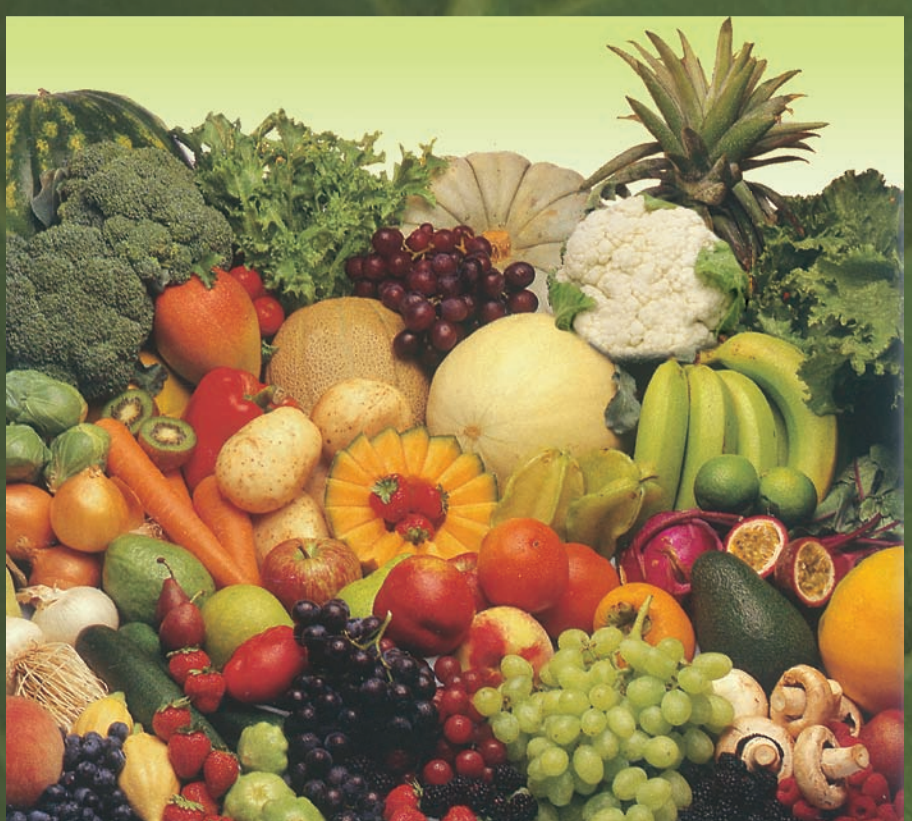
Journal of Applied Horticulture

ISSN 0972-1045
Vol. 12, No. 1, January-June, 2010



Journal of
THE SOCIETY FOR ADVANCEMENT OF HORTICULTURE

JOURNAL OF APPLIED HORTICULTURE
Vol. 12, No. 1, January-June, 2010

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Journal of Applied Horticulture, 12(1): 3-9, *January-June, 2010*

Anthocyanins accumulation and genes-related expression in berries of cv. Tannat (*Vitis vinifera* L.)

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Abstract

Anthocyanins accumulation and gene expression in berries of *Vitis vinifera* L. cv. Tannat trained in Lyre system was investigated. Expression of genes involved in anthocyanins biosynthetic pathway as chalcone synthase (*CHS*), flavonoid 3-hydroxylase (*F3H*), dihydroflavonol 4- reductase (*DFR*), and UDP-glucose flavonoid-3-O- glucosyl transferase (*UFGT*) was examined. On the other hand, the influence of plant architecture, Lyre and vertical shoot positioned (VSP) trellis systems, on anthocyanins accumulation and gene expression was also analyzed. Final contents of total anthocyanins were not affected by trellis systems but varied in two years with different water deficit imposition period. However, the individual profile of the genes and anthocyanidins modified according to the moment of water deficit imposition (*veraison* or harvest) and by the trellis systems (Lyre or VSP). Analysis of gene expression in Lyre along the berry development period showed that low leaf water potentials after *veraison* cause an earlier and greater induction compared with expression in a year with low leaf water potential at harvest. At harvest, the hydric deficit induced an increase in the expression of *CHS*, *F3H* and *DFR* genes and a higher total anthocyanins content. The study revealed that plant architecture affect the expression of anthocyanins related gene in berries possibly by modifying the canopy microclimate.

Key words: Anthocyanins, anthocyanidins, berries, hydric deficit, Lyre, plant architecture, raisin, transcripts profile, Tannat, veraison, *Vitis vinifera* L.

Introduction

Polyphenols are the main components involved in the colouration of grapes and provide the gustative characteristics. Given the inquisitiveness of humans and the importance of wine colour, flavour, and astringency, the management of phenolic compounds is becoming important (Conde *et al.*, 2007). Phenolic compounds of the grape are divided in flavonoids and non- flavonoids groups. Flavonoids include the anthocyanins that are responsible for the characteristic red, blue, and purple colour of plant tissues. Anthocyanins play an important role in plant reproduction and also in protection from different stresses, including photo-oxidative stress (Winkel-Shirley, 2002).

