the end of May

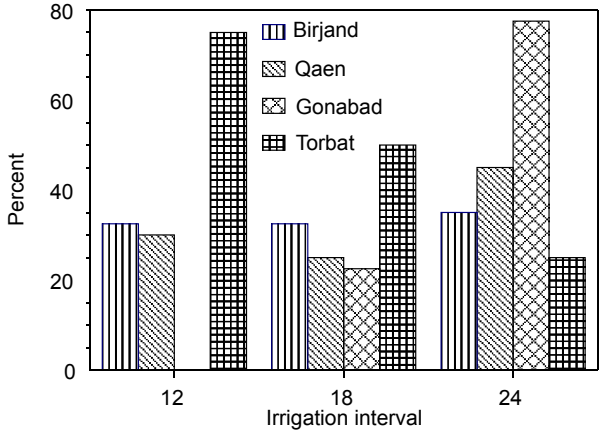

Fig 4. Percentage of farms with different irrigation intervals in different counties.

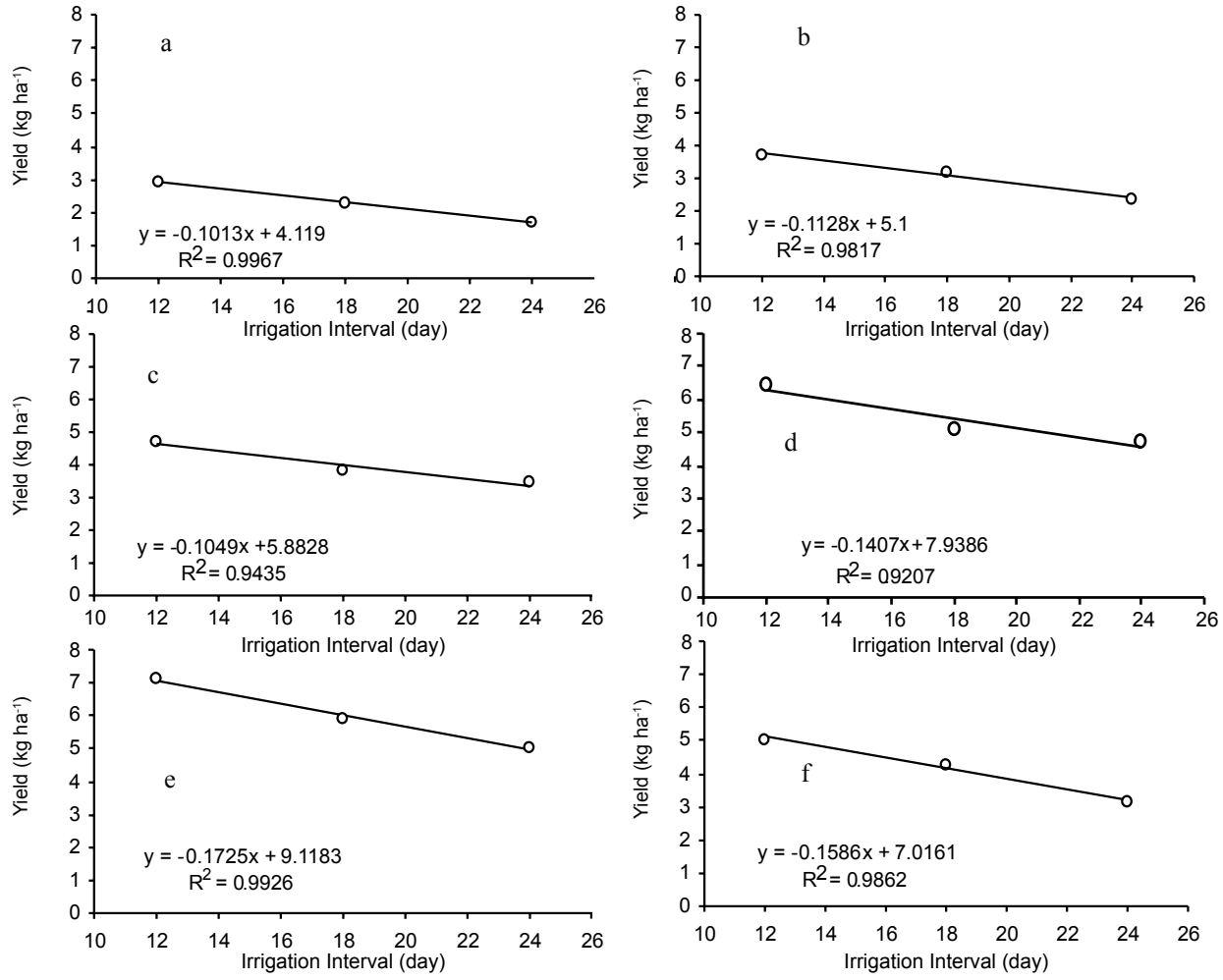

Fig 3. Relationship between irrigation intervals and yield of saffron in different aged farms. a, b, c, d, and e, represent farms with 1, 2, 3, 4, and 5 years old, respectively. f illustrate the mean of different aged farms in whole surveyed area.

leaves are dried and the plant goes under dormancy. Flower initiation start in early July (Farooq and Koul, 1983). Therefore, first irrigation is normally applied at the beginning of the fall without any summer irrigation applied. However, in recent years one summer irrigation is practiced between end of July to mid August. It is believed that application of one irrigation at the time of early flowering helps this process and results higher rate of flowering (Farooq and Koul, 1983; Sadeghi, 1993).

Investigation shows that 45% of the farms, in the area, received one summer irrigation (Table 4) except in Gonabad. Mean yield for the farms with summer irrigation was higher than those with no summer irrigation (4.9 and 3.35 kg/ha, respectively) and as a whole, nearly 60% of yield was obtained from the farms in which summer irrigation was practiced. There are references showing that one irrigation in mid Augest led to yield increase while one irrigation in mid July resulted in 17% reduction weight of flowers (Mosaferi, 2001).

Table 4. Frequency (%) and yield of saffron farms (kg ha^{-1}) with and without summer irrigation

County	Farm/yield characters	No summer irrigation	Summer irrigation is applied
Birjand	Farms (%)	17.00	23.00
	Frequency	42.50	57.50
	Yield	3.17	3.89
	% From total yield	44.87	55.12
Qaen	Farms (%)	25.00	15.00
	Frequency	62.50	37.50
	Yield	3.96	4.88
	% From total yield	44.50	55.50
Gonabad	Farms (%)	40	0
	Frequency	100	0
	Yield	0	0
	% From total yield	0	0
Torbat Haydarieh	Farms (%)	15.00	25.00
	Frequency	37.50	62.50
	Yield	4.32	5.83
	% From total yield	42.58	57.42
Total area	Farms (%)	97.00	63.00
	Frequency	60.62	39.37
	Yield	3.35	4.90
	% From total yield	40.64	59.36

In conclusion, farm practices related to irrigation interval, summer irrigation, age of farms and corm size are the main attributes of saffron yield. Increasing irrigation intervals decrease yield however, a summer irrigation at flower differentiation stage will led to yield increase. While saffron farms are used up to 10 years after establishment, the highest yield is obtained from 5 years old farms and yield will be decreased in the following years. Large corms also have promotive effect on flower emergence, yield and also on producing bigger daughter corms.

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Effect of bud scale removal and AOA on bud break and ACC content of 'Muscat Bailey A' grapevines

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Abstract

A study was carried out to examine the effect of bud scale removal (BSR) and aminooxy acetic acid (AOA) on bud break and 1-aminocyclopropane-1-carboxylic acid (ACC) content of 'Muscat Bailey A' grapevines using single-eye cuttings. Samples were collected monthly from October to February. Single-eye cuttings were subjected to these treatments; BSR, BSR + AOA, control and AOA. The results show that in October and November, BSR and BSR + AOA were more effective on bud break without big difference between the two treatments. Whereas, control and AOA were found to be more effective from December up to February. In October, ACC content recorded a marked increase after one week and decreased afterwards under BSR and BSR + AOA. However, it showed a continuous increase under control and a reverse trend under AOA. In November, it increased after one week and decreased in the fourth week under all treatments. A continuous increase was recorded in December under all treatments. In January, there was no significant change under control with time and AOA treatment exhibited decline with time, while BSR and BSR + AOA treatments recorded small increment and then decreased. In February, it decreased under all treatments with time. The results indicate that bud break of grapevine seems to be associated with the promotion of ethylene biosynthesis caused by wounding stress.

Key words: Aminooxy acetic acid, AOA, 1-aminocyclopropane-1-carboxylic acid, bud break, bud scale removal, dormancy, ethylene biosynthesis, grape.

Introduction

In temperate zone deciduous fruit trees, the endo-dormancy period is overcome by exposure to low temperatures (Samish, 1954; Saure, 1985; Kawamata, *et al.*, 2002; Kuroda *et al.*, 2002). The amount of chilling required for bud break is specific for species and cultivars. Dormant grapevine buds have chilling requirements. However, these requirements for grape are generally thought to be less than those of most deciduous fruit species. Chilling is not an absolute requirement for bud break, because high temperatures (Tohbe *et al.*, 1998a), bud scale removal (Iwasaki and Weaver, 1977; Iwasaki, 1980; Mizutani *et al.*, 1995) which implies wounding stress, and anaerobic conditions (Erez *et al.*, 1980) can replace the chilling requirements. Ethylene production increases following various disturbances or stresses in plants. These disturbances can be induced by abiotic or biological agents (Abeles *et al.,* 1992). Environmental stresses that induce ethylene production include physical wounding and cutting, chilling, drought, and water flooding (Yang and Oetiker, 1998). 1-Aminocyclopropane-1-carboxylic acid (ACC), a precursor for ethylene synthesis, increased during the transition from dormancy to the active state in *Prunus avium* L. and *Prunus serrulata* Lindl. (Wang *et al.*, 1985).

Aminooxy acetic acid (AOA) has aminooxy groups that are effective inhibitors of ethylene production via inhibiting ACC synthase (Abeles *et al.,* 1992). Tohbe *et al.* (1998b) reported that aminooxy acetic acid was found to inhibit bud break. In the present study we examined the effect of bud scale removal and AOA on bud break and ACC content of grapevine using single-eye cuttings to know whether bud break of grapevine is associated with the promotion of ethylene biosynthesis caused by wounding stress or not.

Materials and methods

Canes were collected monthly from mature 'Muscat Bailey A' grapevines (Bailey x Muscat Hamburg) grown at Ehime University Farm from 28 October, 2003 until 24 February, 2004. On each sampling date, single eye-cuttings were prepared from the collected canes and mounted through a sheet of styrene foam, which was floated on water in a plastic container and placed in a growth chamber under continuous white fluorescent light at 24°C.

These single eye-cuttings were subjected to the following treatments: 1) Control (buds without removing scales), 2) Aminooxy acetic acid (AOA), 3) bud scale removal (BSR), 4) BSR + AOA. Bud scale removal was done using forceps and the treatment of AOA was done using aqueous solution (10mM) of AOA by employing absorbent cotton. Each treatment was represented by 50 single-eye cutting.

The percentage of bud break was calculated every week. Bud break was indicated by the presence of green tissues beneath the bud scales.

ACC content was determined as follows: Buds (scaleless buds) were dissected from cuttings, weighed and extracted with 80% ethanol containing 0.05% (v/v) 2-mercaptoethanol. The extract was filtered and evaporated *in vacuo* to dryness. The residue was taken up in 4 mL distilled water and an aliquot of the solution was assayed for ACC content according to the method of Lizada and Yang (1979). ACC content was determined at the sampling

date (0 week), after 1 week and 4 weeks with an exception in January and February samples. It was determined at 0, 1 and 2 weeks because of rapid bud break in these two months.

Results and discussion

In October and November, BSR and BSR + AOA treatments recorded the highest percentage of bud break and bud break started earlier than those of control or AOA. From December until February the highest percentage was recorded under control and AOA treatments without much differences between both of them (Fig. 1).

The time required to achieve 50% bud break was shorter under BSR and BSR + AOA than control or AOA treatments in October and November (Fig. 2). However, the reverse was true from December to February.

These results are in agreement with those of Iwasaki and Weaver (1977), Iwasaki (1980) and Mizutani *et al.* (1985). They reported that bud scale removal was much more effective in stimulating bud break of grape cuttings than leaving them intact. Spires (1972) found that bud scale removal in tung trees greatly reduced bud dormancy at an early collection period. However, this effect decreased with time and completely disappeared two months after the onset of treatments. Also, Iwasaki (1980) found that bud scale removal was more effective in bud break than control from August to November. Whereas, in December they recorded the same bud break percentage and it was higher under control thereafter until February. In this study the same tendency was observed as in Spires (1972) and Iwasaki (1980) results.

Bud scale provide not only a protective covering for the bud, but also act as a buffer against resumption of growth, where it was found that inhibitive substances exist in the scales and its levels increased gradually, and then dropped until bud break (Iwasaki and Weaver, 1977). Therefore, the effect of bud scale

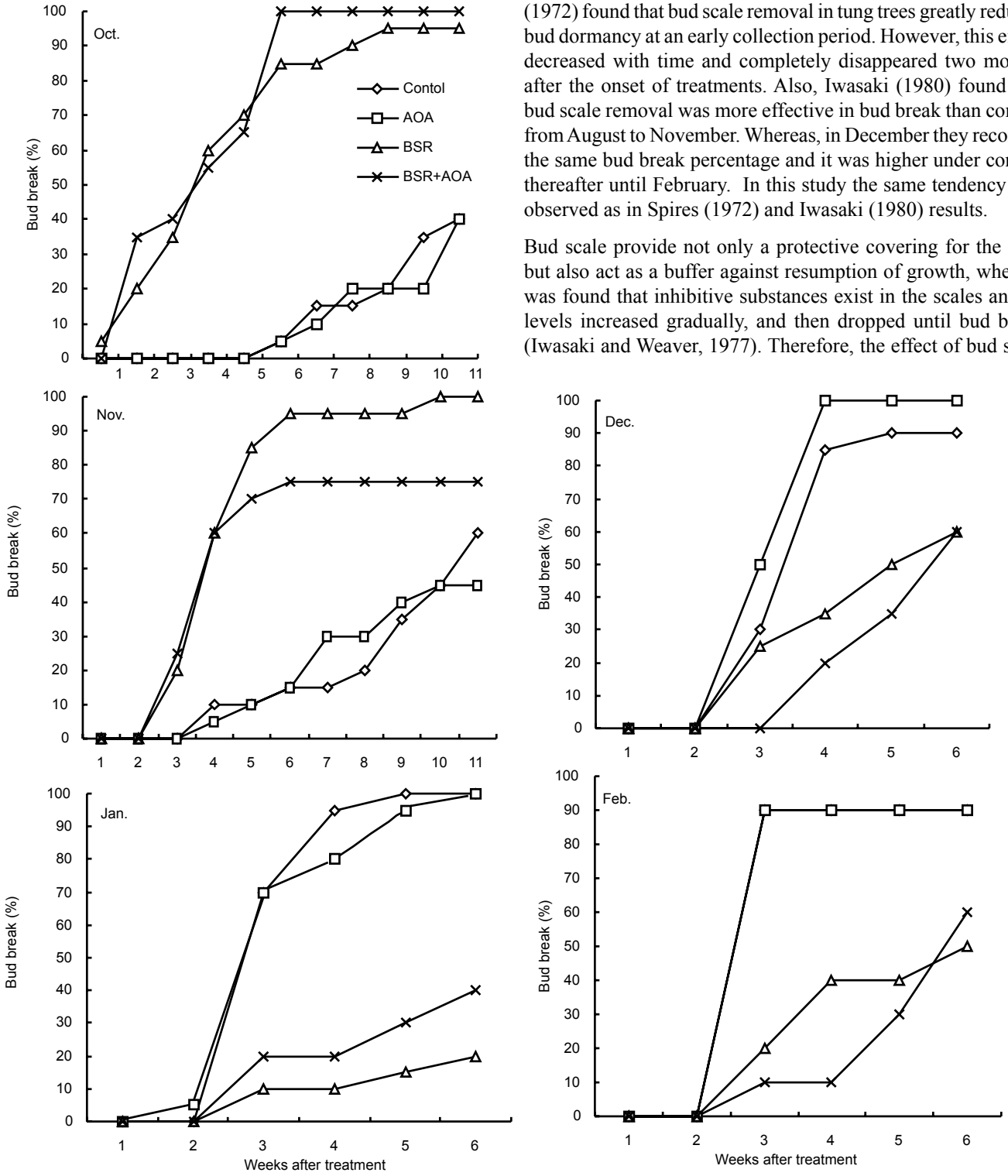

Fig. 1. Effect of bud scale removal (BSR) and AOA on bud break percentage of grapevine buds

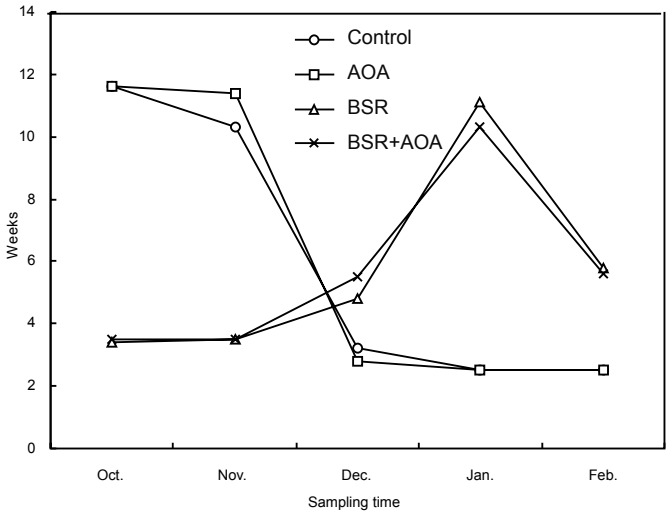

Fig. 2. The time required for 50% bud break of grapevine buds as affected by bud scale removal (BSR) and AOA treatments.

removal seemed to be mostly due to mechanical injury, greater ease of gas exchange and removal of inhibitory factors involved in the scales.

Fig. 3 indicate that the effect of the treatments on ACC content was dependent on sampling date or the intensity of bud dormancy. ACC content recorded a marked increase in October after one week and decreased afterwards under BSR and BSR + AOA. However, it showed a continuous increase under control and a reverse trend under AOA. Whereas, in November it increased after one week and decreased in the forth week under all treatments. A continuous increase was recorded in December under all treatments and there was no significant difference between control and BSR + AOA and between AOA and BSR especially after one week. In January, there was no significant change under control with time and AOA treatment exhibited decline with time, while BSR and BSR + AOA treatments recorded small increment after one week and then decreased to be lower than control in the second week. In February, the general trend was the decrease under all treatments with time.

The effect of bud scale removal on increasing ACC content is supported by Mizutani *et al.* (1995). They found that a sharp increase in the ACC content in the scale removed buds and the high ACC levels in treated buds were maintained until day 20 but the content decreased to the control level at day 30. Also they stated that bud scale removal implies wounding stress. Wounding, in general, is known to produce ethylene in plant tissues, whereas Iwasaki (1980) reported that it is difficult to assume that the termination of bud rest is hastened by ethylene released by bud scale removal. May be the bud break was enhanced because of HCN which is a co-product of the conversion of ACC to ethylene.

These results indicate that bud scale removal which implies wounding stress is more effective within the paradormancy and endodormancy stage and less effective thereafter. AOA inhibits ethylene biosynthesis effectively when it is combined with bud scale removal than with intact buds. Also, it indicate that bud break of grapevine seems to be associated with the promotion of ethylene biosynthesis caused by wounding stress.

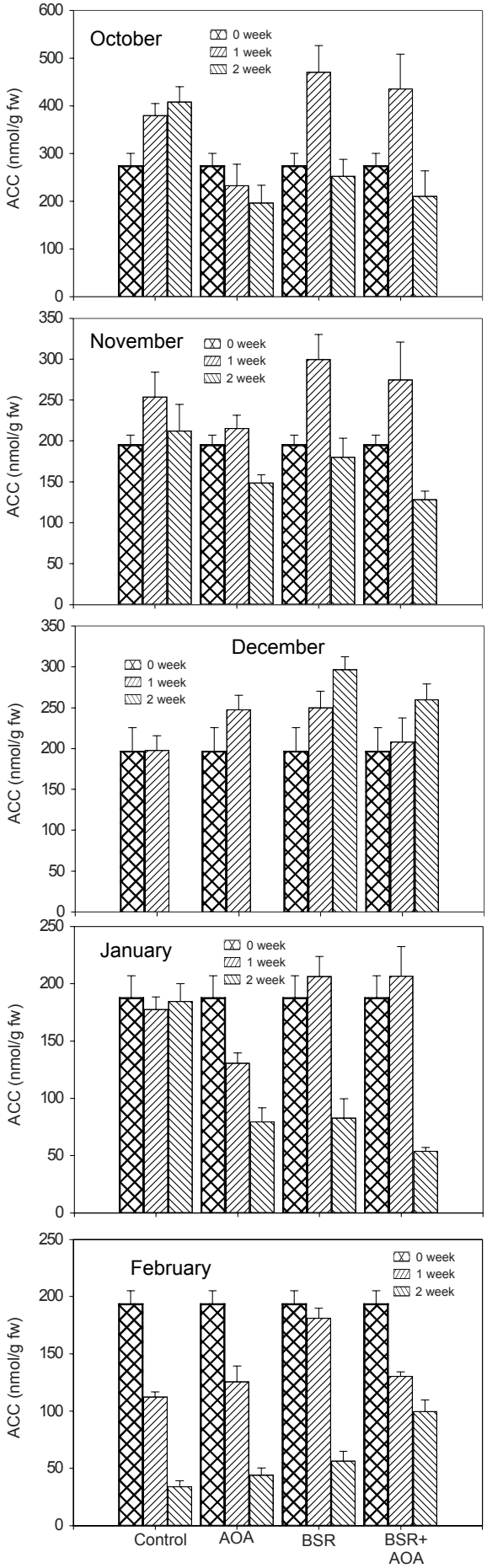

Fig. 3. Effect of bud scale removal (BSR) and AOA on ACC content of grapevine buds. Vertical bars indicate SE.

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Effect of exogenous application of anti-stress substances and elemental sulphur on growth and stress tolerance of tissue culture derived plantlets of date palm (*Phoenix dactylifera* L.) cv. 'Khalas' during acclimatization

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Abstract

There is a high demand for date palm plantlets regenerated via tissue culture techniques. However, such plantlets require a long acclimatization period extending 12-18 months before transplanting in the open field. The effect of foliar and soil application of anti-stress substances and elemental sulphur, respectively, on growth and survival percentage of tissue culture-derived 'Khalas' date palm plantlets during acclimatization were studied. The results showed that application of salicylic acid, acetyl salicylic acid (aspirin), elemental sulphur, plantacur-E (a vitamin E formulation containing 25% □-tocopherol) at 1%, and oleic acid at 100 ppm, significantly increased plantlet survival percentages compared to the control. In this respect, gamma aminobutyric acid (GABA) at 20 mM was the most effective treatment compared to 10 mM and the control. Salicylic acid, aspirin, elemental sulphur and plantacur-E (at 2%) significantly increased the concentrations of Fe, Mn, Zn, and Cu in leaflets compared to the control. However, the macro nutrients showed no clear response to the applied treatments. Application of 250 ppm of the ethylene biosynthesis blocker, ABG-3168 (ABG), inhibited the growth of plantlets, and completely suppressed growth at 500 ppm, suggesting the potential role of ethylene biosynthesis in subsequent plantlet development. Irrigation with 10,000 ppm sea water for two months decreased chlorophyll concentration and increased electrolyte leakage by 2-3 fold compared to the control and the other treatments. GABA at 20 mM significantly increased chlorophyll concentration and decreased electrolyte leakage of leaflets compared to all the saline water treatments. In contrast, ABG at 250 ppm significantly decreased chlorophyll concentration and increased electrolyte leakage of leaflets by about 3-fold compared to all the saline water treatments. These results show potential role of GABA, salicylic acid, aspirin and oleic acid conducive for improved survival percentage of plantlets and stress tolerance during acclimatization.

Key words: Tissue culture, acclimatization, elemental sulphur, gamma aminobutyric acid, salicylic acid, aspirin, vitamin E, oleic acid, ABG-3168, *Phoenix dactylifera* L.

Introduction

The number of naturally produced offshoots by fruiting palms are not sufficient to meet the high demand for the new plantations. Thus, there is a special interest in date palm plants regenerated via tissue culture since this technique can provide a large number of homogenous plants that are true to type and free of diseases and can be produced in large scale (Zaid and de Wet, 1999). Generally, these plantlets require an acclimatization period of about 12-18 months before transplanting in open field conditions. The VP1-stage (*in vitro* plant in a stage-1) is the most sensitive and critical stage due to the stress caused by moving the plantlets from the controlled laboratory conditions and transplanting in pots under greenhouse conditions for 2 to 4 months. During this period, the survival rate of plantlets is low which might, in the long run, lead to economic losses (Zaid and de Wet, 1999; Farag *et al.*, 2002).

Anti-stress substances may enhance the plantlets tolerance to environmental stresses thus increasing the survival rate (Sreenivasulu *et al.*, 2000). Salicylic acid has been found, among many other functions, to control ion uptake by roots, stomata conductivity and to increase the antioxidant capacity of plants (Raskin, 1992). Alpha tocopherol (vitamin E) and ascorbic acid (vitamin C) are antioxidant substances concentrated in the chloroplast and protect the photosynthetic apparatus when a plant is subjected to stress, by scavenging the excessively reactive oxygen species known as free radicals (Fryer, 1992; Kranner *et al.*, 2002). Environmental stresses *e.g.* heat, drought, salt and mechanical stress increase GABA accumulation in plant tissues. The mechanical damage in soybean leaves increased GABA levels by 10 to 25-folds within 1 to 4 min of the start of the stimulus (Kathiresan *et al.*, 1997). Also, it has been reported that the kinetics of GABA accumulation in plants reveals a stress-specific pattern of accumulation that is consistent with a physiological role for GABA in stress mitigation (Kinnersley and Turano, 2000). Increase in free unsaturated fatty acids, *e.g.* oleic acid, due to hydrolysis of membrane phospholipids, often occur during plant senescence and under adverse conditions including wounding, freezing, drought, salt, and pathogen elicitation. The oleate-stimulated phospholipase-D and phosphatidic acid decreased H_2O_2-induced cell death in arabidopsis (Wang and Wang, 2001; Zhang *et al.*, 2003). Ethylene has a significant effect on plant development and influences the stages from

seed germination to organ senescence. However, because of the diversity of ethylene action, it was difficult to assign a definitive role for it in growth responses (Naqvi, 1994). Generally, salinity and drought stress increase ethylene level in vegetative plant tissues (Nilsen and Orcutt, 1996). Elemental sulphur is strongly involved in improving nutrient assimilation and in stimulating the anti-oxidative defense system of plants through its metabolite glutathione (Gondent and Ullman, 2000; Tausz *et al.*, 2000). Also, the acidity produced during elemental sulphur oxidation increases the availability of nutrients such as P, Mn, Ca, and SO_4 in soils which may enhance growth performance of plants (Marschner, 1995).