In grapes, the berry skin accumulates anthocyanins and the relative abundance is determined by the cultivars and also strongly influenced by the environmental conditions under which the vines are cultivated (Keller *et al.*, 2004; 2005). Drought, temperature and light are the main factors affecting the anthocyanins biosynthesis in grape fruits (Spayd *et al.*, 2002; Castellarin *et al.*, 2007a). The expression of the anthocyanin biosynthetic genes is induced by low temperature and repressed by high temperature in various plant species (Dela *et al.*, 2003; Lo Piero *et al.*, 2005; Ubi *et al.*, 2006).

The anthocyanin biosynthesis pathway (Fig. 1) is complex with branches leading to the synthesis of many different metabolites (Boss *et al.*, 1996). The coordinate induction of the expression of genes of the anthocyanin pathway in grapevine after *veraison* suggests that the pathway is tightly controlled by regulatory genes, as has been observed in other plant species (Deluc *et al.*, 2008).

There is extensive literature indicating that temperature has a greater influence on anthocyanin biosynthesis than light (Mori *et al.*, 2007 and references therein). Thus, it is reported that optimum temperature for anthocyanins synthesis in grapes occurs between 15 and 27ºC, and temperature above 30ºC induces degradation of these compounds. That is increased during the light period because of the activation of the corresponding enzymes during the day (Haselgrove *et al.*, 2000; Spayd *et al.*, 2002; Downey *et al.*, 2004). It is also known that cool temperatures at nights, during the maturation period, have a positive effect in the final polyphenols content in the berry skin (Mori *et al.*, 2005).

Cultivation of grapes under restricted water supply has long been regarded as an agronomic tool for increasing polyphenol content in berries. Although the concentration of anthocyanins and other phenolics compound has consistently increased in response to water deficit, it is unclear whether this higher concentration is only due to inhibited berry growth, lowered water content and higher concentration of solutes, or water stress actively triggers by itself the biosynthesis of phenolics compound (Castellarin *et al.*, 2007b and reference therein).

Vine training affects the foliage architecture and the plant shape, both of them greatly influence the plant - environment interaction. Plant responses to radiation, air temperature, and soil water content are modified by the training system, which has also an important impact on the phenolic compounds content of raisins (Boss and Davies, 2001; Conde *et al.*, 2007; Smart *et al.*, 1991).

In Uruguay, the environmental and culture conditions are such that vine management in Lyre training system produces higher yield than the vines managed in vertical shoot positioned (VSP)

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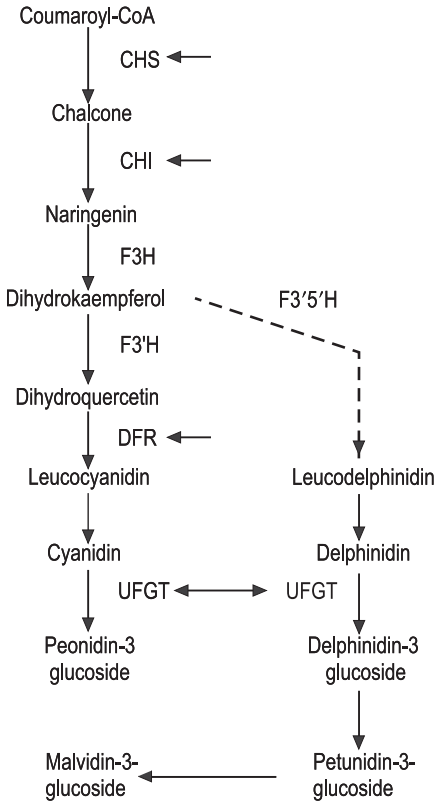

Fig. 1. A simplified diagram of anthocyanin biosynthesis pathway. Arrowheads indicate the gene coding for the enzymes analyzed in this study.

trellis system, without decrease in the enological quality of the berries (González-Neves, 2005). Berries of Tannat, the main black - red *Vitis vinifera* in Uruguay, have higher total anthocyanins content than Cabernet Sauvignon and Merlot (González-Neves *et al.*, 2004, 2007).

In the present investigation, fruit anthocyanins accumulation in grapevine (*V. vinifera* L) cv. Tannat and the expression of anthocyanins biosynthesis-related genes in a vineyard trained in Lyre and VSP system was studied.

Material and methods

Field plots and physiological measurements: Field experiments were conducted in commercial vineyards localized in the main viticulture region (Canelones; 34º 35´ S, 56º 15´ W; Uruguay), with a climatic condition classified as $IS_{A1}IH_{A4}IF_{A2}$ (climate moderated drougth, template warm. Vineyards of *V. vinifera* cv. Tannat were trained in Lyre and VSP trellis systems, the agronomic information about the vineyards is shown in Table 1.

Leaf water potential (LWP) was monitored by pressure chamber technique (Scholander *et al.*, 1965). Twenty adult, fresh and healthy leaves around the raisin (10 from east side, 10 from west side) were selected to perform the water potential measurements at flowering, *veraison* and harvest period. Measurements conducted at sunrise were scored as basal water potential.

Phenological stages scored from bud break were performed as proposed by Eichhorn and Lorenz (1977).

Grape samples: Samples of berries were taken from *veraison* until the harvest time, using the method described by Carbonneau *et al.* (1991), and sampling fractions of bunches in the middle zone of the spurs. Each fraction of the bunch had 3 to 5 berries randomly selected from the upper and lower parts of bunches, until 250 berries were collected. Samples were taken twice from each vineyard.

Fresh grape analysis: The harvest took place at the "technological maturity", according to the relationships between sugars, total acidity and pH, that were analysed according to O.I.V. (2007).

Berries were weighed and crushed in a mortar in order to remove the skins, seeds and pulp. The skins and seeds were rinsed and separated from the pulp, dried afterwards with filter paper and weighed. Skins were frozen and kept at -80°C until their analyses.

Grape extract analysis: In order to determine the total content of anthocyanins, macerations at pH 1 were carried out according to the methodology proposed by Glories and Augustin (1993). The anthocyanins contents of the grape extracts were determined according to Ribéreau-Gayon and Stonestreet (1965), and were calculated according to González-Neves (2005). All measurements were performed twice.

Anthocyanins profiles: The frozen skins from the samples of grapes were macerated in a 12% (v/v) ethanol pH 3.2 solution. The volume of the solution was calculated according to González-Neves (2005), in a ratio 1:1 to the volume of the must. Macerations were carried out using the skins of 50 berries, with two replications per sample of grapes.

The anthocyanins contents of the extracts were determined by HPLC according to Revilla *et al.* (2001). The extracts were previously filtered with a 0.45 µm membrane. A chromatographic system equipped with two pumps (Waters 510 and 515), a Rheodyne 7725i injector, a detector UV-VIS (Waters model 490E) with measurements at 313 and 530 nm, and a C18 column (Phenomenex Luna) (150 x 4.6 mm) of 5 µm (Waters Corp. USA) was used. Gradient conditions were carried out using two solvents. Solvent A: water-formic acid (90:10), solvent B: methanol-water-formic acid (45:45:10). Flow: 0.8 mL/min. Gradient: 35 to 95% of solvent B in 20 min, 95 to 100% of solvent B in 5 min, and isocratic with 100% of solvent B during 5 min.

Anthocyanins were identified taking into account the retention time of each peak and their relationship with the peak corresponding to malvidin-3-glucoside. The concentration of the 3-glucosides of delphinidin, cyanidin, malvidin, petunidin and peonidin were calculated considering a calibration curve with malvidin-3-glucoside chloride (Extrasynthèse, France).

Gene expression analysis: A subsample of berries from the same sampling for anthocyanins analysis was used for extracting the

Table 1. Characterstics of selected vineyards

Age (years)	Rootstock	Distance (m)	Density (plants ha^{-1})	Trellis system	Training
19	SO4	3.20 x 0.90	3472	Lyre	Cordon Royat
21	SO4	2.30 x 1.25	3478	VSP	Cordon Royat

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RNA. Total RNA was extracted from berry skin following the procedure described by Chang *et al.* (1993). Ten µg of total RNA extracted from different sampling dates were resolved in agarose (1.2 % w/v) under denaturing conditions and then transferred to a nylon membrane (Hybond N+). Gene expression was analyzed by RNA blot analysis using ^{32}P-ATP labeling (Amersham Pharmacia) of cDNA sequence from flavonoid 3-hydroxylase (*F3H*), chalcone synthase (*CHS*), dihydroflavonol reductase (*DFR*), and UDP-glucoflavonoid 3-o-glucosyl transferase (*UFGT*) (Sparvoli *et al.*, 1994).

Filters were exposed to X-ray film and the images were scanned in order to perform the image analysis (Image J 1.39 analysis software free access in http://rsb.info.ncb.gov/ij/). Relative expression was plotted using the comparison with the expression of a constitutive expressed gene (accession number BN000705; Bogs *et al.*, 2005).

Statistical analyses: Differences between the treatments were tested for significance using the Statgraphics Plus package 4.1 (Statgraphics Corp., USA) and SAS/Stat Software Release 8.2 (S.A.S. Corp., USA). Means were separated by Tukey test.

Results

Anthocyanins accumulation and individual anthocyanidins profile in Lyre: When total anthocyanins content of grape extracts at pH 1 were compared in two years analyzed, a difference at harvest date was observed (Fig. 2A). The evolution of the contents of the main individual anthocyanidins was quantified by HPLC in the extracts (pH 3.2) from the skins (Fig. 2 B and C). The main anthocyanidins in the two years was the malvidin-3-glucoside, which reached at the same relative amount 28 DAV. The relative amount of malvidin in the year 1 was significantly higher than year 2 between 7 and 21 DAV (Fig. 2 B).