The aim of the present study was to investigate the effect of exogenous application of anti-stress substances and elemental sulphur on survival percentage, nutrient uptake and salinity tolerance of tissue culture derived date palm plantlets cv. 'Khalas' during the acclimatization.

Materials and methods

Plant materials and experimental procedure: This experiment was conducted during the period from 2004 to 2005 on tissue culture-derived plantlets of the commercially important 'Khalas' date palm cultivar at the Date Palm Research and Development Unit (DPRDU), and the Horticulture Laboratory, Department of Aridland Agriculture, College of Food and Agriculture, UAE University, Al-Ain, UAE. Healthy and uniform tissue culture-derived plantlets were transplanted in paper pots (9 x 8 cm), maintained in greenhouse under controlled temperature (28°C and 80-90% relative humidity) and received the normal acclimatization program (irrigation and fertilization, pest and disease control) developed by DPRDU. The plantlets were subjected to one of the following treatments with elemental sulphur alone as soil application and the other treatments as foliar spray. A complete randomized design with 3 replicates (15 plantlets each) per treatment was adopted (Steel and Torrie, 1980). Elemental sulphur was applied as fine particles in pots at three different levels of 0.0283, 0.1415, or 0.283g/pot (these rates correspond to 1, 5 and 10 ton/ha, respectively). Salicylic acid and acetyl salicylic acid were applied at 0.5 mM or 1.0 mM. Plantacur-E was applied at 1.0 or 2.0%. Oleic acid was applied at 100 ppm. GABA was applied at 10 mM or 20 mM, only in the second experiment (GABA was not available at the time of conducting experiment 1). ABG was applied at 250 ppm or 500 ppm. Elemental sulphur was applied once at transplanting, whereas, the other chemicals were applied twice, at 2-days and at 2-weeks from transplanting. All treatments, except elemental sulphur, were combined with 0.1% Tween-20 (polyoxyethylene sorbitan monolaurate) as a wetting agent. In control, plantlets were sprayed with only water plus 0.1% Tween-20. This experiment was repeated to confirm the obtained results.

During the VP1-stage of acclimatization (*in vitro* plantlets under acclimatization procedure extending 0-4 months at 28°C according to the definition provided by the DPRDU), the number of survived, well rooted plantlets and suitable to move to VP2-stage were recorded at 2 and 4 months from transplanting. The survived plantlets were transplanted in plastic pots (25 x 16 cm). At the end of the VP2-stage (*in vitro* plantlets under acclimatization procedure extending 4-12 months at 30°C), all the plantlets in all treatments survived. These plantlets were transplanted in larger plastic pots (40 x 24 cm). During the VP3-stage (*in vitro* plantlets under the acclimatization procedure extending 12-15 months at about 35°C), leaflets at middle age were excised from plantlets and transferred to the horticulture laboratory for nutrient analysis.

During the VP3 stage, the plants of each treatment were irrigated with sea water (diluted to 10,000 ppm) (800ml/pot/week) for two months. The control treatment was subdivided into two equal groups, one group irrigated with diluted sea water and the other group irrigated with normal water. At the end of the two months, leaflets at middle age were excised from the plantlets and immediately transferred to the horticulture laboratory for chlorophyll and electrolyte leakage measurements.

Nutrient analysis of the leaflets: During the VP3-stage, before the start of sea water irrigation, random leaflet samples from only experiment 1 were collected from each replicate of each treatment for nutrient analysis. The collected leaflets were washed with deionized water and oven dried for 48 h at 65°C. The samples were crushed and passed through 20-mesh stainless steel sieve. Samples were digested by the dry ashing method as described by Jones and Case (1990). Total content of micronutrients (Fe, Mn and Zn) were determined by the atomic absorption spectrophotometer Varian, model SpectrAA 220 FS. Sulphur content was measured using ICPAES, Varain model Vista MPX. Phosphorus was determined colorimetrically according to the method described by Kuo (1996). Total nitrogen concentration in leaves was determined, after the wet digestion according to Jones and Case (1990), by steam distillation using the semi automatic Kjeldahl method.

Total chlorophyll measurement: Total chlorophyll was measured in leaflets by the procedure of Hiscox and Israelstam (1979). Two hundred milligrams of leaf tissue in fractions was placed in a vial containing 14 mL dimethyl sulphoxide (DMSO) and chlorophyll was extracted into the fluid without grinding at 65°C by incubating for 8 hours. The extract was then transferred to a graduate tube and made up to a total volume of 20 mL with DMSO. The OD values at 645 and 663 nm were read in Beckman DBG spectrophotometer against a DMSO blank.

Electrolyte leakage measurement: During the VP3-stage two leaflets of middle age, each from different plantlet, were collected for each replicate/treatment. The collected leaflets were directly washed with tap water and rinsed in deionized water to remove the dust and electrolytes adhering to the surfaces and then lightly cleaned with tissue papers. Leaf segments of 3 x 3 cm were cut from the middle of each leaflet (pinna), cut into 2-pieces, placed in each test tube containing 40 mL of deionized water then loosely covered with aluminum foil. Three replicates, each with two leaflets were used for each treatment. The tubes were then incubated in the refrigerator at 6°C over night before electrical conductivity of each solution was determined. In the next day, the tubes were taken from the refrigerator, warmed up to room temperature (22°C±2), placed in a shaker (Gesllschaft FurLabortechnik, GFL, mbh-model 3015, Germany) for one hour to diffuse electrolytes, vortexed for a few seconds. Electrolyte leakage before killing was measured with digital electrical conductivity meter (Orion- model 150- USA). The leaflet

samples were killed by autoclaving (JKA-J.39 Autoclave, Japan) at 121°C for 20 min to release all electrolytes, cooled to 22±2°C after which they were left on the shaker for 1 hour, vortexed for a few seconds. Total electrolyte leakage was measured by using the same digital conductivity meter. Percentage of electrolyte leakage was calculated for each sample using the ratio of the initial (before killing) to the final (after killing) measurements (Ingram and Buchanan, 1984; Jiang and Huang, 2001; Farag *et al.*, 2002).

Statistical analysis: The data were subjected to analysis of variance (ANOVA) using the statistical package MSTATC Program (Michigan State University, East Lansing, MI). Comparisons between means were made by least significant differences (LSD) at 5% level.

Results

Percentage of successful plantlets at VP1-stage: Most of the applied substances positively influenced 'Khalas' plantlets growth during the VP1-stage of the acclimatization period (estimated as the percentage of successful plantlets that were ready to move to the next stage of the acclimatization program or VP2-stage) (Table 1). In experiment 1, elemental sulphur application, especially at the low and the moderate levels, increased plantlets growth after 2 months during the VP1-stage. Aspirin and salicylic acid were the most effective treatments, especially at 0.5 mM, in increasing growth and development of plantlets both after 2 and 4 months. However there was no significant difference between the high and the low concentration of aspirin and salicylic acid on the survival percentage of plantlets. The ABG treatment at 250 ppm significantly inhibited subsequent plantlet growth and completely prevented plantlet development at 500 ppm. Similarly, plantacur-E and oleic acid also had no significant effect on the survival percentage of plantlets both after 2 and 4 months (Table 1). In experiment 2, a similar trend was observed, however, the positive effect of sulphur, aspirin and salicylic acid was less pronounced on the survival percentage of plantlets than in experiment 1. In contrast to the results of experiment 1, plantacur-E (especially at 1%) and oleic acid at 100 ppm application had significant positive effects on the survival percentage of plantlets both after 2 and 4 months. Interestingly, GABA at both concentrations (10 and 20 mM) showed a clear positive effect on the survival percentage of plantlets. In this respect, GABA at the high concentration of 20 mM was more effective (91% survived plantlets) than at the low concentration of 10 mM (73% survived plantlets) (Table 1). At the end of the VP2-stage all plantlets in all the treatments survived.

Table 1. Successful plantlet percentage of 'Khalas' date palm during the VP1-stage of the acclimatization period as affected by elemental sulphur and anti-stress substances

Treatments	Successful plantlet (%) during the VP1-stage			
	Experiment 1		Experiment 2	
	After 2 months	After 4 months	After 2 months	After 4 months
Control	47.0	64.4	44.4	62.2
Sluphur at 0.0283g sulphur/pot	62.2	71.1	44.4	65.5
Sulphur at 0.1415g sulphur/pot	64.4	74.6	55.6	62.2
Sulphur at 0.283g sulphur/pot	57.8	73.3	55.6	67.7
Aspirin at 0.5 mM	60.0	80	48.9	68.9
Aspirin at 1.0 mM	73.3	77.8	42.2	67.7
Salcylic acid at 0.5 mM	66.7	84.4	57.8	66.7
Salcylic acid at 1.0 mM	64.4	80	51.1	77.8
Plantacur-E at 1%	42.2	57.8	73.3	75.5
Plantacur-E at 2%	37.8	53.3	64.4	68.9
ABG at 250 ppm	14.4	35.5	18.7	32
ABG at 500 ppm	0.0	0.0	0.0	0.0
Oleic acid 100 ppm	48.9	62.2	75.5	86.7
GABA 10 mM	-	-	62.2	73.3
GABA 20 mM	-	-	82.2	91.1
F-test	***	***	***	***
LSD (P=0.05)	10.8	13.1	10.9	9.6

*** significant at level $P = 0.001$; - not calculated.

Nutrient concentration of leaflets at the VP3 stage: Salicylic acid and its derivative acetyl salicylic acid (aspirin) application, especially at the lower concentration, significantly increased the concentrations of Fe, Mn, Zn, and Cu in the leaflets compared to the control (Table 2). Also, elemental sulphur application significantly increased the concentration of Fe, Mn, Zn and Cu. However, the concentration of Cu was significantly decreased at the highest level of sulphur application compared to the control. Plantacur-E application, only at the higher concentration, significantly increased the concentration of Fe, Mn, Zn, and Cu in the leaflets compared to the control (Table 2). Oleic acid application significantly increased the concentration of N, Fe and Mn but the other nutrients were not affected. The untreated (control) plantlets contained significantly higher concentration of sulphur than all other treatments. However the concentrations of N, P and K were only slightly affected by the applied treatments (Table 2).

Chlorophyll concentration and electrolyte leakage of leaflets after 2-months of irrigation with sea water: After two months irrigation with sea water (diluted to 10,000 ppm salinity) all plants survived in all treatments including the control. However, the irrigation with sea water significantly decreased the concentration of chlorophyll in all the treatments compared to the control in both experiments (Table 3). ABG application at 250 ppm significantly decreased chlorophyll concentration compared with the control and most of the other treatments in both experiments. Plantlets treated with 0.5 mM aspirin contained significantly higher concentration of chlorophyll than those treated with 1.0 mM in only experiment 1. There was no significant difference in chlorophyll concentration between the different sulphur treatments in both the experiments. GABA application at 20 mM significantly increased the concentration of chlorophyll compared to the sea water treatment and most of the other treatments (Table 3).

Electrolyte leakage of leaflets significantly increased (2-3 fold) by sea water treatment in both experiments (Table 3). ABG application at 250 ppm significantly increased electrolyte leakage (about 3-fold) compared with the control and the other treatments in both experiments. In experiment 1, elemental sulphur at both low and high levels significantly decreased electrolyte leakage compared to the sea water treatment. Plantlets treated with aspirin and salicylic acid at both 0.5 mM and 1.0 mM showed significantly lower percentages of electrolyte leakage than those

Table 2. Nutrient concentration of 'Khalas' date palm plant leaflets at the VP3-stage of the acclimatization period as affected by elemental sulphur and anti-stress substances

Treatments	N%	P%	K%	S%	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)
Control	1.70	0.33	1.84	0.34	26.52	5.22	3.13	1.38
Sluphur at 0.0283g sulphur/pot	1.67	0.34	1.95	0.32	34.01	5.35	3.23	1.67
Sulphur at 0.1415g sulphur/pot	1.78	0.28	1.81	0.32	38.01	6.53	4.14	1.73
Sulphur at 0.283g sulphur/pot	1.83	0.27	2.03	0.32	36.22	6.13	4.36	1.48
Aspirin at 0.5 mM	1.72	0.29	1.82	0.27	35.53	7.16	4.08	2.08
Aspirin at 1.0 mM	1.79	0.3	1.88	0.3	31.6	6.74	4.39	1.67
Salcylic acid at 0.5 mM	1.73	0.24	1.81	0.32	31.81	5.89	4.34	2.05
Salcylic acid at 1.0 mM	1.8	0.28	1.83	0.3	29.06	6.2	3.95	1.47
Plantacur-E at 1%	1.66	0.28	1.96	0.28	29.7	5.13	3.23	1.6
Plantacur-E at 2%	1.79	0.31	1.78	0.27	34.15	5.87	4.34	1.83
Oleic acid 100 ppm	1.83	0.3	1.76	0.26	33.02	6.54	3.27	1.35
F-test	***	***	***	***	***	***	***	***
LSD (*P*=0.05)	0.05	0.05	0.09	0.05	2.27	0.43	0.48	0.26

(***)-significant at level $P = 0.001$; (-)-not calculated.

Table 3. Total chlorophyll concentration and electrolyte leakage (%) of 'Khalas' date palm plant leaflets treated with sea water at the VP3-stage of the acclimatization period as affected by elemental sulphur and some anti-stress substances

Treatments	Experiment (1)		Experiment (2)	
	Chlorophyll (mg/g fw)	Electrolyte leakage (%)	Chlorophyll (mg/g fw)	Electrolyte leakage (%)
Control (normal water)	1.68	2.69	1.55	2.87
Sea water	1.19	4.98	0.91	4.91
Sluphur at 0.0283g sulphur/pot	1.25	4.26	1.11	4.51
Sulphur at 0.1415g sulphur/pot	1.31	4.44	1.14	4.87
Sulphur at 0.283g sulphur/pot	1.26	4.17	1.12	4.10
Aspirin at 0.5 mM	1.39	4.18	0.94	4.72
Aspirin at 1.0 mM	1.24	4.30	0.91	4.77
Salcylic acid at 0.5 mM	1.24	4.63	1.19	4.78
Salcylic acid at 1.0 mM	1.21	4.68	1.10	4.53
Plantacur-E at 1%	1.25	4.84	1.19	4.56
Plantacur-E at 2%	1.18	4.97	1.10	4.85
ABG at 250 ppm	1.11	6.74	0.92	6.25
Oleic acid 100 ppm	1.31	3.88	1.11	4.50
GABA 10 mM	-	-	1.06	4.90
GABA 20 mM	-	-	1.25	4.10
F-test	***	***	***	***
LSD (*P*=0.05)	0.14	0.68	0.15	0.63

(***)-significant at level $P = 0.001$; (-)not calculated.

treated with only sea water in experiment 1. Among all the treatments, oleic acid significantly decreased electrolyte leakage. In experiment 2, elemental sulphur at the high level significantly decreased electrolyte leakage compared to the sea water treatment. Interestingly, GABA application at 20 mM significantly decreased electrolyte leakage than the sea water and most of the other treatments (Table 3).

Discussion

Our results showed that the application of anti-stress substances GABA, plantacur-E, salicylic acid, aspirin, oleic acid, and elemental sulphur increased survival percentage of the plantlets during the VP1-satge of the acclimatization period which would otherwise lead to economic loss (Table 1). The plantlets during VP1-stage was most sensitive to stress since during both VP2 and VP3 stages all the plantlets survived in all the treatments including the control. The results showed that, in both experiments, irrigation with sea water for two months significantly decreased the concentration of chlorophyll and increased electrolyte leakage of the plant leaflets compared to the control (Table 3). In this context, most of the applied substances especially GABA, salicylic acid and aspirin increased chlorophyll concentration and clearly decreased the electrolyte leakage in the plant leaflets compared to those treated only with sea water (Table 3). Such effects might be due to protecting the endogenous anti-oxidant systems often correlated with increased resistance to oxidative stress and/or controlling the level of free radicals within plant tissues (Sreenivasulu *et al.*, 2000). It is also possible that the anti-stress substances were effective in maintaining the membrane integrity to reduce the leakage of electrolyte through its positive effect on the antioxidant enzymes system as has been demonstrated by Pinhero and Fletcher (1994) in corn seedlings. It is generally known that most of environmental stresses have in common a similar mechanism in affecting plant growth and performance. Under stress conditions, the generation of free radicals and low nutrient uptake are believed to be the main cause for damaging and dis-functioning of plant cells (Bohnert *et al.*, 1995; Sreenivasulu *et al.*, 2000). Elemental sulphur is strongly involved in improving nutrient assimilation and in the anti-oxidative defense systems of plants through its metabolite glutathione. Glutathione is also necessary for the efficient-functioning of other defense systems such as ascorbate and □-tocopherol regeneration and carotenoids and protein thiols conservation (Gondent and Ullman, 2000; Tausz *et al.*, 2000). Hence, in the present study we propose that the protection confined by elemental sulphur was due to a similar mechanism

of enhanced free radical scavenging systems in the plant.

Our results showed positive effects of most of the applied anti-stress substances especially salicylic acid, aspirin and elemental sulphur on the concentrations of Fe, Mn, Zn, and Cu in the leaflets compared to the control (Table 2). These nutrients have been reported to be connected with the improved tolerance of several plants (Kinnersley and Turano, 2000). Elemental sulphur also increased the level of these nutrients in the leaflets probably by increasing their availability and uptake via modifying soil pH as reported by Marschner (1995). The results on salicylic acid and aspirin are in accordance with those of Raskin (1992) who reported that salicylic acid, among many other functions, controls ion uptake by roots and stomatal conductivity and increases the antioxidant capacity of plants. These results suggest salicylic acid and aspirin as mediator of mineral acquisition in stress-related metabolism. It has been reported that salicylic acid and its derivative aspirin induced multiple stress tolerance in bean and tomato plants against drought, heat, chilling, and salinity when seeds imbibed in aqueous solutions (0.1-0.5 mM) or when applied to the plants as foliar spray or even as soil drenches (Senaratna *et al.*, 2000). Moreover, salicylic acid has been found to protect wheat plants from drought and salinity stress, diminished the alteration of phytohormones, and increased the level of cell division within the apical meristem of seedling roots which caused an increase in plant growth (Shakirova *et al.*, 2003). Our results on the positive effects of GABA application (Tables 1 and 3) confirm those of Kinnersley and Turano (2000) who reported that the kinetics of GABA accumulation in plants reveals a stress-specific pattern of accumulation that is consistent with a physiological role for GABA in stress mitigation. To the best of our knowledge, this is a pioneer study investigating the role of GABA as a potential anti-stress substance for improving growth and stress tolerance ability of date palm plantlets (Tables 1 and 3). Such positive effects of GABA have been confirmed on 'Khadrawy' date palm plantlets, however, the lower concentration (10 mM) was more effective than the higher one (20 mM), indicating a cultivar dependent concentration (unpublished data).

The application of the ethylene biosynthesis blocker, ABG at 250 ppm greatly inhibited and completely suppressed subsequent plantlet development and growth at 500 ppm (Table 1). Also, the ABG-treated plantlets contained a lower chlorophyll concentration and showed a higher electrolyte leakage, after sea water irrigation, than the control (Table 3). These results suggested an important role of ethylene in subsequent plantlet development. This study might provide certain recommendations to benefit both the tissue culture-based date palm industry and growers.

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Impact of *Anthurium* spp. genotype on callus induction derived from leaf explants, and shoot and root regeneration capacity from callus

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Abstract

In this study, the most critical step in *Anthurium* micropropagation was the induction of primary calli from leaf segments. Genotype played an important role during *in vitro* multiplication of *Anthurium*. Callus induction from leaf segments was examined in ten *Anthurium* cultivars: 'Carnaval', 'Neon', 'Choco', 'Sonate', 'Midori', 'Pistache', 'Tropical', 'Safari', 'Arizona' and 'Cancan' on MS medium supplemented with 1 mg L^{-1} BA, 0.08 mg L^{-1} 2,4-D, 30 g L^{-1} glucose, 8 g L^{-1} agar and adjusted to pH 6.0. After 100 days, leaf segments of eight genotypes formed calli, among them, cultivar 'Pistache' had the highest callus induction ratio (65.1%) and two genotypes, 'Carnaval' and 'Cancan', showed no response. After multiplication, calli were subcultured on shoot regeneration medium, 1/2 MS with NH_4NO_3 level adjusted to 0.206 g L^{-1}, added with 20 g L^{-1} glucose, 1 mg L^{-1} BA, 8 g L^{-1} agar and adjusted to pH 6.0. Shoots were obtained from all cultivars with different potential of shoot regeneration. The average number of shoots per explant in 'Tropical' (10.1) was higher than that of 'Choco' (4.3) and 'Pistache' (3.5), and shoots (at least 10 mm high) were excised and cultured on rooting medium, 30 g L^{-1} glucose, 8 g L^{-1} agar and 1 g L^{-1} activated charcoal added to 1/4 MS medium. All shoots consistently formed roots after 30 days and plantlets developed well after being transferred to the nursery. The propagation process took 10 and a half months to complete.

Key words: *Anthurium andraeanum*, genotypes, leaf explants, callus induction, shoot regeneration, root regeneration.

Introduction

Anthurium sp., a perennial herbaceous plant, is an economically important genus of Araceae. It is native to the tropics of Central and South America, and is enjoyed for its colorful attractive, luxurious flowers and exotic foliage. It is cultivated commercially as cut flower and potted plant throughout the world. The necessity to find a rapid and effective propagation method is required to satisfy the increasing demand of *Anthurium*.

Different explants, leaves, immature petioles, inflorescence stalks, spathes, spadices and axillary nodes have been used for micropropagation. Hamidah *et al.* (1997) induced somatic embryogenesis from leaves, petioles and roots on medium to which various concentrations of sugar and several types of growth regulators were added. For somatic embryos, leaf pieces from micropropagated plants were found to be best on MS medium (Murashige and Skoog, 1962) with 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and a high sucrose concentration (6%), but a low glucose concentration (3%) was better to induce callus. MS with 1 mg L^{-1} 6-benzyladenine (BA) was the best medium for shoot regeneration and 1/2 MS plus 0.1 mg L^{-1} α-naphthalene acetic acid (NAA) for shooting (Hamidah *et al.*, 1997). Pierik (1976) developed a method of *Anthurium* propagation using solid and liquid media alternatively. Geier (1986) used culture media derived from Nitsch's basal medium (1969), which contained minerals, vitamins, sucrose and agar. Of the various media tested, including NH_4NO_3 concentration and the presence or absence of different auxins and cytokinins, NH_4NO_3 level had the most significant effect on callus and shoot formation, a low level being best for regeneration in all the genotypes investigated. Cytokinin was also necessary to induce callus from leaf explants, but if auxin and cytokinin were combined, shoot induction and development were better in subculturing. Kuehnle and Sugii (1991) examined the influence of growth regulators on callus induction derived from leaf segments. Callus was produced most successfully on a modified Pierik medium containing 2,4-D and BA, while sucrose was better than glucose as a carbon source.

Another factor that plays an important role during *Anthurium in vitro* propagation is the effect of genotype. Pierik (1976) examined 38 genotypes of *Anthurium andraeanum* and observed moderate to strong callus formation from leaf segments in 31 genotypes, very poor callus formation in four and no response in the remaining three genotypes. In another study, Geier (1986) found that out of 18 genotypes, three did not show any regeneration, five produced only callus and ten formed caulogenic callus when leaf explants were cultured on Nitsch medium. Not only callus induction but also shoot regeneration from callus was dependent on the genotype. Observing the average number of shoots per culture, Geier (1986) obtained <1 in five genotypes, 1-10 in three genotypes, and >10 in two genotypes. This result showed that different genotypes had different responses to the same medium and the identical procedure could not be applied for the *in vitro* propagation of all *Anthuriums*. Although many researches have already been conducted, the main objective of this study was to investigate the effect of genotype on callus induction derived from leaf explants and its influence on shoot and root regeneration capacity, which allows establishing a

suitable procedure suited to each *Anthurium*, which can be used for commercial production.

Materials and methods

Plant material: Leaf segments of ten valuable cultivars of *Anthurium andraeanum*: 'Carnaval', 'Neon', 'Choco', 'Sonate', 'Midori', 'Pistache', 'Tropical', 'Safari', 'Arizona' and 'Cancan' were used as material. Young leaves having approximately 1/2-2/3 of the final length were surface-sterilized with $HgCl_2$ 0.1%, and then sectioned to about 1 cm^2 pieces.

Callus induction and multiplication: To investigate the effect of genotype on callus induction derived from leaf explants, 100 leaf explants from each genotype were transplanted onto callus induction medium: 1/2 MS basal medium supplemented with 30 g L^{-1} glucose, 1 mg L^{-1} BA, 0.08 mg L^{-1} 2,4-D and 8 g L^{-1} agar. Two leaf segments were placed in each culture vessel, and callus was induced in darkness at 20 ± 2°C, 75-80% relative humidity. When callus had formed and achieved the required size (about 2.0 to 2.5 cm^2), it was divided and three 0.5 cm^2 pieces were transferred to callus multiplication medium (1/2 MS medium with 20 g L^{-1} glucose, 1 mg L^{-1} BA and 8 g L^{-1} agar) to get a large amount of calli.

Shoot regeneration: To investigate the effect of genotype on shoot regeneration from callus, calli obtained in four month subcultured 'Tropical', 'Choco' and 'Pistache' were transferred to shoot regeneration medium: 1/2 MS with NH_4NO_3 lowered to 0.206 g L^{-1}, 20 g L^{-1} glucose, 1 mg L^{-1} BA and 8 g L^{-1} agar. Friable, etiolated calli were chosen to increase the frequency of shoot regeneration and each culture vessel contained three 0.7 cm^2 callus pieces.

Rooting: For more rapid and consistent rooting, 10 mm tall shoots of 'Tropical', 'Choco' and 'Pistache' were isolated and transplanted to rooting medium: 1/4 MS medium supplemented with 30 g L^{-1} glucose, 8g L^{-1} agar and 1 g L^{-1} activated charcoal.