In general trisubstituted molecules (delphinidin, petunidin and malvidin) were predominant over the bisubstituted molecules (peonidin and cyanidin) (Fig. 2 C). In year 1 the relative content of malvidin increased during the ripening process, whereas the relative contents of the cyanidins and peonidins decreased in the same period (Fig. 2 B and C). During the year 2 a decrease of malvidin was detected from 21 to 35 DAV, and in the same period an increase of delphinidin was observed (Fig. 2 B and C).

Leaf water potential and anthocyanins-related gene expression: Values lower than -1 MPa in leaf water potential (LWP) were detected in the vineyards during *verasion* and near harvest periods in the year 1 and 2 respectively (Fig 3).

Analysis of gene expression was performed in order to analyze the relationship between the genes expression of anthocyanins pathway and the low leaf water potential. When levels of transcripts of the four genes studied were compared in two years, one of them with hydric deficit during *veraison* (year 1) and the other with hydric deficit during harvest (year 2), a great difference in the expression profiles was observed (Fig. 4).

The low LWP in year 1 during the *veraison* induced an earlier expression of all genes studied (Fig. 4). However, in year 2, low LWP during the last days of berries ripening process, near the harvest time, was related with a higher expression of *CHS*, *F3H*

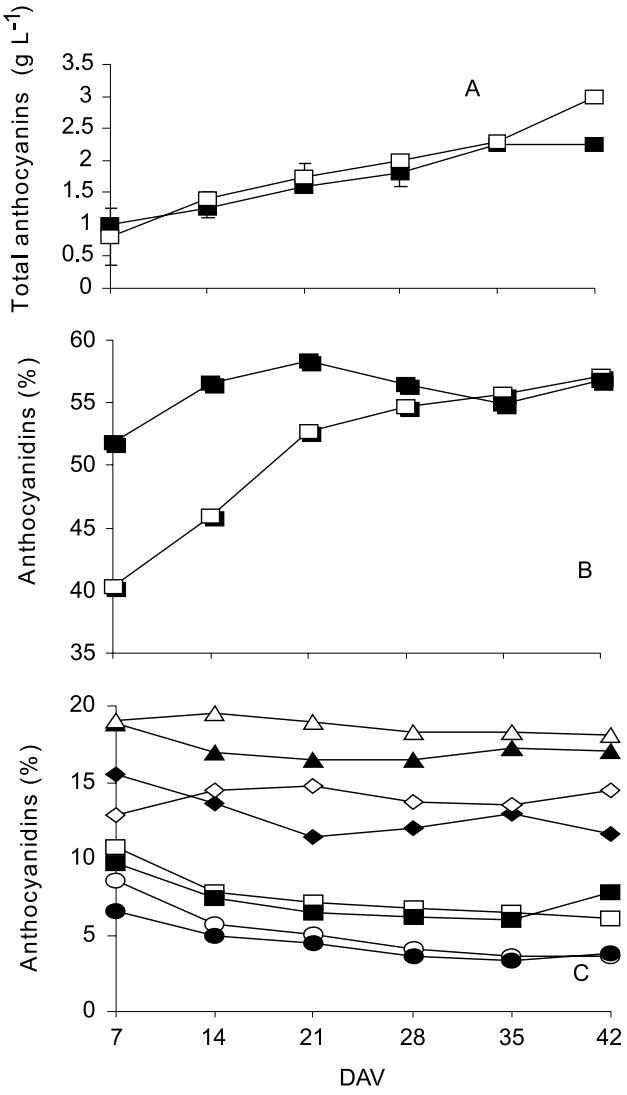

Fig. 2. Accumulation of total anthocyanins and relative amount of five anthocyanidins in berry of Lyre vines. White symbols year 1 and black symbol year 2. (A) total anthocyanins, (B) malvidin and (C) petunidin (triangles), delphinidin (diamonds), peonidin (squares) and cyanidins (circles). DAV indicates days after *veraison*. Vertical bars indicate SD.

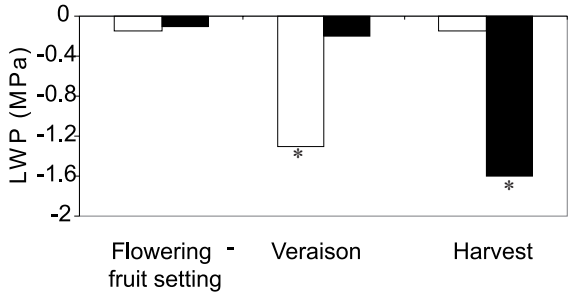

Fig. 3. Leaf water potential (LWP) in three stage of berry development (flowering-fruit setting, *veraison* and harvest) in Lyre managed vine. White bars year 1 and black bars year 2. Asterisk indicates differential significance $P \leq 0.05$.

and *DFR* in the last sampling date compared with the same date of year 1 (Fig. 4).

In case of the *UFGT,* the expression pattern was the same in both years but a delay of 15 days was observed when no hydric deficit occurred during the *veraison*.

Anthocyanins accumulation and individual anthocyanidins profile in two trellis systems: In order to analyze the influence

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of plant architecture on anthocyanins profile, we compared the Lyre with vertical shoot positioned (VSP).

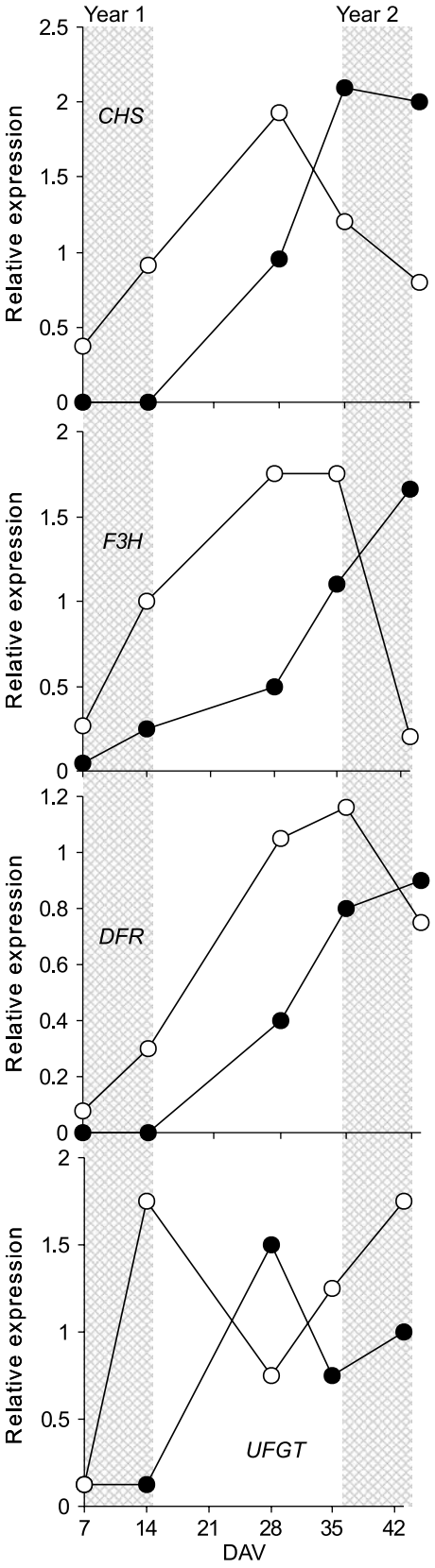

Fig. 4. Transcripts profiling of genes of the flavonoids pathways (*CHS*, *F3H*, *DFR* and *UFGT*) in berry skins of Lyre managed vines. White circles year 1 and black circles year 2. DAV indicates days after *veraison*. Grey background indicate period of leaf water potential lower than -1 MPa. Relative gene expression represents fold differences in gene expression relative to constitutive gene, for more details see materials and methods.