Culture conditions (*in vitro* and *ex vitro*): pH was adjusted to 6.0 in all media except for the rooting medium (pH 6.2) and media were autoclaved for 30 minutes at 121°C and 1 atm. Callus multiplication, shoot regeneration and root formation were carried out under a 10-hour photoperiod, 45 µmol m^{-2} s^{-1} illumination, and at 25 ± 2°C and 70-75% relative humidity.

Plantlets of 'Tropical', 'Choco' and 'Pistache' with more than 2 well-developed roots and 2.5-3.0 cm in height were isolated and transferred to a 50-70% of daylight shaded nursery. Plantlets were cultivated on tree fern fiber substrate at 22 ± 2°C and 80-85% relative humidity, sprayed with water 2 times a day, Komix BFC as fertilizer 3 times a week and 5 g L^{-1} fungicide solution mixture including Zodiac 80 WP and Manozep 80 WP 2 times a week.

Data was analyzed for significance by ANOVA with the mean separation by Duncan's multiple range test (Duncan, 1995).

Results and discussion

Effects of genotype on callus induction from leaf segments: Two 1 cm^2 leaf pieces were cultured in each callus inducing medium vessel. After 30 days of culture in darkness, callus formation was first observed in 'Midori' and 'Safari' with 18.2% and 12.5% explants inducing callus, respectively (Table 1). Leaf pieces of other cultivars were green but without traces of callus.

Table 1. Effect of genotype on callus induction of *Anthurium* after different periods

Cultivar	Callus induction rate (%)		
	30 days	60 days	100 days
'Carnaval'	0	0	0
'Neon'	0	2.0	2.0
'Choco'	0	12.5	12.5
'Sonate'	0	28.6	28.6
'Midori'	18.2	20.0	35.2
'Pistache'	0	61.8	65.1
'Tropical'	0	34.3	35.0
'Safari'	12.5	16.7	20.8
'Arizona'	0	9.8	10.7
'Cancan'	0	0	0

Explants were continuously left in the dark. After 60 days of culture, all cultivars induced callus except for 'Carnaval' and 'Cancan'. While the callus formation rate of cultivars 'Midori' and 'Safari' was slow (18.2 to 20.0% in 'Midori' and 12.5 to 16.7% in 'Safari'), some cultivars which did not form callus after 30 days of culture, had high rates of callus induction (28.6% in 'Sonate', 34.3% in 'Tropical' and 61.8% in 'Pistache'), even higher than those of genotypes inducing calli early. Explants of other genotypes also formed callus but at lower rates: 12.5% in 'Choco', 9.8% in 'Arizona' and 2% in 'Neon'. And after 100 days, the regeneration rate of the ten cultivars gradually increased with a corresponding rise in fresh weight of calli.

In 'Choco', 'Pistache' and 'Safari', leaf explants started to turn yellow, yet calli still developed. 'Safari' explants induced callus early but had a low induction rate (20.8%) while 'Pistache' had the highest callus induction rate (65.1%). The induction rate of 'Midori' and 'Tropical' were similar (about 35.2 and 35.0%, respectively), however the size of 'Tropical' calli were profusely larger. In addition, 28.6% of 'Sonate' leaf explants induced large calli, which developed quickly afterwards. Other cultivars showed little change: a low induction rate in 'Choco' (12.5%), 'Arizona' (10.7%) and 'Neon' (2.0%). 'Carnaval' and 'Cancan' did not induce callus.

These results indicate that ten *Anthurium* genotypes had different responses to the same callus induction medium and culture conditions. They could be clustered into four groups: group of 'Pitasche' having high callus induction rate; group of 'Midori', 'Tropical', 'Sonate', 'Safari', 'Choco' and 'Arizona' having average callus induction rate; group of 'Neon' having low callus induction rate and group of 'Carnaval' and 'Cancan', which could not induce callus.

Effect of genotype on shoot regeneration from callus: Shoot formation was investigated in 'Choco', 'Pitasche' and 'Tropical', which had different callus induction rates. After 30 days of culture, shoot regeneration was first observed in 'Tropical', while other cultivars did not form shoots.

Calli of three cultivars still survived after 100 days of culture. Among them, the highest number of regenerated shoots was obtained in 'Tropical' (10.1 shoots per explant), which were firm with small leaves; moreover, much caulogenic calli was induced. 'Choco' had lower number of regenerated shoots (4.3 per explant), which had large leaves and developed slowly, but its calli turned darker. The lowest number of shoots formed in 'Pistache' (3.5 per explant) (Table 2).

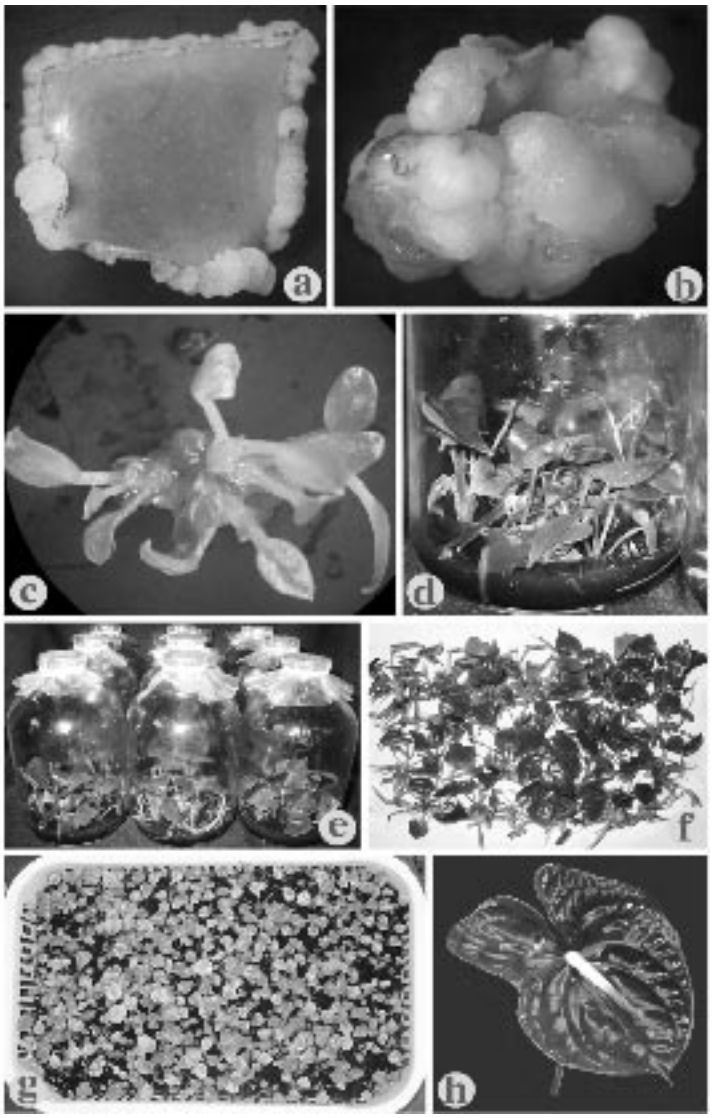

Fig. 1. Micropropagation by leaf explant culture of *Anthurium* spp.: (a), (b) callus induced from leaf segment; (c) shoots regenerated from callus; (d), (e) root formation; (f) plantlets; (g) *ex vitro* plantlets; (h) *Anthurium* 'Choco' flower.

Table 2. Effect of genotype on shoot regeneration from callus of *Anthurium*

Cultivars	Callus survival rate (%)	Number of shoots per explant
'Choco'	100	4.3b[x]
'Pistache'	100	3.5c[s]
'Tropical'	100	10.1a[s]

[x] Different letters within a column indicate significant differences at $P \leq 0.05$ by Duncan's multiple range test.

Effect of genotype on root formation from shoots: Shoots of 'Choco', 'Pistache' and 'Tropical' were isolated and transferred to shoot formation medium without plant growth regulators (10 shoots per vessel), which is necessary for firm and rapid root formation. Although shoots and roots could regenerate naturally when cultured over a long period and under illumination on MS medium, root formation depended on the size and developmental stage of shoots, and culture media. Shoots formed roots when placed on media with low concentration of macroelements and without a cytokinin, BA. Results showed that all shoots of the three cultivars formed fully developed root systems after 30 days of culture with a comparable number of roots. However, based on the characteristics of each cultivar, formed roots had different shapes and sizes. Shoots with small leaves of 'Tropical' formed long and thin roots, while shoots with larger leaves of 'Choco' and 'Pistache' formed short but thick roots.

Effect of genotype on *ex vitro* plantlet development: Five hundred homomorphous plantlets each of 'Choco', 'Pistache' and 'Tropical' were placed in the nursery. All of them survived and developed well after 30 days of culture. Some plantlets with very short roots or even without roots also grew and formed roots post planting, although it took more time.

Anthurium micropropagation by leaf explant culture was characterized by the use of different media at different stages of morphogenesis. The most difficult step in this propagation process was callus induction from leaf explant. This is due to the restricted dedifferentiation of mature tissue and depends strongly on genotype. *Anthurium* 'Pistache' could best induce calli on 1/2 MS medium supplemented with 30 g L^{-1} glucose, 1 mg L^{-1} BA, 0.08 mg L^{-1} 2,4-D, 8 g L^{-1} agar and adjusted to pH 6.0 (65.1% explants induced calli after 100 days of culture), whereas 'Carnaval' and 'Cancan' could not. Genotype affected shoot regeneration slightly but did not affect root formation. The critical factor in shoot regeneration stage was the effect of NH_4NO_3 concentration. 'Tropical' had the highest number of regenerated shoots per explant (10.1 shoots per explant after 100 days) when its calli were cultured on 1/2 MS with NH_4NO_3 lowered to 0.206 g L^{-1}, 20 g L^{-1} glucose, 1 mg L^{-1} BA, 8 g L^{-1} agar (pH 6.0), and all genotypes formed roots normally (after 30 days) on 1/4 MS medium supplemented with 30 g L^{-1} glucose, 8 g L^{-1} agar, 1 g L^{-1} activated charcoal (pH 6.2). All plantlets developed well when transferred to the nursery. According to the results of this study, the propagation process took ten and a half months to complete: four months for callus induction, two and a half months for mass multiplication, three months for regenerating shoots and one month for forming roots. This process was best for 'Pistache', appropriate for 'Tropical', but had to be adjusted to obtain more regenerated shoots of 'Pistache' and 'Tropical' calli. More researches are required to define the appropriate requirements for each of the other cultivars.

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A one step *in vitro* cloning procedure for Red Globe grape: The influence of basal media and plant growth regulators

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Abstract

Earlier studies have shown that the degree of success at each stage of micropropagation in grapevine is genotype dependent; hence it becomes imperative to optimize culture conditions for rapid propagation of a variety. Present report describes two approaches of *in vitro* propagation of a *Vitis vinifera* cultivar, Red Globe. In one approach, whole plants could be developed from single node segments by bud break and direct rooting *in vitro*. Eight different basal media tried showed different morphogenetic responses**.** In second approach, multiple shoots were induced in nodal segments cultured on MS basal medium supplemented with BA (8.88 µM). Also, second crop of shoots could be induced in left over nodal segments devoid of shoots. Rooting of shoots could be induced *in vitro,* both in semi-solid or liquid media and also *ex vitro* by pulse treatment of IAA (2.85 µM) + NAA (2.70 µM). Plant establishment in later case was 80%. A simple procedure described here can complement conventional methods, currently being used in propagation of this important grape variety.

Key words: Auxin pulse, benzyladenine, grape, micropropagation, Red Globe, *Vitis vinifera*

Introduction

Due to heterozygous nature grape varieties are mostly propagated by vegetative means. Application of plant tissue culture techniques in propagation and improvement of grapevines has been reviewed by several workers (Krul and Mowbray, 1984; Gray and Meredith, 1992; Torregrosa *et al.*, 2001). The technique has been used to propagate pathogen free grapevine stock (Duran-Vila *et al.*, 1988). Micropropagation complements the conventional technique when a large number of propagules of a particular variety are required in a shorter time. Earlier studies on *in vitro* propagation of *Vitis* have indicated that the degree of success at each stage of culture is genotype dependent and varies under a given set of culture conditions (Barlass and Skene, 1980; Monette, 1988; Botti *et al.*, 1993). Hence, it becomes essential to optimize culture conditions for a particular clone / cultivar / rootstock or newly bred line that needs large scale planting but availability of sufficient planting stock is a limitation. The present communication describes influence of eight basal media and growth regulators on micropropagation of Red Globe, a *Vitis vinifera* cultivar. The variety is in great demand due to its attractive reddish-purple colour; taste bud arousing flavour and appealing large plum-size berries with uniform bunches.

Material and methods

Twigs of field grown, disease free vines of Red Globe were collected from the vineyard of National Research Centre for Grapes, Pune. These were defoliated and cut into single node segments (2 cm). The explants were dipped in 1% Labolene solution for 10 min; rinsed with tap water; submerged in 0.1% Bavistin solution; kept on a shaker (120 rpm) for 2 h and thereafter rinsed three times with sterile water in a laminar flow hood. These were then disinfected with 0.1% mercuric chloride solution for 10 min and rinsed three times with sterile water. The explants were finally blotted dry on sterile filter paper and inoculated on medium in glass test tubes (150 X 25 mm).

For budbreak, eight different basal media – MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980), NN (Nitsch and Nitsch, 1969), B5 (Gamborg *et al.*, 1968), ER (Eriksson, 1965), LS (Linsmaier and Skoog, 1965), C_2d (Chee and Pool, 1987) and GNMG (Galzy *et al.*, 1990) devoid of growth regulators were tested. Another experiment with MS medium and range of BA concentrations (0.04 to 11.1 µM) was undertaken to maximize budbreak. To obtain second crop of shoots, primary nodal segments left after excising the grown axillary shoot (hereinafter referred to as mother explant) instead of its discard, were transferred to WPM or MS with or without BA (4.44 and 8.88 µM).

For induction of multiple shoots, axillary shoots obtained from primary nodal segments were inoculated (S0) in test tubes having MS medium with BA (2.22 to 8.88 µM). After 30 d, explants showing multiple shoots were transferred to fresh medium in glass bottles. This was continued at an interval of 30 d until five transfers (from S1 to S5). To test the effect of inoculum's density per culture vessel, two, three, four or five shoot clumps per culture bottle were inoculated on MS with BA at 4.44 or 8.88 µM. Two sets of experiments were carried out for elongation of *in vitro* shoots. In the first set, shoots less than 3 cm in length were inoculated on WPM supplemented with or without BA (2.22-8.88 µM). In the second set, multiple shoot clumps with shoots of <1.5 cm in length were kept for elongation. These were inoculated in glass bottles containing MS supplemented with BA (2.22 or 4.44 µM) and NAA (0.54 µM).

For *in vitro* rooting, shoots more than 3 cm were inoculated in test tubes containing half or full strength MS or WPM supplemented

with NAA (0.54 - 1.07 µM) or IAA (0.57 - 1.14 µM) or IBA (0.49 - 0.98 µM) or IPA (0.53 - 1.06 µM) with or without agar. Liquid media had filter paper bridges. *In vitro* raised shoots, more than 7 cm in length were given pulse treatment of different auxins, IAA (2.85 - 5.71 µM) or IBA (2.46 - 4.90 µM) or IPA (2.64 - 5.29 µM) or NAA (2.69 - 5.37 µM) either singly or in combination for 10 min and then planted in plastic cups containing a mixture of coco-peat + soil + sand (1:1:1). Untreated shoots served as control. Shoots rooted were taken out of the culture tubes, their roots gently washed with water to remove adhering medium and were transferred to plastic cups containing the above mentioned mixture. Plants were acclimatized by the Sachet technique (Ravindra and Thomas, 1985). Plants after transfer to cups were kept in continuous light of 24.4 µmol m^{-2} s^{-1} at 25 ± 2^0C. Thereafter, these were shifted to another growth room at ambient temperature (35 ± 2^0C). Establishment of plants was recorded after 30 d.

All the media were supplemented with sucrose 20 g L^{-1} and gelled with agar 7 g L^{-1}. The pH of the media was adjusted to 5.8 before autoclaving at 121^{0}C for 20 min. Cultures were incubated under 16 h photoperiod obtained with cool light fluorescent tubes with light intensity of 24.4 µmol m^{-2} s^{-1} at 25 ± 2^0C. Experiments were repeated at least three times. Observations were recorded at monthly interval. The experiments were conducted in Completely Randomized Design and the results were subjected to analysis of variance.

Results and discussion

Bud break in nodal segments commenced from the fifth day of inoculation and continued up to 20th day and thereafter shoots put forth rapid growth. Among the eight basal media tested, C_2d, LS and WPM without any growth regulators induced 92, 90 and 84% budbreak, respectively. Induction of two or more shoots in maximum explants (76%) was observed in C_2d medium. NN induced the minimum response (Table 1). In addition to budbreak, nodal explants both in WPM and B5 induced rooting at the base in 70% of explants. In case of NN, it was 46%. In all other media rooting was very low. These rooted nodal segments with primary shoots could be established on potting and were hardened by the Sachet technique. Thus, no special difficulty was faced with nodal culture producing entire plantlet.

Eight different nutrient media induced different morphogenetic responses in nodal segments. Shoots in C_2d were found to be stunted, succulent with light green, thick leaves and glossy in appearance. Basal media LS and Eriksson showed necrosis in shoot tip, which continued to the entire shoot and caused drying of the whole shoot. Shoots in NN lacked vigour, had thin stems with dark green leaves. MS resulted into comparatively better shoots with normal internode and light green leaves. Also, shoots on MS were most vigourous as compared to other media tested. The shoots in B5 were similar to those observed in MS except that the internode was slightly thicker. The shoots in WPM lacked vigour and had thin, lanky stems showing twining habit with thin foliage. Of the eight media tested, MS was found to be the most suitable medium resulting into vigorous shoots. Hence, for multiple shoot induction experiment, only MS was used.

In a similar study on basal medium, Reisch (1986) observed significant differences in growth in grape cultivar White Riesling with MS half and MS full medium. However, in contrast to the present study, Gray and Benton (1991) observed stunted growth in shoots of Muscadine grape cultivars when WPM was used. Genotypic variability within *Vitis vinifera* cultivars cultured *in vitro* has earlier been reported (Harris and Stevenson, 1982; Chee and Pool, 1983; Galzy *et al.*, 1990). Varying response of different genotypes to different basal media could be due to variations in nutrient compositions. For example, amount of $CaCl_2$ is higher in MS, LS and Eriksson as compared to WPM and NN, while in C_2d and GNMG, it is substituted by $Ca(NO_3)_2$. Similarly, Potassium Iodide (KI) is absent in WPM, NN, C_2d and Eriksson while it is present in GNMG, B5, LS and MS though in different quantities. Also amounts of $MnSO_4$ vary in the eight basal media tested.

Galzy (1969) demonstrated that mineral requirement varied with the morphogenic process: strong K and N concentrations proved favorable to shoot development but impeded root growth. Chee and Pool (1987) working with grape tissues have reported that lower concentrations of KI and $MnSO_4$ in the medium were good for maximum shoot production and incorporation of $Ca(NO_3)_2$ instead of $CaCl_2$ produced shoots of good quality. Present study corroborates these findings since maximum results of budbreak of shoots were obtained from explants inoculated on C_2d though shoots obtained from explants inoculated on MS medium were comparatively healthy and vigorous. Besides nutrients, differences in *in vitro* response between genotypes of different species may be related to differences in endogenous

Table 1. Effects of eight basal media on morphogenesis in nodal segments of *Vitis vinifera* cv. Red Globe

Basal medium	Explants showing bud-break* (%)	Explants showing single shoots (%)	Explants showing 2 or more shoots(%)	Total number of shoots obtained	Soots elongated (%)	Average shoot height (cm) ± S.D.	Explants showing rooting at the base (%)
NN	42	38	04	23	82.6	2.13 ± 0.10	46
C_2d	92	16	76	97	44.0	3.52 ± 0.46	02
B5	70	32	38	58	52.0	3.90 ± 0.23	70
MS	80	18	62	96	37.0	1.86 ± 0.35	10
LS	90	24	66	98	38.0	1.82 ± 0.36	14
ER	68	04	64	83	39.0	1.19 ± 0.10	04
WPM	84	40	44	68	44.0	4.25 ± 0.50	70
GNMG	62	56	06	25	96.0	1.49 ± 0.18	00
LSD (*P*=0.01)						0.53	

* Based on 50 explants, ± SD = Standard deviation

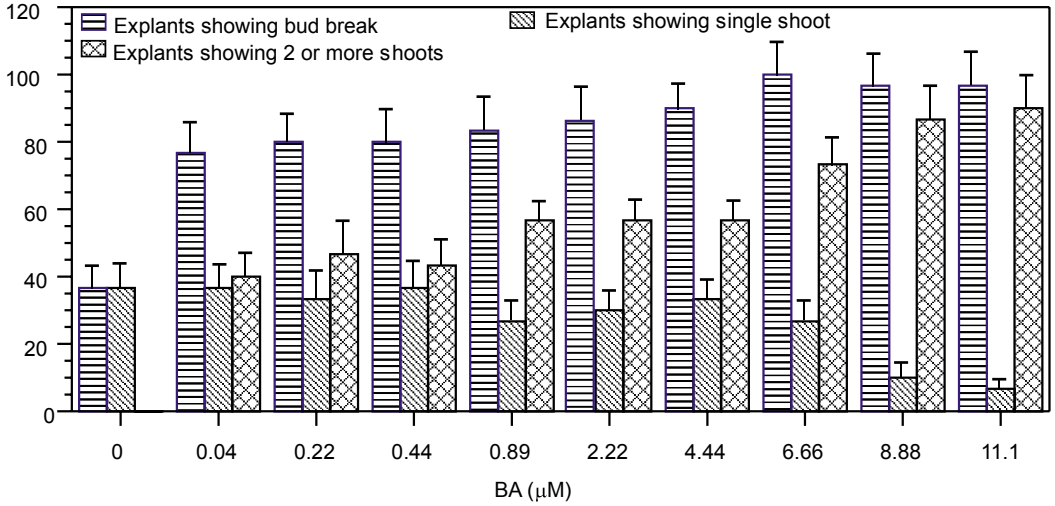

Fig. 1. Effects of BA concentration on budbreak and number of shoots in nodal segments of Red Globe following 30 d of culture, basal medium – Murashige and Skoog, (1962); bars indicate standard error.

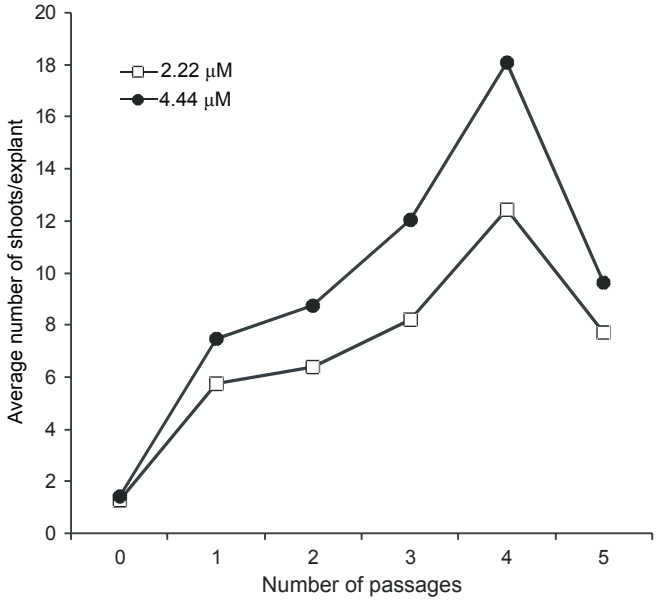

Fig. 2A. Effects of BA concentration and number of passages on shoot proliferation in Red Globe grape.

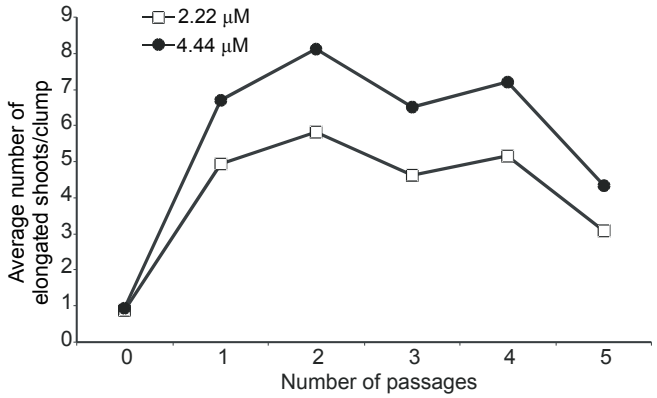

Fig. 2B. Effect of BA concentration and number of passages on elongation of shoots in Red Globe grape.

levels of phytohormones (Looney *et al.*, 1988; Alvarez *et al.*, 1989; Gronroos *et al.*, 1989).

In the second experiment, BA at 6.66, 8.88 and 11.1 μM resulted into 100, 96.66, and 96.66% of explants showing budbreak. There was marginal difference in the response of BA levels from 0.04 to 0.89 μM. However, a linear increase in number of two or more shoots per explant was observed on increase in BA concentration from 0.04 to 11.1 μM. Maximum response (90%) of two or more shoots per explant was recorded with BA at 11.1 μM (Fig. 1). Addition of BA in MS not only induced bud break in higher number of nodal explants but shoots were of better quality in terms of vigour and leaf colour. Positive influence of BA in establishment of axenic shoots in grapes has earlier been documented in several reports (Chee and Pool, 1983; Reisch, 1986; Lee and Wetzstein, 1990; Robacker and Chang, 1992; Torregrosa and Bouquet, 1995; Mhatre *et al.*, 2000). A second crop of shoots could be induced in mother explants cultured on WPM or MS with BA at 4.44 or 8.88 μM. The maximum shoot induction was obtained in MS with BA (8.88 μM) considering both single and two or more shoots in explants (data not shown).

Primary shoot used as explant, induced maximum multiple shoots (2.27) per explant on an average in MS supplemented with BA (8.88 μM) after 30 days of inoculation (S0). Though marginally higher, a linear increase in number of shoots was observed on increase in BA concentration from 2.22 to 8.88 μM though reverse was true for number of shoots elongated per explant. Medium without BA (served as control) showed the least number of shoots as well as least number of elongated shoots per explant. On transfer of these shoots to fresh media (S1) in glass bottles, number of multiple shoots increased several fold and showed linear increase with increase in BA concentration (Fig. 2A). The same trend was observed with number of elongated shoots per explant (Fig. 2B).