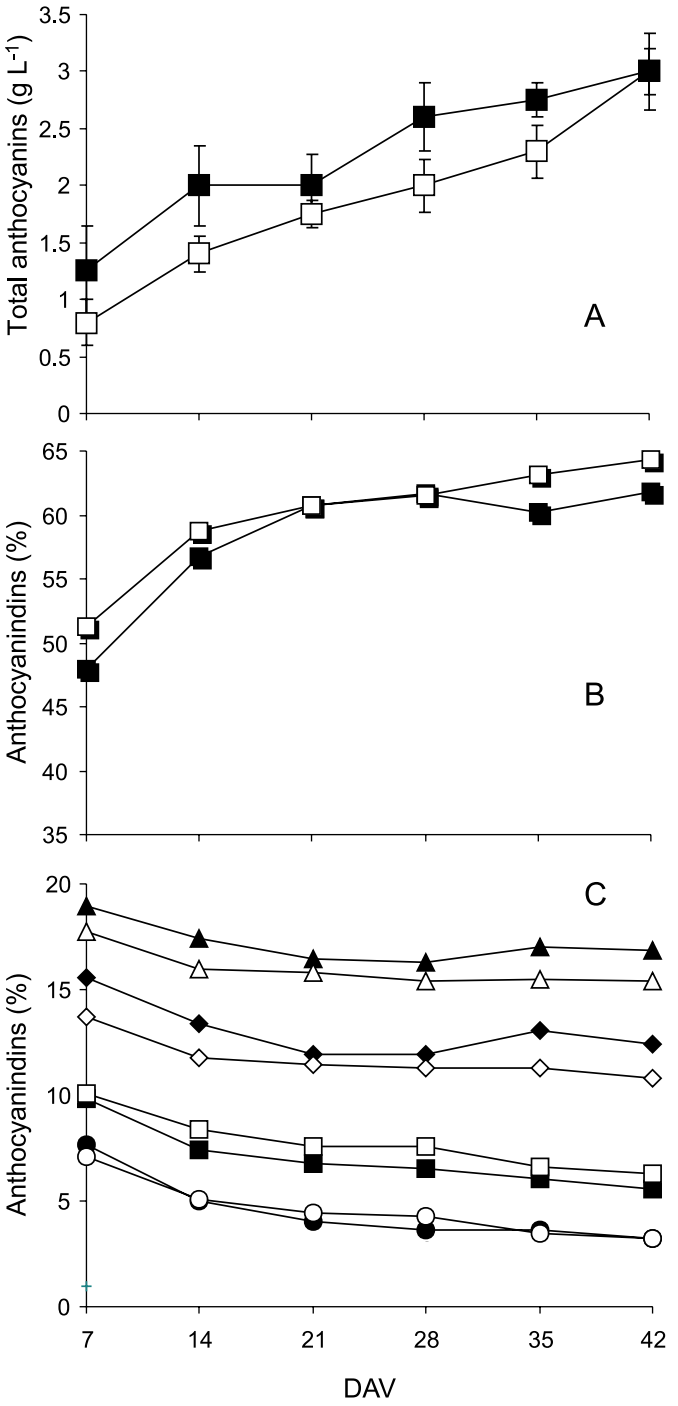

Fig. 5. Accumulation of total anthocyanins and relative amount of five anthocyanidins in berry skins of Lyre (white symbols) and vertical shoot positioned (VSP) (black symbols). (A) total anthocyanins, (B) malvidin and (C) petunidin (triangles), delphinidin (diamonds), peonidin (squares) and cyanidins (circles). DAV indicates days after *veraison.* Vertical bars indicate SD.

Berries of VSP system reached a higher concentration of anthocyanins at three of six sample dates compared with the values found in the grapes of Lyre system (Fig. 5 A). The berries of both training systems accumulated the same anthocyanins content at harvest, 42 days after *veraison* (DAV) (Fig. 5 A). When the changes of anthocyanins content between 14 and 21 DAV was analyzed for both training systems, no changes in the accumulation was observed in the VSP system in that period (Fig. 5 A). The anthocyanidins profile showed a similar pattern between both training systems (Fig. 5 B and C). The malvidin relative

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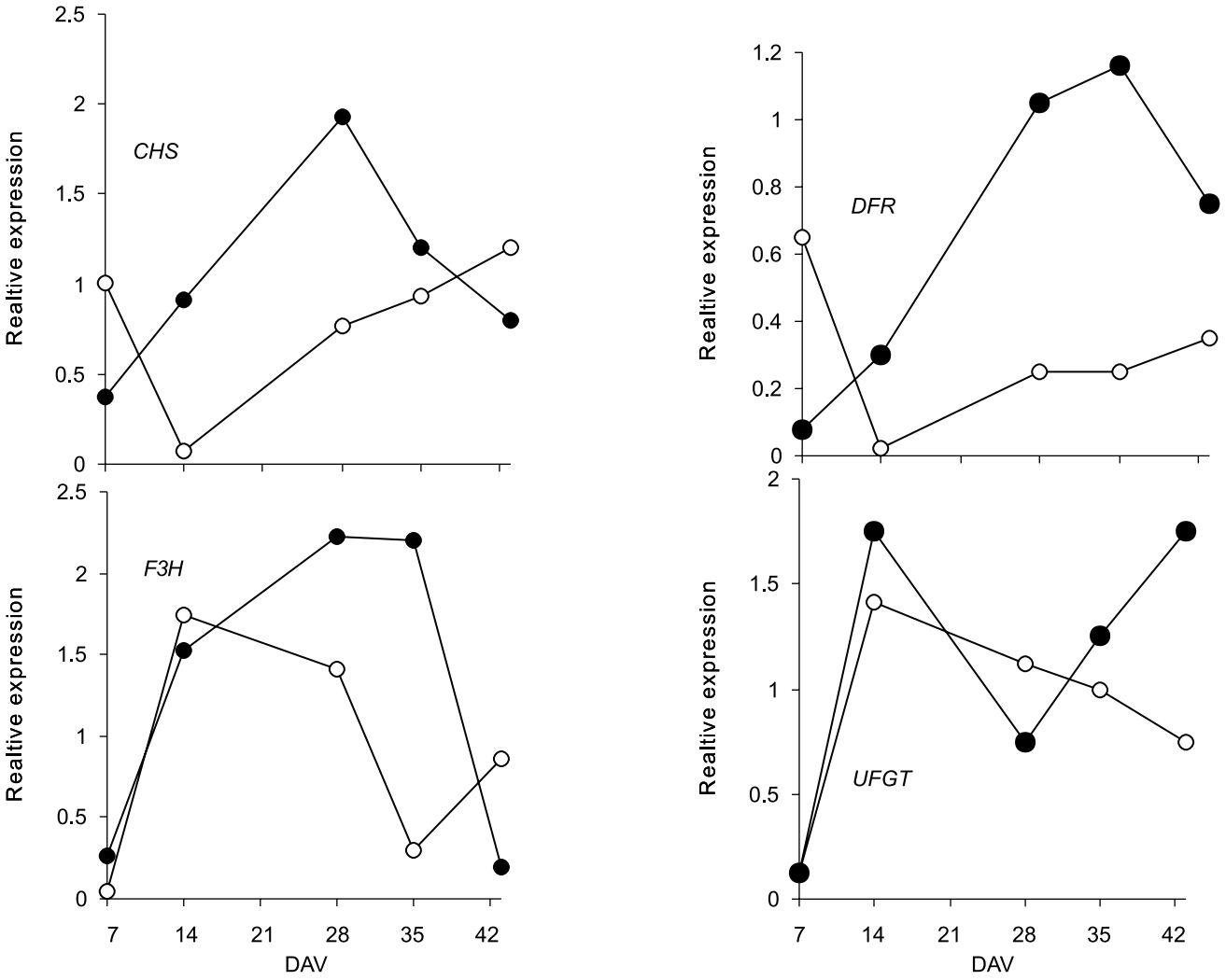

Fig. 6. Transcript profiling of genes of the flavonoids pathways (*CHS*, *F3H*, *DFR* and *UFGT*) in berry skin of Lyre (black circles) and vertical shoot positioned (VSP) (white circles) managed vines. DAV indicates days after *veraison*. Relative gene expression represents fold differences in gene expression relative to constitutive gene, for more details see Materials and methods.

content in 35 and 42 days was related negatively with petunidin and delphinidin relative contents in both training systems (Fig. 5B and C).

Training system and anthocyanins-related gene expression: In order to compare the possible incidence of training systems in the transcription of genes of the flavonoids pathways, the expression of four structural genes encoding for enzymes of the flavonoids pathway were analyzed from *veraison* to harvest. In general, increases in the expression of the four genes were detected in both training systems during the berry growth, and that was in agreement with the changes in the skin colour and the anthocyanins accumulation in the berries (Fig. 6). However, when the genes were analyzed individually, different gene expression profiles were found between both training systems.

CHS and *DFR* showed similar expression pattern in each training system. In Lyre, at 28 DAV and 35 DAV a maximum expression in *CHS* and *DFR* respectively was observed followed with a decrease in the two genes until harvest date (Fig. 6). In VSP a minimum in the expression of both genes was observed 14 DAV, which was reverted in next sampling dates. Between 14 and 35 DAV the expression levels for these genes were significantly lower in the VSP than in the Lyre system (Fig. 6).

F3H transcript profile was different in the two training systems analyzed. In VSP maximum expression was at 14 DAV and in Lyre maximum occurred at 28 DAV (Fig. 6).

The gene related with anthocyanins synthesis and storage, the *UFGT* gene, showed a strong induction at 14 DAV and similar time-course expression pattern in both training systems. However, differences in the transcripts accumulation at the last date of sampling was observed between Lyre and VSP trellis (Fig. 6).

Discussion

Viticulturists know that canopy management is essential to obtain a good quality of grape, and the trellis system is the main practice with influence on this parameter. There are many factors that are influenced by the training systems, as quality and quantity of sunlight interception, canopy density, fruit temperature and water use efficiency (Smart and Robinson, 1991).

Higher yield under Lyre than other trellis systems, without loss of quality, has been observed for several years in cv. Tannat under the environmental conditions prevalent in the Uruguay viticulture region (González-Neves *et al.*, 2004).

The interaction with the environmental factors like light exposure,

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the water use availability, and temperature defines the ultimate composition of anthocyanins of the fruit. Many environmental factors affect the gene expression in the berry however the water deficit during the berry ripening process is one the most important factor. There are three stages of the vine growth and development when drought could considerably affect the berry size and composition; these are at flowering, at *veraison* and at harvest time (Keller *et al.*, 2005; Ojeda, 2005; Roby *et al.*, 2004).

Our study showed that total anthocyanins of berries of Tannat vines managed in Lyre were similar in both years of the study during the ripening process, but the content was different at harvest (Fig. 2 A). In the year 2 a hydric deficit around harvest time (Fig. 3) could explain the higher anthocyanins content in this year than to year 1 (Fig. 2 A).

It has been reported that water deficit stimulates hydroxylation and methoxylation of the flavonoid B-ring, accelerates particularly tri-substitued anthocyanins biosynthesis, and also promotes the conversion of hydroxylated anthocyanins into their methoxylated derivatives (Castellarin *et al.*, 2007b). The same study indicated similar consequences as results of early and late water deficits, in transient expression pattern that favours trisubstitued anthocyanins accumulation. However, the malvidin proportions found in Tannat reflects more the early hydric deficit noted in the vines in the year 1, whereas petunidin and delphinidin proportions were increased in the grapes obtained in year 2 when the hydric deficit occurred near the harvest (Fig. 2 B).