It was observed that BA concentrations at 6.66 and 8.88 μM showed higher number of shoots and elongated shoots per explant from subcultures S0 to S1 however, shoots produced were hyperhydric and showed abnormalities in leaf shape. The leaves were dark green with glossy appearance. Also, shoots

Table 2. Effect of basal media and BA on shoot elongation in cv. Red Globe

Basal medium + BA (µM)	Percent of shoots elongated*	Average shoot length (cm) ± SD
WPM + BA (4.44)	66.67	7.50 ± 0.45
WPM + BA (8.88)	53.33	6.38 ± 0.45
WPM	13.33	1.50 ± 0.17
MS + BA (4.44)	93.33	6.75 ± 0.26
MS + BA (8.88)	61.67	4.50 ± 0.30
MS	30.00	1.00 ± 0.06
LSD (*P*=0.01)		0.56

* Based on 60 explants, ± SD = Standard deviation

were short and compact in the form of clumps. Hence for further subcultures, these two BA concentrations were discontinued. On repeated sub-cultures of these shoot clumps from S1 to S4 at an interval of 30 d, a drastic and linear increase in number of shoots was observed (Fig. 2A). As observed earlier this could be due to axillary branching in shoots clusters or occurrence of adventitious organogenesis (Chee and Pool, 1985). However, in S5, number of shoots per clump decreased drastically indicating toxicity.

It was found that the density of clumps inoculated per culture vessel influenced the rate of shoot proliferation for all the BA levels. An inverse correlation was observed between BA concentration and density of clumps. Higher the BA concentration more the number of shoots per clump while reverse was true with density *i.e.* higher the number of clumps per vessel lesser the number of shoots per clump. The maximum number of average shoots (15.4) per clump was obtained with two clumps per culture vessel in medium containing BA 8.88 µM (data not shown).

Since all the shoots in multiple shoot clumps did not elongate, it was necessary to carry out a separate experiment for elongation of shoots. In one experiment, shoot clump was kept as explant on two media containing MS as basal medium supplemented with NAA at 0.54 µM and BA at two concentrations 2.22 and 4.44 µM. At both the BA concentrations, there was very marginal difference in average number of shoots elongated 12.95, 13.63 with average shoot length of 5.90 and 5.33 cm, respectively. Inoculation of shoot clumps for elongation of shoots has advantage since several shoots elongated simultaneously instead of one, saving time and labour.

In the other experiment, single shoots (2-3 cm) isolated from multiple shoot clumps were kept on WPM and MS basal media without BA or with BA at 4.44 and 8.88 µM. Maximum percentage of shoots (93.33%) elongated on MS with BA (4.44 µM) giving an average shoot length of 6.75 cm followed by WPM (66.67 %) giving an average shoot elongation of 7.50 cm. BA concentrations lower than 4.44 µM showed bolting of shoots while BA at higher than 8.88 µM resulted into thick, stunted, succulent and hyperhydric shoots (data not shown). This is in conformity with observations recorded in three grapevine cultivars by Mhatre *et al.* (2000). Basal medium without growth regulators showed shoot necrosis and was not effective in shoot elongation.

Rooting of *in vitro* shoots is influenced by several factors, of which growth regulator requirement is of major importance. Though rooting of *in vitro* raised shoots could be induced in MS half or full strength basal media (agar solidified or liquid)

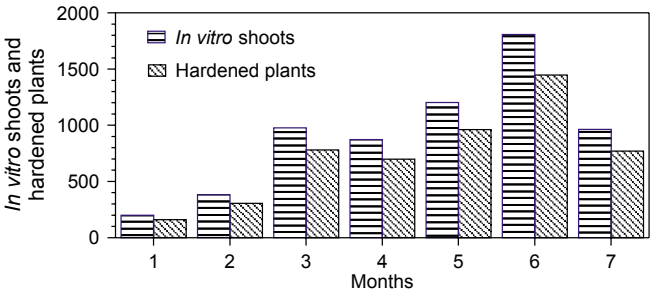

Fig. 3. Number of *in vitro* shoots and hardened plants obtained in a span of seven months (starting with 100 nodal segments).

supplemented with or without NAA (0.54 - 1.07 µM) or IAA (0.57 - 1.14 µM) or IBA (0.49 - 0.98 µM) or IPA (0.53 -1.06 µM) however, quality of roots was better on incorporation of NAA at 0.54 - 1.07 µM in the medium. Number of days required for rooting was less for the shoots inoculated in the liquid medium as compared to the solidified medium. In MS half or MS full medium devoid of growth regulators, the quality of roots was poor and shoots lacked vigour. Addition of NAA in the rooting medium induced longer roots with primary and secondary branching. This was reflected in the higher survival of rooted shoots (83%) when treated with NAA (data not shown). In earlier reports on grapevine, it was documented that auxin stimulated root initiation but inhibited subsequent root growth (Galzy, 1969), and that its appropriate concentration was of critical importance. In previous studies, it was observed that effects of auxins on rooting depend on mineral composition of the nutrient media (Novak and Juvova, 1983; Zlenko *et al.*, 1995). Root initiation was not influenced by salt concentration, but root growth was enhanced when salt concentration of rooting media was reduced (Harris and Stevenson, 1979).

Ex vitro auxin pulse treatment of *in vitro* shoots for 10 min induced direct roots. Shoots given a pulse treatment with auxin mixture of IAA (2.85 µM) and NAA (2.70 µM) showed 80% plant establishment. Pulse treatment of IAA (5.7 µM) or NAA (5.4 µM) alone gave rise 73 and 70% establishment, respectively. A mixture of IAA (5.7 µM) and NAA (5.4 µM) resulted into lower percent (53%) of establishment. Shoots directly transferred to potting mixture without any auxin pulse did not induce roots and could not establish. Plants could be acclimatized by the sachet technique which is simple, effective and does not require any sophisticated set-up. It was found that shoots planted in a mixture of coco-peat + soil + sand (1:1:1) showed a plant survival of 75%.

Thus, present communication describes two routes of micropropagation in grapevine cultivar Red Globe. In one route, whole plants could be developed from single node segments by bud break and direct rooting. To our knowledge no such systematic study on basal media has been reported so far for tissue culture of grapevines. In second route, larger number of plants could be obtained by multiple shoot induction, shoot proliferation and *ex vitro* rooting by auxin pulse treatment. Within seven months period, about 100 single node segments could give rise to about 5442 *in vitro* shoots and 4354 established plants compared to conventional vegetative cutting method where each three to five node cutting yields only one plant (Fig. 3). Tissue culture plants produced, have been supplied to National

Research Center for Grapes (NRCG), for its performance in the field. A simple *in vitro* propagation procedure described here can complement conventional methods, currently being used in propagation of this important grapevine variety.

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Anthocyanin accumulation in the hypocotyl and petal of Red Agati (*Sesbania grandiflora*), an ornamental legume

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Abstract

Seeds of Red Agati (*Sesbania grandiflora*), an ornamental leguminous tree, were germinated *in vitro* under both light and dark conditions for 7, 10, 15, 20 and 25 days. The localization of anthocyanin-containing cells and level of total anthocyanin content of hypocotyl from several developmental stages were determined. In the hypocotyl of light-grown seedlings, anthocyanin-containing cells were observed in epidermal and sub-epidermal layer and peripheral cortex while none was found in that of dark-grown seedlings. On day 7, the hypocotyl of light-grown seedlings had the highest anthocyanin content (290 µg/g FW). Moreover, Red Agati's petal at various developmental stages was also examined for the total anthocyanin content. It was found that the petal of 3 cm length had the highest total anthocyanin level (455 µg/g FW). It is concluded that the hypocotyl of light-grown Red Agati seedlings is an attractive alternative source of anthocyanins to the petal as the seedlings can be raised and be made available throughout the year.

Key words: Red Agati, anthocyanin, *Sesbania grandiflora*, histology, development

Introduction

Red Agati (*Sesbania grandiflora*), a perennial nitrogen-fixing ornamental tree, belongs to the family Fabaceae. The origin of this leguminous plant is not known but it is considered native to Southeast Asian countries. In Thailand, it is commonly grown in backyard gardens. This plant and a closely related species, Agati (*Sesbania grandiflora*), are useful for reforestation as they have extremely fast growth rate. They are also used for forage, firewood, pulp and paper, food, green manure and landscape decoration (National Research Council, 1979; Thiengburanathum, 1993; Bray, 1994; Gutteridge, 1994 and Jensen, 2001). The petal of Red Agati is large, distinctively rose pink or red in color while that of a related species, Agati, is white or yellowish, reflecting the differing anthocyanin content in the vacuole (Markham *et al.*, 2000).

Anthocyanins are plant phenolic compounds in the flavanoid group. They generally are responsible for the red, purple and blue colors in plant parts such as fruits, vegetables, flowers, leaves, roots and tubers. All natural anthocyanins are glycosides called anthocyanidin. Anthocyanins are receiving renewed attention for their potential health benefits as antioxidants and anti-inflammatory agents (Kalt and Dufour, 1997; Wang *et al.*, 1997; Wang *et al.*, 1999).

Plant tissue culture is one of the important biotechnological tools which has been applied in various aspects, such as, micropropagation (Bodhipadma and Leung, 2002a; Haw and Keng, 2003), *in vitro* flowering and fruiting (Bodhipadma and Leung, 2002b; Bodhipadma and Leung, 2003) and secondary metabolite, especially anthocyanin production (Rao and Ravishankar, 2002; Filippini *et al.*, 2003).

Though there were some publications on tissue culture of *Sesbania*, the objective of most of them was to propagate this plant (Shanker and Ram, 1990; Shanker and Ram, 1993; Sinha and Mallick, 1993; Detrez *et al.,* 1994). Unlike Agati, Red Agati has a remarkably red petal that would be a good source of anthocyanin. However, the hypocotyl of a Red Agati seedling is also red. This could be a more attractive and convenient source for anthocyanin production than petal. With this goal in mind, the present study was undertaken to compare the amount of anthocyanin extracted from the hypocotyl and petal of Red Agati and investigate the time during their development when maximal accumulation of anthocyanin might occur in the appropriate organ.

Materials and methods

Plant materials: Mature dry seeds of Red Agati were obtained from a local garden of Nonthaburi province. To avoid possible complications from microbial contamination during raising seedlings from seeds for the present experiments, the Red Agati seeds were germinated under aseptic conditions: they were immersed in sterile distilled water overnight before they were surface-sterilized by soaking in 70% (v/v) ethanol for 30 seconds, 1.5% (v/v) sodium hypochlorite for 15 minutes and rinsing them three times with sterile distilled water. After seeds germinated *in vitro* on the medium (see Medium) under both light and dark conditions for 7, 10, 15, 20 and 25 days, hypocotyl from these seedlings were cut and examined for total anthocyanin content

and its localization. Flowers of Red Agati were collected from local garden of Nonthaburi province. Only petal parts 3, 6 and 9 cm in length were used to analyze for total anthocyanin content. The collection was made after the opening of the petals in 4, 7 and 12 days, respectively.

Medium: MS basal medium (Murashige and Skoog, 1962) in this study were adjusted to pH 5.7, gelled with 0.8% (w/v) agar, and autoclaved at 121 °C and 15 psi for 20 minutes. All the cultures were kept in a growth room at 25 °C under 16 hours illumination from white fluorescent lamps (3,180 Lux) or in the dark.

Determination of total anthocyanin content: Total anthocyanin content was measured using a method modified from that of Fuleki and Francis (1968). 1.5 g of hypocotyl from seedlings grown *in vitro* under both light and dark conditions after 7, 10, 15, 20 and 25 days, or petal (3, 6 and 9 cm in length) were blended with a mixture of 95% (v/v) ethanol and 1.5 N HCl (1.28: 0.23 mL). One mL of the extraction mixture was mixed with 95% ethanol and 1.5 N HCl (10.75: 1.88 mL) in a 20-mL vial wrapped with aluminum foil. This was gently shaken, kept overnight at 4 °C and then centrifuged at 6,870 x g for 10 min. The absorbance of the supernatant was read at 535 nm. Total anthocyanin content was calculated according to Fuleki and Francis (1968).

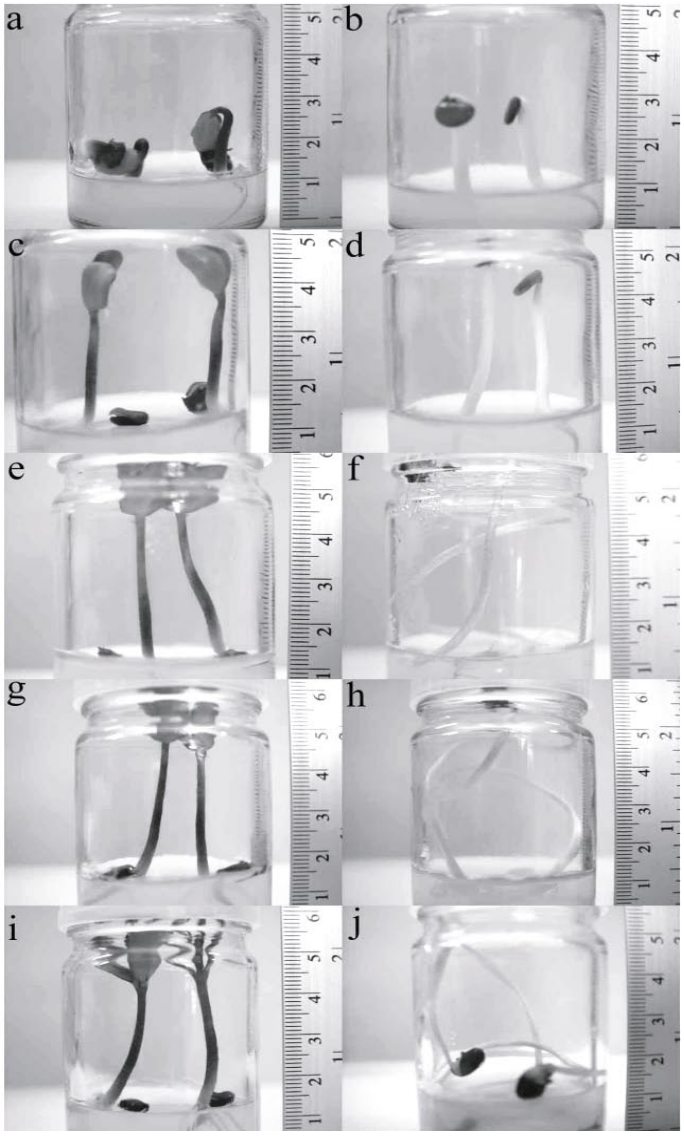

Fig. 1. Red Agati grown *in vitro* on 7, 10, 15, 20 and 25 days under light (a, c, e, g and i, respectively) and dark (b, d, f, h and j, respectively) conditions.

Anthocyanin localization: Hypocotyls of Red Agati seedlings grown *in vitro* under both light and dark conditions for 7, 10, 15, 20 and 25 days were cut independently with a razor blade. The free hand sections were then placed in a drop of water on a glass slide, covered with a cover slip and examined under a light microscope to localize the anthocyanin-containing cells.

Results

Morphology of Red Agati seedlings: Red Agati seeds, germinated *in vitro* developed normally into healthy seedlings in the light but not in the dark (Fig. 1). On day 7 in the light, the cotyledons started expanding and emerging from the seed coat while in the dark by day 25, the cotyledons still remained inside the seed coat. On day 10, true leaves could be found on some seedlings under light conditions. It was clearly seen that plant height was greater in the dark than in the light throughout the experiment (Fig. 2). Hypocotyls of dark-grown seedlings were white while those grown in the light were red. However, the red color appeared lighter as the hypocotyls grew.

Localization of anthocyanin in hypocotyl of Red Agati: When free hand sections of Red Agati hypocotyls, cultured under both light and dark conditions *in vitro,* were examined under a light microscope, it was revealed that anthocyanin could not be found in hypocotyls of dark-grown seedlings at all developmental stages. In contrast, anthocyanin was evidently present in the hypocotyls of light-grown seedlings (Fig. 3). The anthocyanin-containing cells were found in the epidermal and sub-epidermal layer. Besides, some could be seen in the peripheral cortex of the hypocotyl. The hypocotyl on day 7 appeared to have the highest number of anthocyanin-containing cells.

Total anthocyanin content in Red Agati hypocotyl: Total anthocyanin content was measured in hypocotyls of germinated seeds *in vitro* under both light and dark conditions for 7, 10, 15, 20 and 25 days. It was revealed that hypocotyls of light-grown seedlings had the highest anthocyanin content (290 µg/g FW) on day 7 (Fig. 4). Then total anthocyanin content decreased sharply. In contrast, anthocyanin were not detectable in hypocotyl of

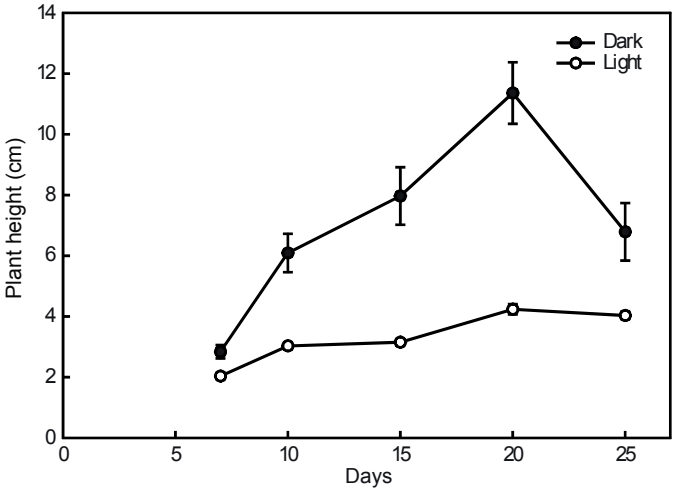

Fig. 2. Height of Red Agati grown *in vitro* at various developmental stages (values are means of 20 replications ± SD). Value in day 25 dropped because explants mostly died and mean of this stage are included all of those 20 replications.

dark-grown seedlings (Fig. 4).

Total anthocyanin content in Red Agati petal: During Red Agati flower development, the petal increased in size and length. The petal of 3 cm in length had the highest total anthocyanin content (455 µg gFW). Total anthocyanin content obviously decreased when the petal length increased further (Fig. 5).

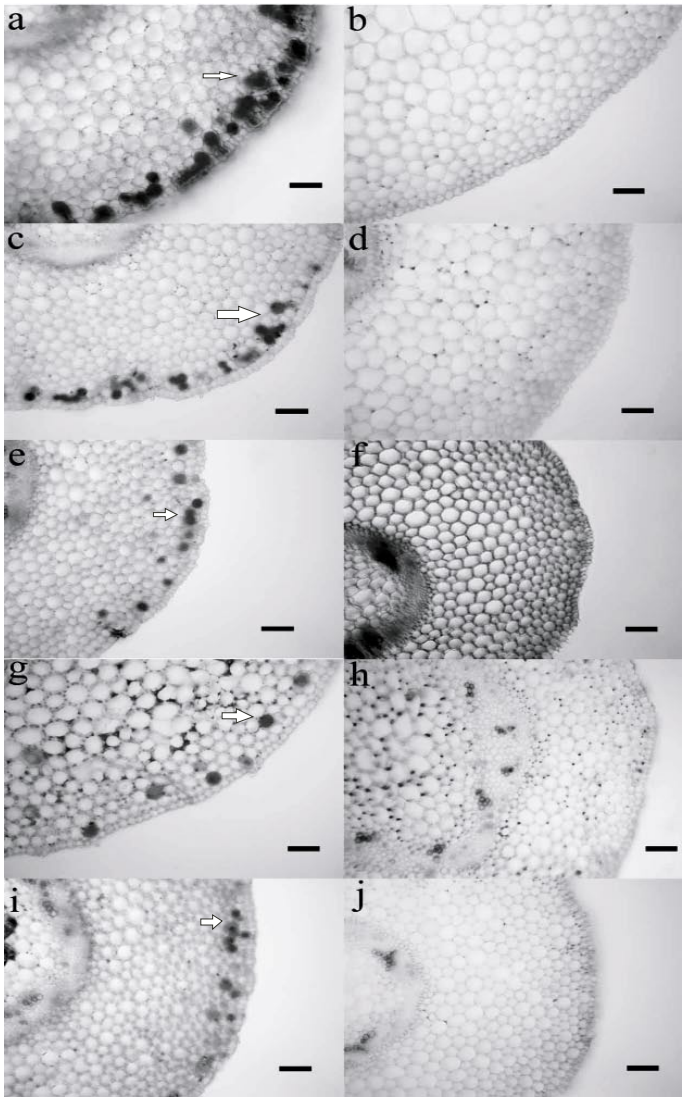

Fig. 3. Cross section of Red Agati's hypocotyl grown *in vitro* on 7, 10, 15, 20 and 25 days under light (a, c, e, g and i, respectively) and dark b, d, f, h and j, respectively) conditions. Scale bar = 100 µm.

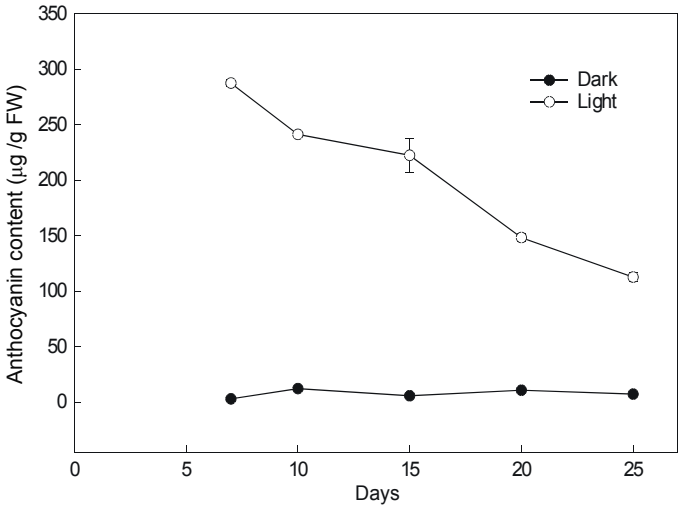

Fig. 4. Total anthocyanin content in hypocotyl of Red Agati at various developmental stages (values are means of 9 replications ± SD).

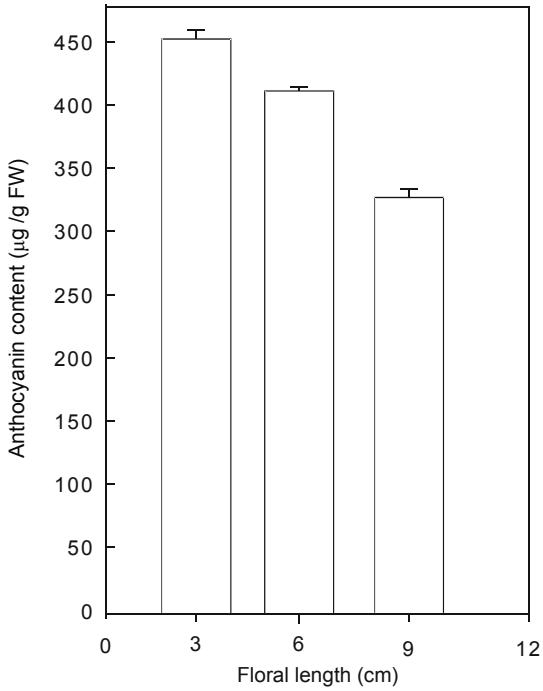

Fig. 5. Total anthocyanin content in petal of Red Agati at various developmental stages (values are means of 9 replications ± SD).

Discussion

The demand of natural anthocyanins has been growing due to their beneficial attributes for human health and well-being when compared to synthetic dyes (Mayer and van Staden, 1995; Stintzing and Carle, 2004). Though leaves and fruits from other plants are the good source of anthocyanins, such as, 188 and 375 µg/gFW of total anthocyanin content were found in purple basil leaf and acerola fruit, respectively (Simon, *et al.*, 1999; Vendramini and Trugo, 2004), hypocotyl and flower of Red Agati could provide high amount of anthocyanins as well. In this research, total anthocyanin content of Red Agati in reproductive (petal) and nonreproductive (hypocotyl) tissues were investigated for the first time. Reddening was found in hypocotyl of Red Agati seed germinated under light condition. This phenomenon, the presence of a red color in seedling, was also discovered in several *Pinus* species and many dicotyledonous epigeous seedlings, especially hypocotyl part which developed a red pigmentation shortly after it emerges from the soil into light (Nozzolillo *et al.*, 2002).

In Red Agati hypocotyl, anthocyanin was observed in the epidermal and sub-epidermal layer and peripheral cortex. There have been many reports showing that localization of anthocyanin varies depending on the species. Mostly, anthocyanins are found in or just below the epidermis (Chalker-Scott, 1999; Nozzolillo *et al.*, 2002). However, Hara *et al.* (2003) found that the location of anthocyanin-containing cells in radish hypocotyls changed during growth. On day 14, these cells were seen in the peripheral cortex but they were mostly found in the pericycle cells on day 21. This finding differed from the present result as the distribution of anthocyanin-containing cells of Red Agati hypocotyl was still in the sub-epidermal layer and peripheral cortex and remained or unchanged throughout the experiment.

There have been a number of reports showing that light is an important factor for anthocyanin synthesis (Matsumoto *et al.*, 1973; Chalker-Scott, 1999). Our results are also in agreement with these studies. Nevertheless, callus culture of *Vitis* and strawberry produced large amounts of anthocyanin in the dark (Yamakawa *et al.*, 1983; Nakamura *et al.*, 1999).

Total anthocyanin content in Red Agati hypocotyl on day 7 under light conditions was comparable to that of the 3-cm long petal. Furthermore, the developmental changes in their total anthocyanin content appeared similar.

Anthocyanin accumulating pattern in Red Agati hypocotyl of seedlings grown under light and dark condition was totally different. The localization of anthocyanin were mostly found in sub-epidermal layer and peripheral cortex at all developmental stages. The early developmental stage of both hypocotyl and petal had the higher amount of total anthocyanin content than the late developmental stage. Thus from the present results, the suitable time to harvest Red Agati hypocotyls for anthocyanin extraction would be on day 7 after sowing seeds under aseptic conditions. Similarly, petal of 3 cm long would also be ideal. Although total anthocyanin content was less in hypocotyl than in petal, the former is more readily available and would be a more favorable source or an attractive alternative to petal of Red Agati to use for anthocyanin production. Further studies would be needed to examine if the anthocyannin profiles in the hypocotyl and petal of Red Agati are comparable.

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Effect of different stalk lengths and certain chemical substances on vase life of gerbera (*Gerbera jamesonii* Hook.) cv. 'Savana Red'

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Abstract

An experiment was carried out to determine the effect of different stalk lengths and certain chemical substances on vase life of gerbera (*Gerbera jamesonii* Hook.) Cv. 'Savana Red'. Twenty treatment combinations consisting of four chemicals *viz*., sucrose 4 %, sucrose 4 % + 8-HQC 250 ppm , sucrose 4 % + aluminum sulphate 100 ppm , sucrose 4 % + citric acid 250 ppm , along with control (distilled water) and four lengths of stalk *viz*., 30, 40, 50, and 60 cm. The vase solution of sucrose 4% + 8-HQC 250 ppm and stalk length of 60 cm, individually and in combination increased fresh weight of flowers by promoting solution uptake. These treatments are also beneficial for improving the vase life of flowers and useful life of flowers, opening of disc florets, with bright, shining red colour and freshness for a longer duration.

Key words: Gerbera, *Gerbera jamesonii* Hook., stalk length, chemicals, vase life, sucrose, citric acid, HQC

Introduction

Gerbera belongs to the family Asteraceae, which consists of many other important cut flowers namely aster, dahlia, chrysanthemum, gaillardia, zinnia, marigold *etc.* (Bose and Yadav, 1989). The genus Gerbera, named in the honour of German naturalist, Traugott Gerber, consists of about 40 species of hardy and perennial flowering plants, out of which only *Gerbera jamesonii* Hook. is under cultivation. It is native to Natal and Transvaal and is commonly known as Transvaal daisy, Barberton daisy or African dasiy. It is an important flower grown through out the world under wide range of climatic conditions.

Keeping quality of flowers is affected by internal and external factors. The internal factors which are responsible for the keeping quality of cut blooms, are the rate of water absorption and transpiration. Both these factors again depend on the relative area of absorption and the total water holding capacity of the tissues. After the flower is detached, the area of absorption is reduced drastically, whereas the rate of proportionate area for transpiration is much higher. Therefore, unless something is done to reduce transpiration, the cut flower will wither in less time. Respiration is another internal factor that affects the life of the cut flower. Besides, some environmental factors also affect cut flower life. These are temperature, relative humidity and wind velocity. Postharvest handling plays an important role in enhancing keeping quality of flowers wherein efforts are made to reduce stem plugging, restrict microbial activity, delay flower senescence through provision of external source of water and nutrients as required by the flower.

Materials and methods

The experiment was conducted at the P.G Research Laboratory, Department of Horticulture, N.M. College of Agriculture, Gujarat Agricultural University, Navsari Campus, Navsari. Twenty treatment combinations consisting of four chemicals *viz*., sucrose 4 % (T_1), sucrose 4 % + 8-HQC 250 ppm (T_2), sucrose 4 % + aluminum sulphate 100 ppm (T_3), sucrose 4% + citric acid 250 ppm (T_4) along with control (distilled water) (T_0) and four lengths of stalk *viz*., 30 cm (L_1), 40 cm (L_2), 50 cm (L_3), and 60 cm (L_4) were tried in Completely Randomized Design with Factorial concept. All the treatments were repeated thrice. The experimental material were kept in glass bottles of 300 mL capacity holding 250 mL treatment solution prepared in the laboratory and a bunch of three stalks of gerbera (Cv. Savana Red), harvested at the stage when the outer two row of disc florets were perpendicular to the stalk. Total sixty glass bottles and one hundred and eighty stalks, having four different uniform lengths of 30, 40, 50 and 60 cm were utilized. The stalks were slightly recut at basal end at every 3rd day during experimentation. The data on uptake of solution (mL) at 3rd, 6th and 9th days, time taken (days) to open 50, 75, and 100 % disc florets, useful life of flowers (days), vase life of flower (days), recorded during the experiment were subjected to statistical analysis. The design with factorial concept as described by Panse and Sukhatme (1967) was used. The significance of the treatment differences was tested by 'F' test on the basis of null hypothesis. The appropriate standard error of mean (S.Em.±) were calculated in each case and the critical differences (C.D.) at 5 per cent level of probability was worked out to compare the two treatment effects.

Results

The data on solution uptake by gerbera flower on third, sixth and ninth day are presented in Table 1. The effect of different chemicals and stalk length on solution uptake by gerbera flower after three, six and nine days were found to be significant. Among different chemicals, maximum uptake of solution (45.0 mL) at

Table 1. Effect of different chemicals and stalk lengths (cm) on the solution uptake by the gerbera flowers

Treatments	Solution uptake (mL)		
	3rd day	6th day	9th day
Chemicals (T)			
T_1	28.25	18.64	1.94
T_2	45.00	41.2	23.94
T_3	30.60	26.36	11.32
T_4	34.84	28.14	17.49
T_0	21.56	14.91	0.00
LSD (*P*=0.05)	0.99	0.70	0.49
Stalk lengths (L)			
L_1	24.14	18.13	0.00
L_2	28.40	22.74	11.26
L_3	33.79	27.36	13.5
L_4	41.86	35.16	18.99
LSD (*P*=0.05)	0.88	0.62	0.44

Zero value indicate that these flowers had completed their vase life before ninth day.

Table 2. Interaction effect of different chemicals and stalk lengths (cm) on the solution uptake by the gerbera flowers

Treatments	Solution uptake (mL)		
	3rd day	6th day	9th day
T_0L_1	14.76	10.13	0.00
T_0L_2	19.56	13.43	0.00
T_0L_3	21.1	15.06	0.00
T_0L_4	30.83	21.03	0.00
T_1L_1	17.96	13.00	0.00
T_1L_2	21.8	16.80	0.00
T_1L_3	33.3	19.16	0.00
T_1L_4	39.96	25.60	7.76
T_2L_1	41.46	32.43	0.00
T_2L_2	43.26	40.43	28.50
T_2L_3	45.40	41.60	29.70
T_2L_4	49.86	46.40	37.56
T_3L_1	22.50	18.43	0.00
T_3L_2	26.50	21.60	10.58
T_3L_3	32.23	26.36	16.40
T_3L_4	41.16	39.06	18.30
T_3L_1	24.03	16.67	0.00
T_3L_2	30.90	21.36	17.23
T_3L_3	36.93	30.80	21.40
T_3L_4	47.50	43.73	31.33
LSD (*P*=0.05)	1.98	1.40	0.99

Zero value indicate that these flowers had completed their vase life before ninth day.

third day, (41.2 mL) at sixth day and at ninth day (23.94 mL) were observed when flower stalks were kept in the solution of sucrose (4 %) + 8 –HQC (250 ppm) (T_2). Among different stalk lengths, maximum amount of solution 41.86, 35.76 and 18.99 mL was absorbed by gerbera flower having L_4 stalk length at third, sixth, and ninth day. The interaction effect of different chemicals and stalk length on solution uptake by gerbera flower on third day (Table 2) was significant. Maximum solution uptake at third (49.86 mL), sixth (46.4 mL) and ninth day (37.56 mL) was noticed when longest gerbera flower (L_4) was kept in solution of sucrose (4 %)+ 8-HQC (250 ppm) (T_2 L_4). The data pertaining to 50, 75 and 100 % opening of florets revealed that all the treatments showed significant difference (Table 3). Among the different chemicals the highest time taken for opening of 50% disc florets (7.18 days), 75 % disc florets (9.08 days), 100% disc florets (10.49 days) was recorded when gerbera stalks were kept in solution of sucrose (4 %)+ 8-HQC (250 ppm) (T_2). The interactions effect of different chemicals and stalk lengths on time taken for opening of 50, 75 and 100 % disc florets was found to be significant and have been presented in Table 4. The data indicated that the maximum time taken for opening of 50% disc florets (7.83 days), 75 % disc florets (10.37 days), 100% disc florets (12.18 days) was resulted when gerbera stalk having longest length were kept in solution of sucrose (4 %)+ 8-HQC (250 ppm) (T_2 L_4). The data presented in Table 5 on the effect of different chemical and stalk lengths on the useful life of gerbera was found to be significant. The data presented in Table 5 revealed

Table 3. Effect of different chemicals and stalk lengths (cm) on the time taken to open 50, 75 and 100 per cent disc florets of gerbera

Treatments	Time taken to open (days)		
	50 % disc florets	75% disc florets	100% disc florets
Chemical (T)			
T_1	5.21	6.90	7.75
T_2	7.18	9.08	10.49
T_3	6.17	7.93	8.77
T_4	6.33	8.70	9.20
T_0	4.25	5.75	6.29
LSD (*P*=0.05)	0.19	0.14	0.28
Stalk length (L)			
L_1	5.30	6.55	7.19
L_2	5.32	7.34	7.93
L_3	6.02	7.98	9.07
L_4	6.67	8.82	9.66
LSD (*P*=0.05)	0.19	0.12	0.25

Table 4. Interaction effect of different chemicals and stalk lengths (cm) on the time taken to open 50, 75 and 100 per cent disc florets of gerbera

Treatments	Time taken to open (days)		
	50 % disc florets	75% disc florets	100% disc florets
T_0L_1	3.62	5.47	5.96
T_0L_2	4.09	5.61	5.98
T_0L_3	4.53	5.72	6.50
T_0L_4	4.79	6.21	6.73
T_1L_1	4.15	6.23	6.52
T_1L_2	5.06	6.47	6.94
T_1L_3	5.17	6.52	8.02
T_1L_4	6.46	8.40	8.80
T_2L_1	6.83	7.19	8.73
T_2L_2	6.76	9.19	10.06
T_2L_3	7.30	9.56	11.0
T_2L_4	7.83	10.37	12.18
T_3L_1	6.40	6.30	6.66
T_3L_2	5.21	7.53	8.00
T_3L_3	6.50	8.66	10.10
T_3L_4	6.59	9.23	10.33
T_3L_1	5.51	7.56	8.10
T_3L_2	5.47	7.90	8.68
T_3L_3	6.63	9.46	9.72
T_3L_4	7.70	9.88	10.3
LSD (*P*=0.05)	0.69	0.28	0.57

Table 5. Effect of different chemicals and stalk lengths (cm) on useful life and vase life of gerbera flowers

Treatments	Useful life of flowers (days)	Vase life of flowers (days)
Chemicals (T)		
T_1	6.46	7.96
T_2	9.87	11.31
T_3	7.85	9.66
T_4	8.31	9.94
T_0	5.04	7.66
LSD (*P*=0.05)	0.26	0.36
Stalk lengths (L)		
L_1	5.82	6.96
L_2	7.11	9.14
L_3	8.09	10.05
L_4	9.00	11.07
LSD (*P*=0.05)	0.23	0.32

Table 6. Interaction effect of different chemicals and stalk lengths (cm) on useful life and vase life of gerbera flowers

Treatments	Useful life of flowers (days)	Vase life of flowers (days)
T_0L_1	4.14	6.48
T_0L_2	4.86	7.69
T_0L_3	5.12	7.88
T_0L_4	6.06	8.60
T_1L_1	5.63	5.79
T_1L_2	6.19	7.86
T_1L_3	6.72	8.50
T_1L_4	7.30	9.70
T_2L_1	6.91	7.98
T_2L_2	10.12	11.30
T_2L_3	10.74	12.30
T_2L_4	11.69	13.65
T_3L_1	6.51	7.76
T_3L_2	7.19	9.39
T_3L_3	7.93	10.2
T_3L_4	9.77	11.2
T_3L_1	5.94	11.3
T_3L_2	7.23	6.80
T_3L_3	9.92	9.46
T_3L_4	10.16	11.36
LSD (*P*=0.05)	0.52	0.73

that among different chemicals, longest useful life of flower (9.87 days) was obtained in sucrose (4 %)+ 8-HQC (250 ppm) (T_2). Among flower lengths, the highest useful life of flower (9.0 days) was recorded from L_4 (60 cm).The interaction effect of different chemicals on useful life of flower was also found to be significant (Table 6). Longest useful life of flower (11.69 days) was recorded when gerbera cut flower having maximum length L_4 (60 cm) were kept in solution of sucrose (4 %)+ 8-HQC (250 ppm) (T_2 L_4). The data furnished in the Table 5 revealed that among the different chemicals, the maximum vase life (11.31 days) was obtained by the use of sucrose (4 %)+ 8-HQC (250 ppm) (T_2). In case of stalk length, the vase life of gerbera increased with increase in length. The maximum vase life of stalk (11.07 days) was obtained when stalk of gerbera was of 60 cm length (L_4). The interaction effect of different chemicals and stalk length on vase life of flower presented in Table 6 was found to be significant. Longer vase life (13.65 days) was obtained when gerbera stalk of 60 cm length were kept in sucrose (4 %)+ 8-HQC (250 ppm) solution (T_2 L_4).

Discussion

From the Table 1 and 2, it is obvious that the solution uptake through stalk was influenced by chemicals, stalk lengths and their combinations. The absorption of water through stalk was maximum at 3rd, 6th and 9th day when stalks were kept in vase solution of sucrose (4 %)+ 8-HQC (250 ppm). Similar results were obtained in gladiolus (De *et al.,* 1996) and in tuberose (Reddy *et al.,* 1997). A beneficial effect of sucrose in absorption was reported by De and Barman (1998b) in tuberose and of 8-HQC by Bhattacharjee (1993) in rose. Other chemicals like aluminum sulphate was beneficial as it acidified the holding solution, which results in greater solution uptake by gerbera. Similar results were obtained in gladiolus (Gowda and Gowda, 1990) and in tuberose (Saini *et al.,* 1997 and De and Barman, 1998a). Aluminum sulphate with sucrose in vase solution significantly influenced the water uptake of the tuberose cut spike (Reddy and Singh, 1996). The maximum solution uptake by cut flower stalks was observed in longest stalk of gerbera at 3rd , 6th and 9th day. Similar result was obtained by Bhattacharjee (1993, 94) in rose. Maximum solution uptake was observed under interaction of 60 cm long stalk length and sucrose (4 %)+ 8-HQC (250 ppm) thus longer stalk length having more carbohydrate and HQC reduced stem blockage which results in increase in the solution uptake.

Results revealed that time taken for opening of the disc florets was significantly influenced by chemicals and stalk lengths individually as well as by their combination (Table 3 and 4). The maximum time taken to open the disc florets was recorded in sucrose (4 %)+ 8-HQC (250 ppm) (T_2) solution. According to Larsen and Cromorty (1967) and Burdett (1970) the microbial growth are checked by the germicidal and bactericidal properties of these chemical substances and thereby were found effective for gladiolus (Lal *et al.*, 1990 and Murali and Reddy, 1991). Other chemical treatments like sucrose (4 %) + aluminum sulphate (100 ppm) and sucrose (4 %) + citric acid (250 ppm) significantly delayed opening of disc florets over control. In case of stalk lengths, maximum time taken for opening of disc florets was recorded for flowers having longest stalk length of 60 cm (L_4). Interaction effect of chemical and stalk length indicated that maximum time taken for opening of disc florets was observed with the stalk having maximum length and kept in sucrose (4 %) + 8-HQC (250 ppm) solution.

Maximum useful life was recorded in longest stalk length alone and in combination with chemicals like sucrose and 8-HQC. This may be due to higher carbohydrate, thus promoting respiration and extending the longevity. Similar results were obtained in tuberose (De and Barman, 1998b). Sucrose is a main source of energy and good respiratory substrate for the maintenance of osmotic potential while 8-HQC helped in controlling harmful bacteria and prevented bacterial plugging of water conducting tissues and there by increased useful life of gerbera stalk.

Maximum vase life was obtained with sucrose (4 %) +8-HQC

(250 ppm) holding soluation. Similar results were recorded in tuberose (Singh and Arora, 1995), rose (Masousky,1969 and Bhattacharjee,1993) and in chrysanthemum (Bhat *et al.*,1999). The increased longevity of flower due to sucrose could be explained from the role of applied sugars in delaying senescence and promoting respiration. It delays the onset of excessive protein degradation as in gladiolus (Gowda and Gowda,1990 and Merwe *et al.,* 1986), tuberose (Pathak *et al.,1979*); Mukhopadhyaya,1982; De and Barman 1998 a) and rose (Borochov *et al.*,1976; Masousky,1969 and Bhattacharjee, 1999)

Sucrose is the main source of energy and good respiratory substrate for the maintenance of osmotic potential in flower. Kaltaler and Steponkus (1976) concluded that the main effect of applied sugar in extending the longevity of flowers was to maintain mitochondrial structure and function. Sucrose could also have antagonist effect on abscisic acid in delaying the senescence as observed in rose (Borochov *et al.,* 1976) and carnation (Mayak and Dilley, 1976). Marousky (1971) explained that sugar improves the water balance in cut flowers, uptake of water is increased due to stomatal closure which reduces the transpiration rate.

The effect of 8-HQ components (8-HQC and 8-HQS) in enhancing vase life of cut flowers had been attributed to its antibacterial property as noted by Serini and Banfi (1974). According to Lal *et al.* (1990) the chelating properties of the Quinoline compounds/esters probably chelated the metal ions of enzymes active in creating the stem blockage. HQC also acidifies the water and affect the flower longevity. Sucrose with HQS has germicidal effect and was beneficial for prolonging the vase life of tuberose (Reddy *et al.*,1997).

The interaction effect of chemicals and stalk lengths was also found significant. The maximum vase life of the gerbera was recorded when gerbera stalk having longest length (L_4) kept in vase solution of sucrose (4 %)+ 8-HQC (250 ppm) solution (T_2 L_4). These favourable effect on vase life of spike might be due to sufficient availability of restorable substrate and 8-HQC which reduced microbial activity and increased and absorption of solution.

The results revealed that the vase solution of sucrose 4% + 8-HQC 250 ppm and stalk length of 60 cm, individually and in combination increased fresh weight of flowers by promoting solution uptake. These treatments are also beneficial for improving the vase life of flowers and useful life of flowers, opening of disc florets, with bright, shining red colour and freshness for a longer duration.

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Micropropagation of *Parthenocissus quinquefolia* (L.) from seedling explants

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Abstract

Parthenocissus quinquefolia L. was successfully micropropagated through axillary bud multiplication from seedling explants. Shoot tips were isolated from seedlings and cultured on B_5 medium supplemented with 1.33-2.22 µM benzylaminopurine (BA) and 0.107 µM α-naphthaleneacetic acid (NAA) to induce axillary buds. The sprouted axillary buds formed multiple shoots when cultured on B_5 medium supplemented with 2.22 µM BA and 0.246-0.49 µM indole-3-butyric acid (IBA). The elongated shoots rooted in B_5 medium containing 0.49 µM IBA and they survived from acclimatizing in soil to grow into healthy plants.

Key words: *Parthenocissus quinquefolia*, axillary buds, micropropagation, B_5 medium, benzylaminopurine (BA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA)

Introduction

Parthenocissus quinquefolia (L.) Planch (family Vitaceae) commonly named as Virginia creeper, has gained considerable attention in ornamental industries and in landscape constructions because of its climbing habit and brilliant foliage that turns scarlet, crimson, or orange in the fall (http://www.floridata.com/ref/p/parth_q.cfm). The creepers are tolerant of most soil conditions and grow well under full sun to full shade. However, *P. quinquefolia* plants do not grow well in cold climate, limiting its application in areas where the climate is cold year-around. *P. quinquefolia* was also found to be a symptomatic alternative host of *Xylella fastidiosa* in habitats surrounding agricultural systems in Florida (Hopkins and Adlerz, 1988). Therefore its common usage as an ornamental and wide distribution is a threat in agricultural and urban systems because of potential disease transmission to susceptible crop and trees (Hopkins and Adlerz, 1988, McElrone *et al.*, 2001). *In vitro* cultures have long been accepted for the production of virus-free plants (Mellor and Stace-Smith, 1977; Wang and Hu, 1982; Tian *et al.*, 1993). *In vitro* cultures are also used in selecting and screening of cultures that exhibit genetic variation, and for tolerance to low or high temperatures (Tomes and Swanson, 1982; Chaleff, 1983; Palonen and Buszard, 1998; Mokotedi *et al.*, 2000). It is therefore highly desirable to use such biotechniques to improve and accelerate *P. quinquefolia* breeding progress and reduce long term program cost.

Furthermore, *P. quinquefolia* show strong resistance to phylloxera. Grape phylloxera (*Phylloxera vitifolli* Fitch) is a serious risk to the viticulture and wine industry (He, 2001). This insect attacks grapevine roots, slowly causing a decline in vine health and ultimately destroying the grapevine. Attempts made to introduce the resistance from *P. quinquefolia* into grape by traditional breeding methods were unsuccessful due to species incompatibility (He, 2001). Somatic hybridization has the advantage of overcoming species incompatibility and improving plant morphological traits or disease resistance by DNA integration (Pandeya *et al.*, 1986; Tian *et al.*, 2002; Zimnoch-Guzowska *et al.*, 2003), and it could offer an alternative to transferring the strong phylloxera resistance of *P. quinquefolia* to grape cultivars. Thus, there is a need to understand the *in vitro* culture characteristics of *P. quinquefolia* to benefit the successful application of biotechnological approaches that have been used for plant modification.

Tissue culture is a basic tool in plant biotechnology. The successful and efficient application of tissue culture is highly dependent on the establishment of a reliable regeneration system. There were only two reports on *in vitro* study of *P. quinquefolia,* which described chitinase isolation from *P. quinquefolia* cell cultures (Bernasconi *et al.*, 1987; Flach *et al.*, 1993). To the best of our knowledge, no report is available on *in vitro* regeneration of *P. quinquefolia.* The objective of this study was to investigate the amenability of *P. quinquefolia* for *in vitro* cultures. The *in vitro* plantlets and methods can be subsequently used as a plant source for the cold tolerant treatment and other *in vitro* plant improvements.

Materials and methods

Establishment of aseptic explant: *Parthenocissus quinquefolia* (L.). Planch seeds were first washed with running tap water for five minutes and soaked in water overnight. The soaked seeds were treated with 70% ethanol for 2 min. After rinsing two times with sterile water, the seeds were further surface sterilized in 1% (w/v) sodium hypochlorite solution for 60 min, followed by four washes in sterile water. The seeds were placed on B_5 (Gamborg *et al.*, 1968) and half strength Murashige and Skoog medium (Murashige and Skoog, 1962). All the media used in this study

contained 2% (w/v) sucrose and were gelled with 0.5% agar. The pH of the media was adjusted to 6.0 prior to autoclaving at 121°C for 20 minutes. Seeds were germinated in light conditions with a 16-h photoperiod of 40-50 µmol m^{-2} s^{-1} at 23±2 °C. The aseptic seedlings that were obtained after 30 days of culture were used as the source of explants for the subsequent experiments.

Axillary shoot initiation and multiplication of *P. quinquefolia*: Shoot tips (include 1 cm hypocotyls and two cotyledons) were excised from the 4 week aseptic seedlings and were placed on shoot induction media. The effects of BA and mineral composition on axillary shoot formation were evaluated by adding various concentration of BA (0.0, 1.33, 2.22, 3.55, 8.87 and 17.74 µM) to B_5 or half strength MS media supplemented with 0.1 µM NAA. Each treatment had ten cultures and all experiments were repeated four times (total of 40 explants per experiment). The number of axillary shoots produced per explants and percentage of explants showing emergence of shoots were counted after 4 weeks in culture.

For the multiplication of shoots, semisolid B_5 medium was used. The B_5 medium was variously supplemented with BA at different concentrations (2.22-13.3 µM) in conjunction with auxins (IAA, IBA and NAA in a range from 0.1 to 5.7 µM). Axillary shoots were excised from clusters and subcultured at 30 day intervals. Multiplication rates were calculated on the basis of the new axillary buds derived from one culture at the end of each passage.

Root initiation and transplantation: After 5 weeks of subculture in multiplication media, 2-3 cm long shoots with two to four leaves were separated from the explants and transferred to semisolid B_5 medium. To study the effect of auxins on root initiation, the medium was variously supplemented with 0.25-0.98 µM IBA and 0.27-1.07 µM NAA. Each rooting treatment consisted of 10 shoots and was replicated three times, representing a total of 30 observations per treatment. The percentage of shoots forming roots was determined after 3 weeks of culture.

Transfer to greenhouse conditions: Rooted plantlets on media were placed in the greenhouse with natural light radiation for one week. Then the jar covers were opened and the plants were allowed to acclimatise for three days. The plantlets were removed from culture, rinsed thoroughly with water to remove media and transferred to potting mixture (perlite : vermiculite 1:1). Individual plantlets were covered loosely with plastic wrap for one week. Thereafter, the plants were grown in normal greenhouse and nursery conditions.

Results and discussion

Initiation of cultures: One hundred seeds were put on B_5 and ½ MS media for *in vitro* germination. The surface-sterilization procedures yielded 97-98% aseptic seed cultures. Seeds started germinating at 12-15 day and grew to 3-4 cm long with fully extended cotyledons at 30 days. B_5 and ½ MS media were equally effective for *P. quinquefolia* seed germination, 65% and 68%, respectively.

All media became brown after 2 days of culture due to the release of color materials from the seed coat. To test the germination effect of the brown media, half of seeds were left in the same brown media and half were transferred to fresh media at one week, to allow these seeds fully release the brown chemicals and make sure there was no bacterial or fungal contamination. There was no difference in germination frequency and seedling growth during 30 days of observation. The results indicated that color materials released from the seed coat were not inhibitory for the seedling growth.

Axillary shoot initiation and multiplication of *P. quinquefolia*: Shoot excised from hypocotyls were transferred to induction media. The B_5 medium was superior to ½ MS for *P. quinquefolia* axillary shoot induction, as 100% explants cultured in B_5 media supplemented with various plant growth regulator (PGR) combinations induced axillary buds, produced more axillary buds per explants and grew longer axillary shoots compared with cultures in ½ MS media (Table 1). It was noted that a high percentage of explants (90-100%) cultured on basic B_5 or half strength MS media without any PGR produced an average of only one axillary shoot but was weaker than PGR media. Among PGR treatments, those cultured on B_5 + 2.22 µM BA + 0.1 µM NAA (BA: NAA 2.22:1) showed the best shoot elongation performance. Though the axillary bud number increased with higher concentrations (over 3.55 µM) of BA and consistent NAA combinations, the shoot elongation was inhibited (Table 1). Higher BA levels also strongly inhibited shoot elongation in grape, which is from same family as *P. quinquefolia* (Lee and Wetzstein, 1990).

Table 1. Effects of BA and mineral composition on axiliary bud induction of *P. quinquefolia* shoots

Medium type	BA (µM)	NAA (µM)	Explants producing axillary shoots (%)	Axillary shoot length (cm±SE)
1/2 MS	0.0	0.0	90.0	1.0±0.32
	1.33	0.1	90.0	1.1±0.36
	2.22	0.1	97.5	1.1±0.33
	3.55	0.1	80.0	0.4±0.18
	8.87	0.1	77.5	0.4±0.15
	17.74	0.1	70.0	0.3±0.16
B_5	0.0	0.0	100	1.5±0.34
	1.33	0.1	100	1.6±0.26
	2.22	0.1	100	1.7±0.24
	3.55	0.1	100	1.4±0.30
	8.87	0.1	100	0.9±0.25
	17.74	0.1	100	0.6±0.17

Each treatment had ten cultures and all experiments were repeated four times (total of 40 explants per experiment). The percentage of explants showing emergence of shoots and shoot length were counted within four weeks.

Shoot multiplication: Shoots induced from the above cultures were excised and transferred to semisolified B_5 medium supplemented with BA and auxins for further proliferation. Different concentrations of BA (2.22-13.3 µM) and NAA (0.1-0.54 µM) were tested in preliminary experiments. Results indicated that a low concentration of BA (2.22µM) and NAA (0.1 µM) in combination were suitable for the new axillary bud induction and growth. High concentrations of BA (13.3 µM) and NAA (0.54 µM) in combination reduced the percentage of explants that formed bud and reduced the bud elongation. Only 70% explants produced shoots and the average shoot growth was 0.3 cm in one month under this culture condition. In

tissue culture, where the balance of cytokinin to auxin is a key morphogenic factor for obtaining maximum multiplication and growth of the shoots (Skoog and Miller, 1957), B_5 medium with 2.22 µM BA showed better effects on multiplication when used alone in preliminary studies. It was then variously supplemented with IAA, IBA and NAA in a range from 0.1µM to 5.7 µM. The effects of auxins were determined after 30 days culture. Of the various plant growth regulator combinations that were tested, B_5 + 2.22 µM BA + 0.25-0.49 µM IBA or B_5 + 2.22 µM BA + 0.1 µM NAA proved to be the best for *P. quinquefolia* axillary shoot multiplication and growth. On these media, over 93% explants produced average of two shoots, which grew more than 1.6 cm in one month. The percentage of explants producing shoots was reduced and the shoot elongation was inhibited when higher concentrations of auxins (IBA greater than 1.48 µM and NAA greater than 0.27 µM) were used. Compared with IBA or NAA, IAA was less effective for *P. quinquefolia* shoot induction and growth, as indicated by a lower percentage of shoot differentiation (maximum 86.7%) and slow shoot growth (maximum 1.2 cm).

On average, a shoot multiplication rate of 2 fold every 30 days was maintained. It is possible to obtain a higher shoot multiplication rate (3-4 fold) on B_5+ 17.74 µM BA + 0.1 µM NAA, but the elevated concentration of BA inhibited the elongation of shoots and possibly lowered the recovery of shoots with rooting potential in the subsequent passage because of reduced shoot length, as is the case of *Anogeissus pendula* and *A. latifolia* culture (Saxena and Dahwan, 2001). Based on our observation of Jujube culture, the number of shoots can increase with every passage and become stable after five to six subcultures (Luo *et al.*, 1996). In a grape axillary shoot proliferation study, Lee and Wetzstein (1990) also observed the rate of proliferation was slow during culture establishment for the first 8 weeks, but increased rapidly thereafter. Harbage (2001) reported that reducing subculture frequency of *Echinacea angustifolia*, *E. pallida* and *E. purpurea* from 4 to 2 weeks can increase total shoots produced per subculture from 2.8 to 23.9 after 12 weeks. We observed slow growth and yellow leaves of *P. quinquefolia in vitro* shoots when the subculture frequency was extended from 30 days to 45 days. It is possible to improve the *P. quinquefolia* multiplication rate and keep high shoot quality by shortening the subculture time while maintaining multiple subculture cycles.

The regenerated shoots longer than 2 cm and with a pair of true leaves were transferred to B_5 medium in the presence of IBA or NAA to initiate roots. *P. quinquefolia* rooting frequency was significantly affected by plant growth regulators. IBA alone at 0.49 or 0.98 µM induced the highest percentage of rooting (100%) with 3 weeks of culture (Table 2). The highest root number per shoot was induced at IBA concentration of 0.49 µM, with three to four vigorous terminal roots and with lateral roots on them. The roots emerged directly from the base of the shoots without callusing. Though rooting efficiency and the root count per shoot was similar when IBA was increased to 0.98 µM, callus formation was observed at this higher concentration IBA treatment. The effects of increasing IBA concentration results in callusing at the shoot base and suppression of root elongation was reported in other plant species. Koroch and coworkers (2002) reported that higher IBA rooted purple coneflower plantlets had significantly lower survival rates after transfer to the greenhouse compared to lower IBA treatments due to the absence of roots or an anomalous adventitious root system originating from callus. On the NAA (0.27-1.07 µM) rooting medium, the rooting frequency was relatively low (60-76.7%), moreover, NAA induced root number per shoot was lower than IBA treatment (averaging 2 vs 3), calli formed at the shoot cut, and some roots were thick and also differentiated as callus-like. Only 76% of plantlets rooted in NAA survived after acclimatization, while over 90% of plantlets rooted in IBA survived with same treatment. Our results showed that IBA at 0.49 µM is ideal for rooting of *P. quinquefolia.*

Table 2. Effect of auxin (IBA and NAA) concentrations on rooting of *in vitro* regenerated shoots of *P. quinquefolia*

Auxin (µM)	Rooting (%)	Performance of root
IBA (0.25)	80.0 ± 10.0	Less lateral roots
IBA (0.49)	100.0 ± 0.0	Normal, no callus, with lateral roots
IBA (0.98)	100.0 ± 0.0	Slim root, thicker callus
NAA (0.27)	63.3 ± 15.3	Thick root
NAA (0.54)	76.7 ± 11.6	Callus
NAA (1.07)	60.0 ± 26.5	Many callus

Acclimatization and transplantation: Sixty-five regenerated plantlets were transferred into pots filled with perlite and vermiculite (1:1). The survival rate of these plantlets was over 90% (30/33) following root initiation with 0.49µM IBA (Fig. 1). Phenotypically, all the *P. quinquefolia* plants produced in this study appeared normal. By contrast, only 75% (25/32) survival rate was achieved with NAA (0.54 µM) treated plantlets, which is significantly lower than the IBA treatment. The result is consistant with previous reports that adventitious roots originating from callus has a deleterious effect on regenerated plant transplantation (Koroch *et al.*, 2002). Our results indicated that *P. quinquefolia* root initiation treatment with IBA is superior to NAA by inducing more robust root systems directly from shoots.

In vitro multiplication of elite plant species via axillary bud proliferation was preferred in many studies by avoiding the tendency of undergoing somatic mutation (Lee and Wetzstein, 1990; Saxena and Dhawan, 2001; Shu *et al.*, 2003). Molecular evidence showed that plants derived from axillary buds were clonally uniform and genetically stable (Shu *et al.*, 2003). We recently regenerated *P. quinquefolia* plantlets from explants of

Fig. 1. Well-established *P. quinquefolia* plants growing in pots

field grown shoot tips using the above regeneration protocols, indicating a practical application for rapidly propagating elite *P. quinquefolia* varieties.

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Effects of organic manure on okra (*Abelmoschus esculentus* (L.) Moench) production

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Abstract

The effect of different organic manures (cow dung, poultry manure and compost) on the yield of okra, soil physical and chemical characteristics was investigated at the University of Ghana, Legon. Results obtained revealed that the application of recycled garden solid waste compost, poultry manure and cow dung improved the soil physical condition, particularly, structure and drainage, increased nutrient and organic matter levels and enhanced the yield components of okra plants. Inorganic fertilizer improved only chemical properties, but soil physical properties such as structure was not improved. There were improvements in pod yield, yield components and pod fibre content on all manured plots. The study clearly inidicated the superiority of poultry manure over cow dung and compost as a source of manure for okra production.

Key word: *Abelmoschus esculentus***,** okra, organic manure, soil characteristics, yield

Introduction

To sustain crop yields, the removal of nutrients from the soil has to be balanced by added amounts. Fertilizers are therefore added to the soil to supply elements essential to the growth of plants. The use of chemical fertilizer is necessary for supplying the nutrient requirements but without recycling of crop residues, yields suffer. Organic manures are normally derived from animal or plant sources and are excellent sources of organic matter, but relatively low in nutrients. Organic manures therefore need to be applied at very high rates (20,000-40,000kg/ha) to make up for their low nutrient content and to supply enough humus to measurably improve the soil physical condition (Mathew and Karikari, 1995).

Animal manure is an important source of N, P and K and its additions to the soil increases the available P and exchangeable K, Ca and Mg content (Magdoff, 1998). In addition to providing nutrients for crop growth, manure has several beneficial effects on soil properties. Several researchers have reported that the application of organic waste leads to improved structural stability, lower bulk density of the soil by increasing both the organic fractioning of the soil and a balance between fine and coarse pores, organic manures improve moisture retention, water infiltration rate and the hydraulic conductivity of soil (Tisdale *et al.,* 1990; Young, 1997).

Most of the okra produced in Ghana and other West African countries are grown on sandy loam and loam soils which, with time, may become deficient in N, P, K, Mg and B (Nelson and Tisdale, 1978). Chemical fertilizers do not sustain soil fertility for longer and after their continual use there is deterioration of soil characteristics. This study was undertaken to investigate the effectiveness of different nutrient supply regimes namely, cow dung, poultry manure and compost and inorganic fertilizer for improvement and maintenance of soil fertility, yield and quality of okra.

Materials and methods

Field experiment was conducted at the University of Ghana, Legon. The soil in the farm belongs to the Adenta series, Ferric Acrisols (WRB, 1998). Poultry manure and cow dung used for the research were collected from the Agricultural Research Station, University of Ghana and dried thoroughly before application. Garden solid waste compost was prepared from elephant grass, lawn clippings, leuceana tree prunings (*Leuceana leucocephala*), topsoil and cow dung. Bulk samples of soil, and a mixture of soil plus manure were collected from the top 0-15 cm at the time of land preparation for physical and chemical characterization. Analyses were carried out at the Soil Science Laboratory, University of Ghana, Legon. Parameters determined included particle size, pH in 1:1 soil:water ratio and 1:2 soil:0.01 $CaCl_2$. Organic carbon was determined by Walkley and Black method and total nitrogen by the macro Kjeldahl's method involving digestion and distillation. Bray's No 1 method was used to determine available phosphorus and exchangeable cations were determined by the flame photometry.

Cow dung, poultry manure, compost, inorganic fertilizer and control (no-manure) were used as treatments in this study. NPK (15:15:15) was applied two weeks after planting (WAP) at the rate of 300 kg ha^{-1}, and ammonium sulphate as side dress at 4 and 6 weeks after planting at the rate of 125 kg ha^{-1}. Treatments were arranged in a randomised complete block design with 3 replications. Okra var: Asontem White was used. Each replicate had 36 plants and 16 record plants. Manure was incorporated into the soil two weeks before planting the test crop at the rate of 25 t ha^{-1}. Soil and a mixture of soil and manure sampling were carried out before planting and at 12 WAP and analysed. Chemical fertilizers were applied to inorganic fertilizer treatment plots. At fruit maturity, yield, yield components and pod fibre were determined.

Results

Table 1 depicts nutrient content of compost, cow dung and poultry manure used in the study. Poultry manure had the highest total nitrogen (N), total phosphorus (P) and potassium (K) levels followed by cow dung and compost.

Initial effects of manure application on some soil physical and chemical properties are shown in Table 2. Both the untreated soil (no manure) and compost-supplemented soil were within the sandy loam texture range. Poultry manure supplemented soil gave higher levels of organic matter, available P and K, exchangeable Ca, Mg than the other manured soil and control. Soil in control plots had a slightly acidic pH, however, the cow dung and poultry manure supplemented soils were near neutral.

The Cation Exchange Capacity (CEC) of soil of the control plot was 14.3 Cmol kg^{-1}, incorporation of organic matter such as poultry manure, cow dung and compost increased the CEC of the soil to 17.1, 16.8 and 16.7 Cmol kg^{-1}, respectively.

Table 3 shows the effect of manure application on soil physical and chemical characteristics at 12 weeks after planting. Soil bulk

Table 1. Chemical characteristics of poultry manure, cow dung and compost used in the studies

Property	Manure		
	Compost	Cow dung	Poultry manure
Kjedhal–Nitrogen(%)	0.7	0.76	2.04
Total P (%)	0.06	0.18	1.54
Total K (%)	0.24	0.55	0.65

Table 2. Physical and chemical characteristics of soil and soil plus manure within a 0-15 cm soil depth (before planting)

Characterstics	Untreated soil (Control)	Inorganic fertilizer + soil	Compost + soil	Cow dung + soil	Poultry manure + soil
Sand (%)	57.4	56.9	56.5	49.6	47.2
Silt (%)	11.6	12.2	11.5	19.4	17.8
Clay (%)	35.0	34.5	30.0	25.0	24.6
Organic carbon (%)	0.72	0.73	0.75	0.96	1.04
Kj-nitrogen (%)	0.10	0.11	0.12	0.13	0.15
Total P (ppm)	188.1	259.8	234.2	398.7	398.9
Total K (%)	0.08	0.13	0.11	0.14	0.16
Available K (mg kg^{-1})	0.60	0.93	0.82	1.20	1.36
Available P (mg kg^{-1})	8.15	13.83	9.43	45.0	50.2
Water holding capacity (WHC)	40.0	40.2	42.0	45.0	49.0
Ca^{2+} (mg kg^{-1})	2.2	2.2	3.6	5.0	5.6
Mg^{2+} (mg kg^{-1})	4.4	4.4	5.8	7.4	8.1
Cation exchange capacity (CEC) [Cmol kg^{-1}]	14.3	13.9	16.7	16.8	17.1
pH	6.1	6.4	6.4	6.6	6.8
Bulk density	1.4	1.4	1.3	1.2	1.2

Table □

Characterstics	Untreated soil (Control)	Inorganic fertilizer + soil	Compost + soil	Cow dung + soil	Poultry manure + soil
Sand (%)	57.5	57.8	56.2	52.9	55.6
Silt (%)	10.8	10.5	15.6	19.6	16.9
Clay (%)	35.0	35.1	29.5	27.5	27.4
Organic carbon (%)	0.78	0.75	0.81	1.02	1.32
Kj-nitrogen (%)	0.11	0.13	0.13	0.14	0.15
Total P (ppm)	198.7	261.7	229.1	415.8	432.6
Total K (%)	0.07	0.12	0.10	0.14	0.16
Available K (mg kg^{-1})	0.58	0.95	0.77	1.24	1.54
Available P (mg kg^{-1})	9.02	13.95	9.58	46.3	51.42
Water holding capacity (WHC)	41.5	41.4	43.1	47.0	51.4
Ca^{2+} (mg kg^{-1})	2.0	2.1	3.4	4.8	5.5
Mg^{2+} (mg kg^{-1})	4.4	4.4	5.9	7.8	8.6
Cation exchange capacity (CEC) [Cmol kg^{-1}]	15.01	14.2	16.91	17.03	17.15
pH	6.2	6.5	6.6	6.8	6.9
Bulk density	1.4	1.4	1.2	1.1	1.0

density decreased in the poultry manure, cow dung and compost treated plots while soil pH, nitrate-N, available P, available K and organic matter contents increased.

Water infiltration rate at 12 WAP was higher in the poultry manure, cow dung and compost treated plots as compared to the inorganic fertilizer and control plots. The increase is probably due to the cumulative effect of applied organic manure on soil structure. The results in Table 3 indicate that inorganic fertilizer supplemented plots showed improvement only in the soil chemical properties, but not on soil physical properties such as bulk density and water holding capacity.

Manure application resulted in significantly greater soil organic matter level and a positive organic matter balance in the soil. In a study to evaluate the effects of organic matter and nutrients in manure on soil organic matter dynamics and crop production, Eghball *et al.* (2002) reported significantly greater soil organic matter level in plots treated with organic manure. In the present study, poultry manure gave the higher organic matter content compared to other manured treatments. The addition of the organic manure might have provided supplemental exchangeable cations such as potassium, calcium, magnesium and ammonium (NH_4^+) in the topsoil (Olsen *et al.*, 1970). The application of manure influenced the soil pH and bulk density. The soil in the farm was slightly acidic but the application of organic manure decreased the acidity as indicated by the increased pH. Magdoff (1998) reported that, organic matter is a reservoir of plant nutrients and exhibits a high cation exchange capacity and buffers the soil against pH changes. Manured soils recorded a significant decrease in bulk density. The decrease in bulk density of the manured soils could be attributed to the increase in organic matter content of the soil. Young (1997) stated that addition of organic matter lowers bulk density, improves structure and increase a balance between fine and coarse pores. The result of the studies indicates that the addition of manure increases the water holding capacity of the soil which might be due to the organic matter in the soil. Organic matter improves water infiltration rate, water holding capacity and the hydraulic conductivity of soil (Cross and Fischbach, 1972; Hafez, 1974).

There was significant effect of treatments on the number, length, girth and fresh weight of pod (Table 4). The application of poultry manure resulted in a significant ($P < 0.05$) increase in the number of okra pods compared to the control. The pods produced by poultry manure treatment were double in number than in the control. Non significant ($P < 0.05$) difference in the number of pods per plant was observed between cow dung and poultry manure. Significant ($P < 0.05$) difference was observed in pod length between pods from manured plants and control. Poultry manure produced the longest pod (6.5cm). Significant ($P < 0.05$) increase in pod diameter was observed in poultry manure treated plot as compared to control (Table 4). There was a significant ($P < 0.05$) difference in pod girth between manure treated plots and the control. Pod fresh weight per plant was significantly ($P < 0.05$) increased with poultry manure application compared to the control.

The increase in okra yield and other yield components such as pod length, diameter and fresh weight apparently resulted from improved soil chemical and physical characteristics under manure application. Plants responded to the improved conditions under manure, especially poultry manure, with an increased yield (Bhangoo *et al.*, 1988; Howard and Albregts, 1981). The significant increase in total yields in manured plots might also be attributed to the increased branching. In okra, more branching accounts for increased yield as pod developed in the axil of every branch once flowering has began. Similarly, the significant difference in pod length, girth and pod fresh weight with manured plots compared to the control might be due to differences in soil structure and fertility. The increase in the water holding capacity and increased availability of nutrients of the soil in manured plots might have provided additional support to the plants (Agarwala *et al.,* 1981; Nelson and Tisdale, 1978).

Table 4. Number of pods, pod length, pod girth and pod fresh weight of okra

Treatment	Pod number plant^{-1}	Pod length (cm)	Pod girth (cm)	Pod fresh weight (g)
Control	2.13	5.30	1.97	9.47
Inorganic fertilizer	2.33	5.53	2.13	11.10
Cow dung	3.53	5.93	2.13	13.03
Compost	2.83	5.77	2.17	11.53
Poultry Manure	4.33	6.50	2.40	15.90
LSD ($P < 0.05$)	1.36	0.39	0.31	1.77

Increased organic matter in the soil from application of poultry manure, cow dung and compost improved both soil physical and chemical properties compared to inorganic fertilizer alone and the control. Poultry manure was identified as a better source of organic manure for okra production than cow dung and compost.

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Economic rationale of commercial organic fertilizer technology in vegetable production in Osun State of Nigeria

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Abstract

The fragility and high susceptibility of the soils in Nigeria to degradation and loss of nutrients make augmentation through the use of fertilizers necessary to obtain reasonable crop yield. The use of market oriented organic fertilizer is being encouraged to improve soil fertility and there is the need to determine the economic rationale of this technology. This study determined the change in net income of users of commercial organic fertilizer (UCOF) relative to non-users of fertilizers (NUF) in vegetable crop production in Osun State of Nigeria to find out if its use should be encouraged based on economic reason only. Nested sampling technique was used in selecting UCOF and NUF respondents. Data on yield, quantities and prices of inputs and output; and reasons for non-use of commercial organic fertilizer were collected and analyzed using descriptive and inferential statistics, partial budgetary technique, sensitivity analysis and importance ranking. Analyses indicated that UCOF applied 610kg ha^{-1} of commercial organic fertilizer resulting in additional yield (3,375kg ha^{-1}) and rate of returns (401%) over and above the NUF, making the use of organic fertilizer technology economically superior to non-use of fertilizers. Constraints to the use of commercial organic fertilizer are doubtful efficacy, offensive odour, heavy weed infestation, bulkiness and lack of funds in descending order of importance which if eliminated will boost demand for commercial organic fertilizer and improve production of vegetable for consumption.

Key words: Vegetable, commercial organic fertilizer, marginal rate of return, constraints, Osun State, Nigeria

Introduction

Nigeria is one of the countries in Sub-Saharan Africa (SSA) where self-sufficiency in food production remains a critical challenge even in the absence of wars and natural disasters (ADB, 1999). It is reported that the population in SSA is rising at about 2.5% which outstrips food production that is growing at about 1.5%. The results of population pressure and the demand of land for non-agricultural uses lead to decrease in available agricultural land and consequently small farm size. Olutawosin and Olaniyan (2001) noted that Nigeria is a nation of smallholder farmers cultivating an average of 2 hectares per household under traditional system of farming. Spencer (1991) opined that about 90% of food production in SSA (Nigeria inclusive) comes from smallholder farmers under traditional system of farming. In a situation of small farm size, agricultural intensification is the key to effectively addressing the problem of self-insufficiency in food production (Pinstrup-Anderson and Pandya–Lorch, 1994). Agricultural intensification is defined as the production of more food per unit area of land. Agricultural intensification is usually portrayed either as an opportunity or as a threat to the environment. The advocates of the concept argue that it holds great promise as an instrument to simultaneously alleviate poverty and meet food needs at all times while the opponents express great concern that it may lead to degradation of natural resources and unparalleled loss of soil nutrients. No doubt, agriculture is the most important user of environmental services including water, forests, pastures and soil nutrients. Hence, intensive land use without appropriate soil management practices leads to environmental degradation (Senjobi *et al.*, 2000). DFID (2002) stated that environmental degradation can compromise with current agricultural productivity, undermine future production and perpetrate poverty. In order to alleviate such threat, the proposed soil management practice must ensure the sustainability of the agricultural production environment. A sustainable agriculture has been defined to be one that over a long term enhances environmental quality and resource base on which agriculture depends; provides for basic human food and fibre needs; is economically viable and enhances the quality of life of farmers and society as a whole (CGIAR, 1988).

Although, various soil conservation practices under different categories of farming systems have evolved over the time (Olayide *et al.*, 1981), it is essential for countries to promote policy measures that will enable farmers to make use of their natural advantages (DFID, 2002). Systems that allow intensification using mainly locally available resources, such as organic fertilizers may play an important role in soil fertility management thereby reducing hunger through increased agricultural productivity. Organic fertilizers are generally made from plant and animal by-products and natural minerals that may originate from the farm itself (crop residue, livestock manure) and is thus a nutrient–saving technology, or they can be obtained from other sectors or from products manufactured elsewhere, and as such constitute a nutrient adding technology. Greg (1996) stated that apart from producing more vigorous growing and high yielding crop, the improvement in overall soil quality resulting from the use of organic soil amendments may reduce the potential for nutrient contamination of ground and surface water. Organic fertilizers have been confirmed to improve the

physical properties of soil (Swarup, 1987), the biological status of soil (Chai *et al.,* 1988); soil fertility and consequently crop yield (Lal and Mathur, 1989).

All these attributes of organic fertilizers serve to eliminate the fears of negative impacts of agricultural intensification in the use of organic fertilizer technology. Traditionally, farmers engage in composting to supply organic fertilizers at the subsistence level to their farms and such organic fertilizer commodity does not pass through the market exchange system. However, there is the recent development whereby organic fertilizers are produced in commercial quantity by organic fertilizer manufacturing enterprises for farmers' use in crop production and its use is being encouraged.

It is therefore necessary to empirically study the economics of commercial organic fertilizer technology in crop production. Preliminary investigations indicated that commercial organic fertilizer was used mostly for vegetable crop production (largely *Amaranths* spp.) and that there were different categories of farmers in relation to the use of fertilizers. These categories are: users of commercial organic fertilizer only, users of inorganic fertilizers only, farmers combining both organic and inorganic fertilizers and non-users of fertilizers. In order to reveal the full effect of use of commercial organic fertilizer, the non-users of fertilizers were compared with users of only commercial organic fertilizer in vegetable production. The goals of the study were to determine if the use of commercial organic fertilizer for vegetable production was economically better than non-use of fertilizer and identify constraints to its use. The specific objectives were to: i) determine and compare the vegetable yields of UCOF and NUF, ii) determine the marginal rate of returns on the use of commercial organic fertilizer, iii) identify and rank the constraints to commercial organic fertilizer use.

Achievements of these objectives will assist the vegetable farmers and the agricultural policy makers on the need to use and encourage, respectively commercial organic fertilizer.

Materials and methods

Study area: The study was conducted in Osun State of Nigeria. Osun State occupies an area of about 10,456km^2 and has a population of about 2,551,522 (FOS, 1997). Osun State is the most urbanized State in Nigeria with a rate of urbanization of 5 percent per annum (UNS, 2001), thereby, constituting a large market for agricultural products. The State has two seasons: wet season that spans from April to October; and dry season starting from November through to March. The wet season supports vegetable production without irrigation water while proximity to perennial water source for irrigation is necessary during the dry season.

Method of data collection and analytical techniques: There were two populations of interest. These are the users of only commercial organic fertilizer (UCOF) and the non-users of any kind of fertilizers (NUF). A nested sampling technique was used to select respondents (UCOF and NUF) for interview. One Agricultural Development Programme (ADP) zone was selected out of the three zones in the State, five Local Government Areas (LGAs) from the selected zone, and five town/villages from each of the five LGAs were chosen using purposive sampling technique at each of the stages. The purposive sampling was based on the relative availability of vegetable farmers. A list each of UCOF and NUF was compiled in each town/village and five each of the UCOF and NUF were selected using simple random sampling technique. In all, a total of one hundred and twenty five each of UCOF and NUF respondents were selected for interview. Primary data were collected from all the respondents on the prices and quantities of vegetable production inputs and output. In addition**,** data on commercial organic fertilizer were obtained from UCOF only; and information on constraints for non-use of commercial organic fertilizer and their ranking from NUF only. Data collected were analyzed using descriptive and inferential statistics, partial budgetary technique, sensitivity analysis and importance ranking.

Descriptive and inferential statistics: Frequency distribution tables, means and standard deviation were the descriptive statistics used to present and summarize yield, organic fertilizer and farm size. Inferential statistics of t-test of difference between two population means was used to establish significant difference in the mean yields of UCOF and NUF as well as in their farm sizes.

Comparison of mean vegetable yields of UCOF and NUF: The null hypothesis (H_0), that the mean vegetable yield of NUF is equal to that of UCOF (equation 1) was tested against the alternative hypothesis (H_1) that the mean vegetable yield of NUF is not equal to that of UCOF (equation 2) using the t statistic stated in equation 3 and at 5% level of significance (Karmel and Polasek, 1977). This is with the intention of determining if using commercial organic fertilizer brings about vegetable yield different from non-use of fertilizers. Sample mean, variance, population mean of NUF and UCOF were calculated.

$$H_O : \mu_1 = \mu_2 \quad (1)$$

$$H_I : \mu_1 \neq \mu_2 \quad (2)$$

$$t_c = \frac{(\overline{X}_1 - \overline{X}_2) - (\mu_1 - \mu_2)}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}} \quad (3)$$

Such that $\overline{X}_1, S_1^2$, μ_1 and n_1 are the sample mean, sample variance, population mean and sample size of NUF, respectively; and $\overline{X}_2$, S_2^2, μ_2 and n_2 are the sample mean, sample variance, population mean and sample size of UCOF, respectively

Partial budgeting, marginal and sensitivity analyses: Partial budgeting and marginal analyses were used to indicate the superiority of the use of commercial organic fertilizer over non-use of fertilizers.

CIMMYT (1988) noted that partial budgeting is a method of organising data and information about the costs and benefits of various alternative treatments/technologies. The alternative treatments in this study are commercial organic fertilizer use and non-use of fertilizers in vegetable production. The relevant costs to use in Partial Budget Analysis (PBA) are costs that vary between alternative technologies, which for this study are material (commercial organic fertilizer) cost (at the farm gate price), and labour (for application of organic fertilizer, weeding and harvesting of vegetable output) cost. These costs are added together to obtain Total Cost that Vary (TCV) which is subtracted

from Gross Field Benefits (GFB) to give Net Benefit (NB). GFB is the product of yield (kg ha^{-1}) and the price per unit (₦ kg^{-1}) of output (gross revenue).

Marginal analysis in PBA is the comparison of change in TCVs with change in NBs. This comparison reveals the change in benefits associated with a given change in cost for using a technology (commercial organic fertilizer). PBA is based on per unit, which in crop farming is on per hectare basis. Thus, in this study, PBA is based on a farm size of one hectare, and variable costs and benefits are assumed to vary directly with farm size. It is basically the computation of Marginal Rate of Return (MRR), which is compared with Acceptable Minimum Rate of Return (AMRR). MRR is the ratio of marginal net benefit to marginal cost. The marginal net benefit is the difference between the NBs of two consecutive treatments while the difference between the TCVs is the marginal cost. AMRR is the minimum return that farmers expect to earn from an enterprise or technology, which technically is the sum of returns to management and capital. In this study AMRR is assumed to be 100 percent of marginal cost. A technology/alternative treatment is considered economically worthwhile if MRR is higher than AMRR.

Sensitivity analysis (determining the break-even level) was performed to show the change in the mean of each of the commercial organic fertilizer price, yield and price of vegetable that will make the use of commercial organic fertilizer in vegetable production uneconomical. It implies redoing a marginal analysis with alternative values of the decision variables (price and yield). Price sensitivity analysis was carried out by varying each of the mean prices of commercial organic fertilizer and vegetable output. The formula for calculating the break-even yield (Alimi and Manyong, 2000) was employed to determine the break-even yield of vegetable (Equation 1). These assist in establishing the degree of economic superiority of commercial organic fertilizer over non-use of fertilizers to justify the encouragement of its use.

$$q^* = \frac{[\Delta TVIC \times AMRR] + TVIC_2 + NB_1}{P} \qquad (4)$$

Where,

q* = level of vegetable yield below which the use of commercial organic fertilizer becomes unviable.

ΔTVIC = change in total variable input costs of the two technologies

AMRR = acceptable minimum rate of return

$TVIC_2$ = total variable input cost of technology 2 (commercial organic fertilizer)

NB_1 = net benefit of technology 1 (no-use of fertilizer)

P = price of vegetable output (₦ kg^{-1})

Importance indices: The importance index was constructed using matrices A, B and C as indicated below. In order to determine the relative importance of constraints to the use of commercial organic fertilizers, importance index was constructed using the methodology adopted by McLean-Meyinsse *et al.* (1994). For the construction of the indices, NUF were asked to give and rank the reasons for non-use of commercial organic fertilizer on an ordinal scale, (1 being assigned to the most important, 2 to the next most important and sequentially in descending order of importance). For analysis, the scale was reversed for ease of index construction. The mean score computed for each identified reason for non-use of commercial organic fertilizer was multiplied by the percent of NUF identifying the reason for non-use as the most important; to obtain the importance index. Jose and Valluru (1997) used importance index to identify price risk as the most important in the opinion of the farming communities in Nebraska. Importance indices method was used by Alimi (2002) to identify regular feed supply as the most important reason for integration in poultry production; and by the same method, Alimi (2005) identified low okra price and moisture stress as the most important constraint to okra production in the rainy and dry seasons, respectively.

The importance index was constructed using matrices A, B and C as indicated below:

$$A = \begin{bmatrix} f_{11} & f_{12} & . & . & . & f_{1n} \\ f_{21} & f_{22} & . & . & . & f_{2n} \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ f_{m1} & f_{m2} & . & . & . & f_{mn} \end{bmatrix}.$$

$$B = \begin{bmatrix} w_1 \\ w_2 \\ . \\ . \\ . \\ w_m \end{bmatrix}$$

$$C = AB = \begin{bmatrix} f_{11} & f_{12} & . & . & . & f_{1n} \\ f_{21} & f_{22} & . & . & . & f_{2n} \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ f_{m1} & f_{m2} & . & . & . & f_{mn} \end{bmatrix} \begin{bmatrix} w_1 \\ w_2 \\ . \\ . \\ . \\ w_m \end{bmatrix} = \begin{bmatrix} c_1 \\ c_2 \\ . \\ . \\ . \\ c_m \end{bmatrix}$$

Matrix A gives the distribution of NUF according to reasons for non-use of commercial organic fertilizer ranks. The matrix indicates that there are m reasons for non-use, to be put in n categories of rank.

Matrix B is the weight attached to each of the ranks, w_i is the weight attached to rank j where i = j, i =1,2….m and j =1,2….n. w_1 is the weight attached to rank 1,w_2 to rank 2 *etc.*.

Matrix C gives the product of matrices A and B, (AB). It is the total value of importance attached to each reason for non-use of commercial organic fertilizer. For example $C_3 = f_{31}w_1 + \ldots\ldots + f_{3n}w_m$ = total value of importance attached to reason 3 for non-use. C_i is the total value of importance attached to reason i for non-use.

Importance rating for reason i = $\frac{1}{\lambda_i} C_i$

Where, $\lambda_i = f_{i.}$ = n = total number of NUF selecting reason i as important.

$$\text{Importance index} = \frac{C_i}{f_{i.}} \cdot \frac{f_{i1}}{\sum_{i=1}^{m} f_{i1}}$$

Such that $f_{i.}$ is the number of NUF (frequency) ranking reason i as the most important (highest rank). This will assist in ordering constraints to the use of commercial organic fertilizer for attention to increase its use if found more profitable (economical) than non-use of fertilizers. Increasing its use will increase demand for commercial organic fertilizer and higher business activity for commercial organic fertilizer enterprises.

Results and discussion

Sample size: Information collected from three and two respondents of the NUF and UCOF, respectively was incomplete and these respondents were dropped from further analyses. Thus, the sample size for NUF was 122 and that of UCOF was 123.

Characteristics of vegetable farm enterprises: Most of the vegetable farm enterprises were located in relatively urban centres where demand for vegetable by the non-farming households is high. The distribution of NUF and UCOF by vegetable farm size is indicated in Table 1. None of the vegetable farmers in the two categories (NUF and UCOF) cultivated smaller than 0.01ha and none as large as 1.00 ha. None of the NUF had larger than 0.79 ha while some UCOF (6%) cultivated between 0.80 and 0.99 ha farm size category. The mean farm size of NUF was 0.424 ha which was just 85.48 percent of the mean farm size of UCOF (0.496 ha). The vegetable farm size of UCOF was significantly larger than that of NUF ($t_c = 2.41$), although both belonged to the smallholder group. The vegetable output from the two sources attracted the same selling price as consumers (buyers) did not discriminate between the vegetable outputs derived from the two sources to justify difference in prices.

None of the UCOF applied less than 300 kg ha^{-1} of commercial organic fertilizer and none as high as 800 kg ha^{-1}. High proportion of the UCOF (52%) used between 500 and 699 kg ha^{-1}. The mean quantity of commercial organic fertilizer applied by the UCOF was 610 kg ha^{-1}. The trade name for the most common commercial organic fertilizers used by the UCOF is Pace setter A (PSG-A) and its nutrients composition (g kg^{-1}) is N- 2.58, P- 1.10, K- 0.68, Ca- 0.36 and Mg- 0.11(Ipinmoroti *et al.*, 2003).

The yield (kg ha^{-1}) obtained by the vegetable farmers varied between 3000 and 10,499kg ha^{-1}. While none of the UCOF obtained yield that was lower than 6000 kg ha^{-1}, NUF obtained yield as low as 3000 kg ha^{-1}. None of the NUF realized yield as high as 7500 kg ha^{-1}. More than half (56%) of the NUF were in the yield class of 4500 to 5999 kg ha^{-1}, and over three-quarters (77%) of UCOF operated in the yield class of 7500 to 8999 kg ha^{-1}. The mean yield of UCOF was 8235 kg ha^{-1} and was 69% higher than the mean yield of NUF (4860 kg ha^{-1}).

Comparison of mean vegetable yields of NUF and UCOF: In testing the null hypothesis of no significant difference in the mean yields of UCOF and NUF (equation 1), and applying the test statistic in equation 3, the null hypothesis is rejected (t_c = 32.1) for the acceptance of the alternative hypothesis. This shows that UCOF obtained larger mean vegetable yield than the NUF and that commercial organic fertilizer assisted in increasing the yield of vegetable crop significantly.

Table 1. Distribution of vegetable farmers by farm enterprise characteristics

Characteristics	Distribution (%)		Mean		Standard deviations		t_c
	NUF	UCOF	NUF	UCOF	NUF	UCOF	
Farm size (ha)							
0.01-0.19	20	09					
0.20-0.39	15	20					
0.40-0.59	48	41					
0.60-0.79	17	24					
0.80-0.99	-	06	0.424	0.496	0.198	0.204	2.81*
Organic fertilizer (kg ha^{-1})							
300-399		05					
400-499		19					
500-599		11					
600-699		41					
700-799		24		610		118.3	
Yield (kg ha^{-1})							
3,000-4,499	35	-					
4,500-5,999	56	-					
6,000-7,499	09	12					
7,500-8,999	-	77					
9,000-10,499	-	11	4860	8235	915.4	719.2	32.1*

* Means significant at P=0.05

Partial budget analysis: In addition to the physical input-output data, the market situation relating to the prices of inputs and output is necessary to measure the economic feasibility of a change in technology. The physical input-output data assist in establishing the technical efficiency. Favourable technical efficiency is not enough for economic feasibility as input-output price ratio may cause the outcome of technical efficiency to be different from that of the economic efficiency. Partial budget analysis combines the information on physical input-output relationship with those of prices of input and output to determine the economic feasibility of a proposed technology (the use of commercial organic fertilizer). Table 2 shows the partial budget analysis of change from no-fertilizer technology to commercial organic fertilizer technology in the production of vegetable. Since there is no discrimination in the output of vegetable from the two sources, the farm gate price remained the same (₦76 kg^{-1}) and the gross farm gate benefit varies with the level of yield obtained from each technology. The gross farm gate benefit which is the product of average yield and farm gate price was ₦369,360 ha^{-1} and ₦625,860 ha^{-1} for no-fertilizer and commercial organic fertilizer technologies, respectively. The variable inputs were commercial organic fertilizer and labour for fertilizer application that were restricted to commercial organic fertilizer technology only, and labour for weeding and harvesting which affected the two technologies. Labour for weeding was higher for commercial organic fertilizer technology than no-fertilizer because the organic fertilizer encourages the growth of weed, thereby higher labour cost. Labour cost on harvesting was higher for organic fertilizer technology as a result of higher yield obtained than no-fertilizer. The total variable input cost was ₦35,796 ha^{-1} for no-fertilizer technology that is smaller than ₦86,943 ha^{-1} for

Table 2. Partial budget and sensitivity analyses for vegetable production under no-fertilizer and organic fertilizer technologies

S.N.	Items	No-fertilizer (Treatment 1)	Organic fertilizer (Treatment 2)	Break-even price of vegetable	Break-even yield of vegetable	Break-even price (₦[z]) of organic fertilizer
	Gross farm gate benefits					
1	Average yield (kg ha^{-1})	4,860	8,235	8,235	6,206	8,235
2	Farm gate price (₦ kg^{-1})	76	76	57.27	76	76
3	Gross farm gate benefits (kg ha^{-1}) (1x2)	3,69,360	6,25,860	4,71,656	4,71656	6,25,860
	Variable input costs (₦ ha^{-1})					
4	Commercial organic fertilizer (₦ ha^{-1})	-	24,400 (40)	24,400 (40)	24,400 (40)	1,01,260 (166)
5	Labour –fertilizer application	-	6,100	6,100	6,100	6,100
	- weeding	15,572	21,111	21,111	21,111	21,111
	- harvesting	20,224	35,332	35,332	35,332	35,332
6	Total variable input costs (4+5)	35,796	86,943	86,943	86,943	163,803
	Net benefits					
7	Net benefit (₦ ha^{-1}) (3-6)	3,33,564	5,38,917	3,84,713	3,84,713	4,62,057
8	Change in net benefits from technology 1 to 2. (₦ ha^{-1})		2,05,353	51,149	51,149	1,28,493
9	Change in total variable input costs from technology 1 to 2 (₦ ha^{-1})		51,147	51,147	51,147	1,28,007
	Marginal rate of return					
10	Marginal rate of return (%) (8/9x100)		401	100	100	100

[z] ₦ = Naira the currency of Nigeria. The mean exchange rate during the study period was: $1US = ₦137.

commercial organic fertilizer, producing a change in total variable input costs of ₦51,147 ha^{-1} between the two technologies. The net benefit was ₦333,564 ha^{-1} for no-fertilizer technology and ₦538,917 ha^{-1} for commercial organic fertilizer technology resulting in a change in net benefit of ₦205,353 ha^{-1} between the two technologies. The resulting marginal rate of return (MRR) is 401%. Since the resulting MRR is greater than the acceptable minimum rate of return (AMRR =100%), the change from no-fertilizer technology to commercial organic fertilizer technology in vegetable production is profitable.

Sensitivity analysis: Sensitivity analysis (Table 2) was used to determine the break-even price of commercial organic fertilizer, break-even yield of vegetable and break-even price of vegetable. The break-even yield obtained using the formula by Alimi and Manyong (2000) is 6206 kg ha^{-1}. This implies that adverse conditions beyond the control of the farmers such as technology failure and inclement weather condition would result in decreased yield obtained from commercial organic fertilizer by more than 2029 kg ha^{-1} or 24.64% to make commercial organic fertilizer technology less lucrative than no-fertilizer. An adverse change in market condition (increase in variable input price such as price of commercial organic fertilizer and or decrease in price of vegetable produced using commercial organic fertilizer) can affect the decision to change from no-fertilizer technology to commercial organic fertilizer technology. A more than 315% rise in price (from ₦40 kg^{-1} to more than ₦166 kg^{-1}) of commercial organic fertilizer will disfavour change from no-fertilizer technology to commercial organic fertilizer technology. If for whatever reason(s) consumers of vegetable develop a distaste for vegetable produced using commercial organic fertilizer, thereby reducing demand for it, and necessitating reduction in price of vegetable from this source, result of break-even analysis indicates that the price of vegetable output will have to decrease below ₦57.27 kg^{-1} for commercial organic fertilizer technology to be less viable than no-fertilizer technology.

Constraints to the use of commercial organic fertilizer: The constraints stated by NUF preventing the use of commercial organic fertilizer were its offensive odour, heavy weed infestation, doubtful efficacy, bulkiness and lack of funds to purchase (Table 3). The order of ranking starting from the most important was doubtful efficacy, offensive odour, heavy weed infestation, bulkiness and lack of fund. NUF were not convinced that commercial organic fertilizer could lead to appreciable yield increase to justify additional expenses on organic fertilizer. It is necessary for agricultural extension agents to mount demonstration plots to convince farmers on the higher profitability of commercial organic fertilizer technology for vegetable production. The issue of offensive odour could be addressed by adding inexpensive and harmless deodorant to make the application and handling of commercial organic fertilizer users' friendly. Commercial organic fertilizer encouraged the growth of both weed and vegetable thereby increasing labour cost on weeding. While rapid growth of vegetable is desirable, that of weed is not; cost saving weed control method(s) must be considered. The bulkiness of commercial organic fertilizer commodity makes its transportation difficult and expensive; research should consider means of reducing its bulkiness at no loss of quality.

Table 3. Constraints to the use of commercial organic fertilizer and their relative ranking

Constraints	Importance rating		Importance index	
	Mean	Standard deviation	Index	Rank
Offensive odour	3.56	1.27	121	2nd
Heavy weed infestation	3.07	1.39	64	3rd
Doubtful efficacy	4.15	0.90	216	1st
Bulkiness	2.33	1.41	23	4th
Lack of funds	1.89	1.10	9	5th

The study examined the relevance of commercial organic fertilizer technology in vegetable production in Osun State of

Nigeria. Primary data on quantities and prices of inputs and outputs were collected from non-users of fertilizers (NUF) and users of only commercial organic fertilizer (UCOF); and in addition data on commercial organic fertilizer from UCOF only and reasons for non-use of commercial organic fertilizers from NUF only. Data collected were analyzed using descriptive and inferential statistics, partial budgetary technique, and sensitivity analysis and importance indices. Results indicated that UCOF obtained significantly higher mean output and higher marginal rate of return than the NUF thereby making the commercial organic fertilizer technology superior to non-use of fertilizers. The constraints to non-use of commercial organic fertilizer in descending order of importance are doubtful efficacy, offensive odour, heavy weed infestation, bulkiness and lack of funds to purchase the commercial organic fertilizer commodity which should be addressed to boost commercial organic fertilizer production enterprise, increase profits to vegetable farmers and produce more vegetable for consumption.

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Technical and economic aspects of utilizing fibrous wool composts in horticulture

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Abstract

Composts produced from a mixture of fibrous wool by-products and other components (*e.g.*, wood-shavings, cotton-gin trash, yard waste, biosolids, *etc.*) have a high concentration of nitrogen and low concentrations of regulated trace elements. Some have low soluble salts content and have slightly acidic to neutral pH. These composts met standards of the US EPA of an exceptional quality product and were successfully used to grow ornamental crops in a greenhouse and to establish turfgrass from seeds. Market research showed that the turfgrass industry and retail garden centers would be the largest and most profitable markets for fibrous wool-based composts and potting mixes. Cost-volume-profit analysis (CVP) indicated that production and sale of about 17,200 tonnes per year of the compost product would be a break-even point in units for a hypothetical compost production and marketing business. Since composting is also a waste management operation, revenues from accepting waste (tipping fees) does improve business profitability.

Key words: Bioconversion, wool, composting, wood wastes, economic analysis.

Introduction

The retail sector, tree farms, nurseries, greenhouses and turf applications are known to be highly profitable market segments for compost use within the horticultural industry (Tyler, 1996; Walker *et al.*, 2006). In addition, it is known that sod production is a large volume market for compost utilization (Tyler, 1996). Retail markets demand high quality potting mixes and soil amendments to grow container plants and ornamentals. Likewise, commercial greenhouses and nurseries also demand consistent potting mixes/soil amendments for their field work. Previous work has shown that nutrients from compost or its extracts can significantly benefit ornamentals nurseries. For example, Jarecki *et al.* (2005) found that marigolds responded well to compost leachate in a hydroponic growth experiment.

Homeowners and business customers value compost products that are consistent and stable and have the right combination of physical and chemical properties (Composting Council, 1995). Although most sod is grown in the field, some sod producers are interested in growing sod on plastic sheets (Decker, 1989), which entails spreading a layer of organic substrate over plastic and growing grass on it. The crop is harvested by rolling up the sod from the plastic. The convenience of this approach is expected to make this an increasingly popular method of sod production.

Tyler (1996) reported that compost sale prices were highest at urban retail garden centers (US$17.6 to 27.5 tonne^{-1}; 16 to 25 tonne^{-1}) and when used in turf applications (US$17.6 to 22 tonne^{-1}). All other low-volume, high-dollar markets had reported sale prices in the range of $4.4 to 17.6 tonne^{-1}. These reported prices suggest that highest profitability for compost producers may occur at retail and turf applications. Therefore, our work on fibrous wool compost products focused on developing potting mixes for growing ornamentals (targeting retail garden centers) and soil amendments for turfgrass establishment.

The objective of this work was to evaluate through greenhouse experiments, the suitability of using fibrous wool-based composts as components of potting mixes to grow ornamental pansies (*Viola wittrakiana* L.) and marigolds (*Tagetes erecta* L.) and to grow turfgrass. In addition the economic feasibility of this product was evaluated through proforma analysis.

Materials and methods

Description of composts and their production: The composts used in this study were made as part of a project to develop useable products from organic waste streams generated around Laurens County, Georgia, USA (Das *et al.*, 1997). The waste streams relevant to this study consisted of wool fiber wastes from a wool fabric manufacturing plant, cotton gin trash from a cotton gin, wood shavings from a lumber yard, yard trimmings from municipal collections, and dewatered biosolids from a municipal wastewater treatment plant using the activated sludge treatment process. Five wool composts blends were made with combinations of wool compost and other potting soil amendments such as pine bark, perlite, and vermiculite. Composts were made either in laboratory scale composting systems (W1 and W2) or in field scale windrows (W3, W4 and W5) using wool fiber waste and agricultural or municipal wastes mentioned earlier.

The individual mixes used in the laboratory are shown in Table 1 and were wool and cotton gin trash at 33 and 67% (w/w, as received) in W1 and wool and wood shavings at 30 and 70% for W2 (Das *et al.*, 1997). Compost mixes used in the field scale windrows included wool, yard trimming and biosolids at 3, 60 and 37% (w/w, as received) in W3, wool and yard trimmings at 4.5 and 95.5% in W4, and wool and cotton gin trash 10.4 and 89.6% in W5 (Das *et al.*, 2000).

Mixes W1 and W2 were composted in research compost vessels as described by Das *et al.* (1997). The compost vessels were

stainless steel containers with a diameter of 38.1 cm, depth of 49.5 cm and a volume of 56.8 L. The vessels were insulated with 7.6 cm of foam insulation to minimize conductive heat loss thereby simulating an environment within a large compost pile. Temperature of the material in the vessel was monitored using T-type thermocouples and vessels were aerated using a temperature feedback control with an adjustable set point. When the compost temperature exceeded the set point, air was continuously introduced to the vessel at a rate of 40.8 m^3 day^{-1}. When the temperature of the compost was below the set point, air was introduced intermittently based on a timer providing aeration for 30 seconds in every 20 minutes. The resulting effective aeration of 1.02 m^3 day^{-1} was designed to provide sufficient oxygen (>6% residual oxygen) to maintain aerobic microbial growth. The composting continued in the vessels for approximately four weeks, and the materials were removed and allowed to cure (at room temperature with no forced aeration) for a period of over 9 months before using in this study.

Table 1. Mixes used in developing the composts used in greenhouse study

Sample	Components in the mixes and percentage by weight as received	Method of compost preparation
W1	33 Wool + 67 Cotton gin trash	Laboratory reactors
W2	30 Wool + 70 Wood shavings	Laboratory reactors
W3	3 Wool + 60 Yard waste + 37 Biosolids	Field windrows
W4	4.5 Wool + 95.5 Yard waste	Field windrows
W5	10.4 Wool + 89.6 Cotton gin trash	Field windrows

Field treatments were placed in windrows, which ranged in weight from 9.5 to 33.7 tons. A tractor-pulled windrow turner (Aeromaster PT120, Midwest Biosystems Inc., Illinois USA) was used to homogenize the materials in the windrow. At the end of two weeks, water was added using a soaker hose assembly that provided water at a rate of 37 L min^{-1} (9.4 gal min^{-1}) to maintain moisture content of approximately 50%. The composting process continued for 96 days of active management.

Ornamental plant establishment: Wool composts W1, W2 and W3 were each blended with a base mix (containing equal parts by volume of pine bark, perlite, and vermiculite) at 25, 50 and 67% (v/v) inclusion and the resulting potting mixes were evaluated for growing pansies in a completely randomized design with five replicates per treatment. Data collected were analyzed using ANOVA and where applicable comparison of means was conducted using the Duncan's Multiple Range test. In a separate experiment with similar experimental design, marigold plants were grown in pots containing a mix of 25% (v/v) of W5 wool compost and 75% of the base mix. A commercially available Fafard 3B potting mix (blend of milled pine bark, sphagnum peat moss, and perlite) was used as a reference or control potting mix to compare the performance of the treatments. Plant seedlings used were approximately 51 to 76 mm tall (2 to 3 inches) and were obtained from a local garden center. All pots were watered with tap water once a day or to field capacity for optimal moisture content. No fertilizer was used on the wool potting mix during the course of the experiment since the mixes' ability to satisfy plant nutrient requirements without using external sources of nutrients was being tested. The experiments were conducted for six weeks. At the end of this time period plant growth observations such as biomass yield, number of buds per pot, and number of open buds were made and above ground plant biomass was collected to evaluate total dry biomass per pot.

Turfgrass establishment: Soil in the turfgrass test plots (1 x 1 m) was amended with a 51-mm (2-inch) layer of the W4 and W5 composts, respectively. Each treatment was replicated in a completely randomized design as described earlier and soil amended with straw mulch and NPK fertilizer (300 kg/ha of 10-10-10 fertilizer) was used as a control. The surface-applied composts were tilled to a soil depth of approximately 100 to 125 mm (4 to 5 inches) and then a 1:1:1 mixture of Bermuda grass (*Cynodon dactylon* (L.) Pers.), perennial ryegrass (*Lolium perenne* L.), and annual ryegrass (*Lolium multiflorum* Lam.) was planted at a sowing rate equivalent to 200 kg ha^{-1}. After six weeks of growth, the grass was clipped at about 25 mm (1 inch) above the soil level. The collected samples were dried in a forced air oven (70°C; 72 h) and weighed to obtain dry mass per plot.

Sample analysis: Dried and homogenized samples (1 g) of composts were digested in three replicates in open vessels with concentrated HNO_3. The resulting digests were filtered through Whatman 42 filters, adjusted to 100 mL volume, and diluted 10 times prior to instrumental analysis. Compositions of regulated elements (As, Cd, Cr, Cu, Hg, Mo, Ni, Pb, Se and Zn) were analyzed by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS). The resulting measurements are regarded as total compositions in the tested materials. Samples of standard reference materials (SRM l570a and 2709) were included in analytical batches to check the accuracy of the analytical method used. Duplicate samples (approximately 10% of all samples) were used to check precision. Total N in composts were obtained by the combustion of a dry sample in a LECO 2000 CNS analyzer. The pH and soluble salt content of composts were determined by using the standard methods typically used in soil science (Sparks, 1996).

Market research and financial analysis: Market research involved direct interview and phone survey of over 50 retail garden stores located around urban centers in Georgia (retail sales census). Demand for compost products and selling prices were obtained from vendors and in some cases internet sites of commercial establishments. Data collected included quantity of different types of potting media and composts sold and their prices. Projected demands in similar urban areas statewide were estimated by multiplying the compost use per capita from sampled areas by known population densities at other urban locations.

The financial analysis included preparation of pro-forma income statements for a hypothetical compost factory and cost-volume-profit (CVP) analysis. CVP analysis (also known as break-even analysis) is commonly used to determine the effect of changes in a company's selling prices, costs, and volume on its profit in the short term (Homgren *et al.*, 1999). Reliable CVP analysis requires a separation of the total costs into variable and fixed costs. Fixed costs remain constant over some relevant range of output and are time related costs rather than volume related costs. In contrast, variable costs vary directly with changes in volume of output. Cost data used were adopted from Tyler (1996) and Das *et al*. (2001) for the composting side of the business. Prevailing tipping fees were obtained from the Georgia Department of Community Affairs' Solid Waste Report.

Table 2. Composition (mg kg^{-1} dry wt.) of regulated metals in the indicated composts and a commercial mix

Sample[1]	As	Cd	Cr	Cu	Hg	Mo	Ni	Pb	Se	Zn
W1	1.7	0.4	24.4	46	<0.1	2.3	5.0	0.6	20.7	182
W2	1.5	0.3	8.5	20	<0.1	0.6	0.2	0.6	12.4	99
W3	1.4	0.1	7.6	8	<0.1	0.4	0.2	0.6	5.2	45
W4	13.5	0.3	30.0	114.5	0.8	2.5	8.3	7.0	5.5	224
W5	13.9	0.2	19.6	99.3	0.1	2.3	5.9	8.8	4.2	218
Control[2]	0.4	1.1	344	16	0.1	0.8	45	1.5	0.1	67
US EPA[3]	41	39	1200	1500	17	18	420	300	36	2800

[1] W1 (30% wool, 70% wood shaving, w/w, as received), W2 (33% wool, 67% cotton gin trash), W3 (3% wool, 60% yard trimmings, 37% biosolids), W4 (4.5% wool, 95.5% yard trimmings), and W5 (10.4% wool, 89.6% cotton gin trash).
[2] Commercial potting mix.
[3] Environmental protection agency's published limits. Source U.S. EPA (1993).

Results and discussion

Individual pH values of the composts measured at the end of composting were 6.6, 7.2, 7.8, 7.8, and 7.8 for W1, W2, W3, W4, and W5, respectively. Compost W4 and W5 had high salt contents of 3.0 and 2.7 dS m^{-1}, respectively, suggesting potential concern when used with soluble salt sensitive plants (Composting Council, 1995). Salt contents of W1, W2 and W3 were found to be 1.1, 0.9, and 1.8 dS m^{-1}, respectively. Nitrogen (N) contents for the five composts were 3.7, 7.5, 1.5, 1.8, and 1.8% for W1, W2, W3, W4, and W5, respectively. The high concentration of total N seen in W1 and W2 is attributed to the high fraction of wool in these mixtures. The wool itself had a N content of 8.6% as a result of being largely keratin protein. Although high in N, this may not necessarily be an excellent fertilizer because the release of this N is expected to be very slow because of the recalcitrance of the wool to biodegradation.

Amounts of regulated trace elements in the composts and the control potting mix were below the regulatory limits established by the US EPA (Table 2). Since properly composted organic materials are class A products as defined by the US EPA, and because the composts and (compost-based) potting mixes have acceptably low metal concentrations, they can be used and distributed without restrictions.

Greenhouse experiments: The mixes with the higher percentages of composts produced bigger plants of pansies that had better foliage and flower quality than the control and the tested mixes with lower proportions of compost (Table 3). Visual evaluation of plant growth and appearance and biomass yield indicated that W1 was the best performing compost. Plant yield measurements showed that the mix containing 67% of W1 produced significantly higher (1.5 times more) biomass than the control treatments. The better performance of the mixes with more compost could be a result of higher N and trace element concentrations (Table 2).

The fertilized control produced larger plant biomass as a result of the easy availability of nutrients but had fewer open flowers than the W5 treatment (Table 4). Marigolds grew and developed well in the W5 potting mix containing 25% wool compost. Relative to the control mix with no fertilizer, the W5 mix produced about 10% bigger biomass of healthy and attractive plants (Table 4). In addition, the marigolds in the W5 mix had a higher percentage of open flowers than the control treatment. Visual evaluation indicated that marigolds grown in the W5 treatments were more vigorous and had more blooms than did the controls. An additional benefit of using the W5 mix instead of a commercial mix is the potential cost savings from growing plants without fertilizer addition.

Table 3. Yield of pansies in three compost types and three expanded mix levels

Test Compost	Expanding mix : Compost	Biomass yield (g pot^{-1})
W1	3:1	8
W2	3:1	6
W3	3:1	4
W1	1:1	8
W2	1:1	6
W3	1:1	4
W1	1:2	9
W2	1:2	8
W3	1:2	7.5
Control	1:2	6

Note: Expanding mix or base mix contained pine bark, perlite, and vermiculite and was used as the base medium for incorporating the compost.

Table 4. Growth response of marigolds to various potting mixes

Treatment	Marigolds[1]		
	Dry biomass (g pot^{-1})	Number total buds per pot	Number open buds per pot
W5 (25%)[2]	9.4^{ab}	36^{a}	12^{a}
Control[3]	8.4^{a}	36^{a}	6^{b}
Control[3] +NPK	12.5^{b}	43^{b}	9^{ab}

[1] Values that superscripted with different letters are significantly different at P<0.05.
[2] Wool compost + base mix in a ratio of 1:3.
[3] Commercial potting mix.

Turfgrass establishment: Both wool compost treatments produced significantly higher biomass than the control (Table 5), confirming the beneficial effect of the nutrients and organic properties brought by the compost. The grass grown in the W4 and W5 treatments established a dense, uniform, and healthy-looking cover. Growth of the control plants was not as uniform as those growing in the wool compost treatments.

Table 5. Growth response of the 1:1:1 grass mixture to wool compost applications

Treatment	Dry biomass (g plot^{-1})[1]
W4	160.1^{a}
W5	155.1^{a}
Control[2] + NPK	116.1^{b}

[1] Values that superscripted with different letters are significantly different at P<0.05.
[2] Commercial potting mix.

Market research and financial analysis: Projections calculated from retail sales census indicate that the market size in Georgia for potting mixes and soil amendments is approximately 450,000 tons year^{-1} (Table 6). Georgia is a state in the southeast United States with warm temperate climate. The estimated population in Georgia in 2005 was 9.07 million with approximately 70%

living in urban areas (US Census Bureau, 2006). There were also an estimated 3.7 million homes in the state. Assuming 50% of the compost demand in the state goes to commercial uses and 50% to residential, the market size translates to approximately 80 kg home^{-1} yr^{-1} or 30 kg capita^{-1} yr^{-1}.

In order to maximize returns, compost products could be offered at the retail level as potting mixes and soil amendments targeting several different markets. The markets for organic by-products include the following: (i) retail markets (garden centers); (ii) sod producers and turf industries; (iii) landscaping industries; (iv) greenhouses and nurseries; and (v) topsoil blenders (Table 6). To minimize transportation costs of bulk sale product, it is recommended that the marketing effort focus on customers within 30-miles of a compost facility in an urban area. The retail market, however, does not have to be limited to the vicinity of a compost factory; once the product is bagged, it can be shipped distances farther than 30-miles and still produce a profit. Expanding markets over the 30-mile radius can also be profitable in high profit margin markets such as golf courses.

Table 6. Estimated markets for compost products in Georgia

Market	Tons per year
Sod production and turf	270,923
Garden centers	113,530
Landscapers	36,123
Topsoil blenders	20,642
Nursery and Greenhouse operators	12,901
Total	454,119

A composting business is different from other businesses in the way that it serves two sets of customers: (i) inflow customers who require disposal of their solid waste (*e.g.*, fibrous wool processing waste, biosolids, cotton gin trash, *etc.*) and (ii) outflow customers who buy compost products. As a result of waste management functions (inflow), a composting operation can be paid to take raw materials. The projected operating income for a compost factory is based on an assumption that 20,000 tons of final products can be sold at a weighted average price of $25 tonne^{-1}, generating manufacturing revenue of $500,000 (Table 7). Typical costs for compost production indicate that total capital and operating costs (for yard trimming composting) range between $9 and 28 tonne^{-1} depending on amount of grinding, duration of composting and location of facility (Steuteville, 1996). In our analysis, we assumed a production cost of $18.5 tonne^{-1} which is an average value within the range reported in the literature. The sale price of $25 was arrived based on selling 90% of production as bulk product at $12 tonne^{-1} and 10% as bagged product at $150 tonne^{-1} ($0.15 kg^{-1} or $2.7 for a 40-lb bag). The weighted average price, at sales levels of 20,000 tons of the product per year, results in an operating income of $20,000. Adding inflow revenue from tipping fee for waste material processing would further increase earnings before income taxes to $286,667. Assuming an effective tax rate of 35%, the net income would be $186,337 (Table 7). Changing both the sale price, cost of operations, and/or the tipping fee would affect the bottom line of a composting business. If the tipping fee were increased to $15 tonne^{-1}, which is not an unrealistically high price, and the (weighted) average sale price dropped to $20 ton^{-1}, there would be an operating loss of $80,000, but net income, resulting from both sides of the business, would still be around $201,500 (Table 7). If operating costs increased by up to 55% at sales of 20,000 tons, the business would still be profitable, provided revenues from both sides are included. For example, if the operating costs were higher by about 10% the net income (from both sides of the business) would be about $232,000. The increase in operating costs, especially selling and marketing costs, is very likely if the products are to be introduced successfully in new and undeveloped markets.

Table 7. Pro forma income statement of composting business Wool Waste Composting Company, Income statement for the year ended December 31, 2006 (value in US $)

Items	Case I	Case II
Sales Revenue	500,000[a]	400,000[a]
Cost of goods sold – manufacturing and interest on capital	370,000[b]	370,000[b]
Gross income	130000	30000
Operating expenses		
Selling & marketing	60000	60000
Administrative & general	50000	50000
Total operating expenses	110000	110000
Operating income	20000	80,000
Other revenues and gains		
Tipping revenues	266,667[c]	390,000[c]
Earnings before income taxes	286667	310000
Income taxes	100,333[d]	108,500[d]
Net income	186333	201500
Earnings per share	1.86[e]	2.02[e]

[a] Assuming sale of 20,000 tonnes of the product at a weighted average price of US$25 and US$20per tonne, for case I and case II, respectively.

[b] The cost of operating a compost facility is estimated at $18.5 per tonne, which is the median in the range reported by Steuteville (1996).

[c] Assuming acceptance for processing 26,667 tons of waste material at a price of $10 and $15 per tonne for case I and case II, respectively. These input materials undergo a 25% mass reduction over the composting period to produce 20,000 tonnes of compost product.

[d] Assuming 35% effective tax rate.

[e] Assuming 100,000 common shares outstanding and no preferred or dilutive securities.

The cost-volume-profit (CVP) analysis (break-even analysis) is used to determine the effect of changes in the company's selling prices, costs, and volume on its profit in the short term. Reliable CVP analysis requires separating the total costs into variable and fixed costs (Table 8). The company's fixed costs include depreciation, insurance, property taxes, advertising, administrative costs, *etc.*, and are projected to be $200,000 for the range of output of 15,000-50,000 tonnes of product (Table 8). Direct materials costs, direct labour costs, some selling costs, and part of the overhead are variable costs and are estimated to be $11.45 tonne^{-1} of the company's product. The difference between unit revenue and unit variable cost, *i.e.* the unit contribution margin equals $13.55. This is the amount that would contribute to the coverage of the fixed cost and net income after covering the company's variable costs. At the projected cost structure, production and sales of 17,200 tonnes of the product would be a break-even point in units, and $429,000 in sales revenue would be a break-even point in dollars for the outflow side of the business.

Table 8. Projected annual manufacturing and sales activities of a compost factory

Items	Amount ($)
Variable manufacturing cost (per tonne)	
Direct materials	5.25
Direct labor	2.40
Overhead	0.80
Total	8.45
Variable selling expenses (per tonne)	3.00
Total variable costs	11.45
Fixed costs:	
Manufacturing overhead	150,000
Administrative expenses	50,000
Total fixed costs	200,000
Selling price per ton	25[a]
Units produced and sold (tons)	20,000
Contribution margin income statement for wool waste compost factory at break-even point	
Revenue (17,160 x $25)	429,000
Less: Variable manufacturer	169,000
Variable selling	60,000
Total contribution margin	200,000
Less: Fixed manufacturing costs	150,000
Fixed administrative costs	50,000
Net operating income	0

[a] Weighted average sale price based on assumption that about 10% of product is sold retail in bags.

Increasing sales over 17,200 tonnes at the same cost structure would generate operating income. It is important for the compost factory operator to assure operation at a profitable production volume. Therefore, if the supply of wool by-products is not large enough to generate enough product volume then inclusion of other raw materials (*e.g.*, biosolids, yard waste, cotton gin trash, *etc.*) in production may be necessary.

The tested composts are materials that meet the U.S. EPA's definition of a Class-A product and have physical and chemical properties suitable to support plant growth in a greenhouse when used as components of potting mixes and in the field when applied as a soil amendment. The wool composts can be successfully used as a component of potting mixes to grow ornamentals. Although mixes of various compositions were successful in supporting plant growth, generally, only mixes with higher percentages of composts surpassed the performance of the control treatment.

The wool composts can be successfully used as soil amendments to establish turfgrass. It is expected that these materials can be beneficially used in field vegetable production as well. The greenhouse experiment has shown that wool composts can be used as topsoil to grow sod on plastic. Because of their balanced nutrient composition and favourable chemical and physical properties, the wool composts produced a better grass coverage than the control mix.

At the projected cost structure (fixed costs: $200,000; variable costs: $11.45 tonne^{-1}) and at an average sale price of $25 tonne^{-1}, production and sales of about 17,200 tonnes of the product would be a break-even point in units, and $429,000 of sale revenue would be a break-even point in dollars for the outflow side of the business. The markets for organic by-products in the state of Georgia in the USA listed in order of decreasing profit margin include retail markets (garden centers), sports turf industries, landscaping industries, greenhouses and nurseries, topsoil blenders, and sod producers. Our analysis indicates that a manufacturing facility that has over 17,200 tonnes of production and sales, would generate a profit and income to the operators.

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Effect of pollen source on productivity, maturity and fruit quality of 'Hayyani' date palm

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Abstract

To study the effect of pollen source on the productivity, maturity and fruit quality of 'Hayyani' date palm, one local and three commercial male varieties were used during 2002. Effect of pollen source on 'Hayyani' fruit-set and yield was statistically not significant, however, trees pollinated with 'Mejhool' pollen recorded the highest fruit-set and yield. The largest fruit weight, length and diameter were obtained when trees pollinated with 'Barakah' male. In addition, pollen source had no or little effects on 'Hayyani' fruit maturity, however, fruits of trees pollinated with 'Jarvis' male matured earlier while in trees pollinated with 'Barakah' showed delayed maturity. Moreover, 'Hayyani' trees pollinated with 'Barakah' pollen gave the highest fruit flesh %.

Key words: Male, pollen, Hayyani, pollination, maturity, fruit quality, date palm, *Phoenix dactylifera* L.

Introduction

Date palm (*Phoenix dactylifera* L.) plantations in Jordan have increased in the last years; however, this has not coincided with the increase in the knowledge of the appropriate cultural practices for date palm such as pollination. In addition, little information is available about date palm males used in pollination and its viability, which could mean the use of low quality males (low pollen viability) in pollinating highly valuable female trees, that will affect fruit set and thus yield and quality of date palm fruits.

Date palm is a dioecious plant with separate male and female trees in which pollination is normally done by wind. However, to ensure and improve fruit setting, pollination is done artificially in which mature male inflorescence are cut off before spathe splits, and male strands are placed in the female flower cluster so pollen will be transferred to female inflorescence (Asif *et al*., 1983; Asif *et al*., 1987; ElMardi *et al.*, 2002; Shabana *et al*., 1985). In addition, pollen of date palm has effects on the resulting seed shape and size (xenia) and on the size, development, quality and ripening time of date palm fruits (metaxenia) (Nixon, 1955).

Pollen source affect ripening time and it ranged from about 10 days when fruits ripen early in hot weather, to as much as 2 month when fruits ripen late in cool weather (Nixon, 1955). In addition, pollen which produced smaller fruits and seeds, also produced earlier ripening (Monselise, 1986). Pollen from both 'Ghannami' and 'Werdi' males shortened the yellowing time of the Khalal stage of 'Zehdi' and increased the percentage of matured fruits. However, pollen from 'Rissasi' and 'Werdi' resulted in increased fruit set compared to other males. Fruits from trees pollinated with 'Werdi' had the lowest seed weight percentage (the highest edible portion), however, those pollinated with 'Ghannami' had the highest percentage of soluble solids (Delaimy, 1969).

This research aimed to study the effect of different sources of pollen (male varieties) on the yield, quality and maturity of 'Hayyani' date palm cultivar.

Materials and methods

The study was conducted in a private farm at Aqaba area on twelve, 18 years-old uniform 'Hayyani' trees divided into three replicates during the 2002 season. A local date palm seedling male was evaluated with respect to its viability (according to germination test in a nutrient media), and named 'Barakah' male according to its location in Jordan. In addition, three commercial males (`Boyer', `Jarvis' and `Mejhool') collected from local date palm farms were used in this study.

At the beginning of the season, pollen germination test was done for all sources in a nutrient media consisted of 20 % sucrose and 1 % agar according to the method followed by Kwan (1969) and Asif *et al.* (1983). Pollen germination was recorded after 24 hour under microscope. Pollen having germination tube longer than pollen diameter were taken as geriminated. Results were statistically analyzed according to Randomized Complete Block Design (RCBD) with three replicates.

At the time of natural opening of female spathe, each tree was pollinated with one source of pollen and the spathe was covered with a paper bag to prevent contamination with other sources. Pollen was diluted by mixing with flour in a ratio of 1:2 to insure maximum pollen distribution. Bags were removed after two weeks of pollination.

Seven female spathes were left on each tree to ensure uniformity. Fruit set percentage was calculated four weeks after pollination. At the end of the season, the following parameters were recorded: Total yield, fruit and seed weight, length and diameter, fruit total soluble solids (TSS %), maturity % (calculated as weight of fruit at Rutab stage to the total fruit weight at harvesting date), and flesh %. Data was analyzed using ANOVA with three replicates.

Results and discussion

Pollen germination: Pollen germination percentage ranged from 60.7% for 'Mejhool' to 76.6 % for 'Boyer' which significantly gave the highest pollen germination (Table1).

Table 1. Pollen germination of male varieties used in the experiment

Treatment	Pollen germination (%)
Boyer	76.6 a*
Barakah	73.8 ab
Jarvis	70.6 b
Mejhool	60.7 c

* Mean separation within columns by LSD test, values that don't share the same letter are significantly different at *P*=0.05.

Fruit set percentage: No significant difference was observed among treatments when trees were pollinated with 'Mejhool' (68.1 %) and the least fruit-set was recorded when trees were pollinated with 'Barakah' (57.4 %) (Table 2).

Total yield: No significant differences was observed among treatments. However, trees pollinated with 'Mejhool' pollens gave the highest yield (78.4 kg) while trees pollinated with 'Jarvis' which was the lowest yield (57.9 kg) (Table 2).

Table 2. Effect of pollen source on fruit set (%) and total yield (kg) of Hayyani fruits

Treatment	Fruit set (%)	Total yield (kg per tree)
Boyer	67.6 a*	66.5 a
Barakah	57.4 a	69.2 a
Jarvis	59.5 a	57.9 a
Mejhool	68.1 a	78.4 a

* Mean separation within columns by LSD test, values that don't share the same letter are significantly different at *P*=0.05.

Fruit weight: Fruit weight was highest when trees were pollinated with 'Barakah' male (12.0 g). This could be due to low fruit set obtained when trees pollinated with this male. But it had no significant differences with those pollinated with 'Jarvis' and 'Boyer' (9.8 and 8.4 g, respectively). Trees pollinated with 'Mejhool' pollen gave the least fruit weight (7.4 g) (Table 3).

Fruit length and diameter: No significant difference was observed among the treatments in relation to fruit length. However, the highest fruit length was observed when pollinated with 'Barakah' male (39.5 mm) while those pollinated with 'Mejhool' pollen had the least (31.9 mm) (Table 3).

Table 3. Effect of pollen source on fruit weight, length and diameter and L/D of Hayyani fruits

Treatment	Average fruit weight (g)	Average fruit length (mm)	Average fruit diameter (mm)	L/D
Boyer	8.4 ab*	34.0 a	21.2 ab	1.6 a
Barakah	12.0 a	39.5 a	24.0 a	1.7 a
Jarvis	9.8 ab	36.0 a	21.0 ab	1.7 a
Mejhool	7.4 b	31.9 a	20.5 b	1.6 a

* Mean separation within columns by LSD test, values that don't share the same letter are significantly different at *P*=0.05.

The same was observed for fruit diameter but the differences were significant. The increase in fruit size could have contributed to the increase in fruit diameter rather than fruit length since no significant difference was observed for fruit length.

Fruit shape results showed that pollen had no effects on 'Hayyani' fruit shape (Table 3).

Seed weight, length and diameter: No significant difference was observed among treatments in relation to average seed weight and length. However, trees pollinated with 'Boyer' and 'Barakah' gave the largest seed diameter (10.3 and 10.2 mm, respectively), and those pollinated with 'Jarvis' gave the least (9.7 mm). In addition, no significant difference was observed among treatments with respect to length/diameter ratio, which meant that there was no effect of pollen source on seed shape. Consumers prefer date fruits with small seeds which could be obtained when 'Hayyani' trees were pollinated with 'Jarvis' male (Table 4).

Table 4. Effect of pollen source on seed weight, length, diameter and L/D of Hayyani fruits

Treatment	Average seed weight (g)	Average seed length (mm)	Average seed diameter (mm)	L/D
Boyer	1.7 a*	24.2 a	10.3 a	2.3 a
Barakah	1.6 a	24.5 a	10.2 a	2.4 a
Jarvis	1.5 a	24.2 a	9.7 b	2.5 a
Mejhool	1.5 a	23.6 a	10.0 ab	2.4 a

* Mean separation within columns by LSD test, values that don't share the same letter are significantly different at *P*=0.05.

Maturity percentage: With regard to maturity, no significant difference was observed among the treatments, however, trees pollinated with 'Jarvis' male had early fruit maturity while trees pollinated with 'Barakah' pollens showed delayed fruit maturity (Table 5).

Flesh percentage: 'Hayyani' trees pollinated with 'Barakah' pollens gave the highest fruit flesh % (86.1 %), but with no significant difference with 'Jarvis' male (83.7 %). Trees pollinated with 'Mejhool' pollens significantly gave the least flesh percentage (78.4 %) (Table 5).

Total soluble solids (TSS): No significant difference was observed among treatments with respect to total soluble solids (TSS), however, trees pollinated with 'Boyer' male gave the highest fruit TSS (32.8 %) while trees pollinated with 'Barakah' pollens gave the least fruit TSS (29.6 %) (Table 5).

Table 5. Effect of pollen source on maturity, flesh percentage and total soluble solids (TSS %) of Hayyani fruits

Treatment	Maturity (%)	Flesh (%)	TSS (%)
Boyer	12.7 a*	79.3 bc	32.8 a
Barakah	6.4 a	86.1 a	28.4 a
Jarvis	12.8 a	83.7 ab	31.9 a
Mejhool	7.8 a	78.4 c	29.6 a

* Mean separation within columns by LSD test, values that don't share the same letter are significantly different at *P*=0.05.

Studying these results, we can conclude that to improve fruit weight and quality, 'Hayyani' trees should be pollinated with 'Barakah' male which increased fruit size by 35-60 % and fruit flesh % by 8-10 % compared to other males. But if high productivity is desired, 'Mejhool' will be the best option.

In addition, if early maturity is desired, 'Hayyani' trees should be pollinated with 'Jarvis' or 'Boyer' males. Moreover, if delayed maturity is desired 'Hayyani' trees should be pollinated with 'Barakah' and 'Mejhool' pollen.

Four date palm varieties used as pollen source influenced fruit size, pulp percentage, quality and ripening time of Hayyani fruits with varying degree and thus indicating that these parameters are influenced at genotypic level also. Such varietal response were also reported by Nixon (1955). Both xenia and metaxenia effects of pollen source were apparent in the present study. Influence of pollen source has been reported in date palm by Monselise (1986). Pollen from both 'Ghannami' and 'Werdi' shortened the yellowing time of the Khalal stage of 'Zehdi' and increased the percentage of matured fruits. However, pollen from 'Rissasi' and 'Werdi' resulted in increased fruit set. Fruits resulting from the trees pollinated with 'Werdi' had the highest edible portion, however, those pollinated with 'Ghannami' had the highest percentage of soluble solids (Delaimy, 1969). Our results indicate that 'Hayyani' also responds differently to various pollen sources and thus there is a scope for selection of pollen source for better and quality production.

Based on the results of the present study, it can be conclude that to improve fruit weight and quality, 'Hayyani' trees should be pollinated with 'Barakah' male which increased fruit size by 35-60 % and fruit flesh percentage by 8-10 %, compared to other males. But if high productivity is desired, 'Mejhool' will be the best option.

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