Taking in account the data obtained under hydric deficit period (*veraison* or harvest) we could propose that a period of water deficit close to *veraison* induce prematurely the expression of the genes involved in the anthocyanins pathways with the results of accumulation of these compounds in the berries (Fig. 2 and 4). Similar results were obtained when a field experimental trail was carried out with two dates of water stress. In that study an early water deficit accelerated sugar accumulation and the onset of anthocyanin synthesis after *veraison* (Casterllarin *et al.*, 2007a).

On the other hand, when hydric deficit period was close to harvest, a higher gene expression in the last stage of berry development was found with the exception of *UFGT* (Fig. 4). Our results indicate the gene expression profile is affected by the hydric deficit period, what could explain the increased accumulation of anthocyanins in the first weeks after *verasion* and at harvest date. The water deficit occurring in two different berry growth periods (*veraison* and harvest) modified the expression profile of the studied genes in Lyre training system. Water deficit at *veraison* induces an earlier and higher expression of *CHS, F3H, DFR*, and *UFGT* genes than in years with a water deficit at harvest.

In Uruguay, periods of high temperature during the summer are common, then the temperature must be considered when studying berry metabolism. On the other hand, it has been reported that anthocyanins accumulation is inhibited in the skins of black - red grapes in hot regions (Haselgrove *et al.*, 2000; Spayd *et al.*, 2002; Downey *et al.*, 2004). We think that possibly a high temperature period (above 35º C) 14 DAV (data not shown) could be the cause of the different behavior between VSP and Lyres vines in anthocyanins accumulation.

Lyre training system could have higher capacity to maintain the thermal homeostasis of the raisin, which is reflected in a lower variation of the slope for the anthocyanins accumulation during ripening (Fig. 5).

A recent study has shown that *CHS, DFR* and *UFGT* expression are negatively affected by high temperatures and provided definitive evidence of anthocyanins loss in the skin of grape berries due to high temperature (Mori *et al.*, 2007).

Individual analysis of the transcription of four genes in Lyre training and VSP trellis systems evidences a higher expression at 14 DAV of *CHS*, *DFR* and *UFGT* in Lyre (Fig. 6). Canopy architecture in VSP could be responsible of the higher raisin temperatures and inhibit of the anthocyanins pathway gene transcription mentioned above (Fig. 6). However, this explanation could not be applied to the *F3H* gene expression, since it showed similar level to Lyre (Fig. 6). A change in the gene expression pattern in different training systems is evidenced by the analysis of expression of flavonoid pathway *CHR*, *F3H*, *DFR* and *UFGT* (Fig. 6).

Previous studies that monitored the temperature inside and outside of the Merlot showed lower values in Lyre compared with VSP trellis system (Ferrer, 2007), which related with the major anthocyanin accumulation found in the berries of Lyre (González-Neves, 2005).

Apart from the effect of temperature and light on anthocyanin accumulation, recent research also shows some shift in anthocyanin composition with temperature. The results indicate that in warm-climate the fruits would tend to have a higher proportion of malvidin, petunidin and delphinidin coumaroyl derivatives, while in cool-climate and shaded fruits would have more non-acylated glucosides and acetyl-glucosides of peonidin and cyanidin (Spayd *et al.*, 2002). Our results showed that in both year and in both training systems the individual anthocyanidins profile in berries of cv. Tannat was similar to those found in vines cultivated in warm-climate.

A future work related with raisin microclimate training system-dependence and gene expression will be useful to improve the understanding of environmental signals which determine the polyphenols content in the grapes.

Acknowledgments

The authors thank Establecimiento Juanicó, Viñas Varela-Zarranz for kindly providing the vineyards for the study. This study was funded by FCE_8054. Authors want to dedicate this manuscript to Ing. Agr. Julio Omar Borsani Estevez (1934-2006) and Dr. Maria Fernanda Agius Guadalupe (1972-2005) for their invaluable collaboration and permanent encouragement for maintaining the excellence of the work.

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Journal of Applied Horticulture, 12(1): 10-15, January-June, 2010

Effects of different photoperiods on flowering time of facultative short day ornamental annuals

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Abstract

An experiment was carried out to study flowering response of six facultative short day plants (zinnia cv. Lilliput, sunflower cv. Elf, French marigold cv. Orange Gate, African marigold cv. Crush, cockscomb cv. Bombay and cosmos cv. Sonata Pink) under four distinct controlled photoperiods (8, 11, 14 and 17 h d^{-1}). A curvilinear facultative response was observed in almost all cultivars studied. zinnia, sunflower, French marigold, African marigold, cockscomb and cosmos took minimum time to flower when grown under 8 h d^{-1} photoperiods however it was significantly ($P<0.05$) increased when photoperiod was increased to 17 h d^{-1}. These findings revealed plant scheduling prospect that is, the flowering time of facultative SDPs grown under long day photoperiod can be extended in order to continue supply of these plants in the market.

Key words: Ornamental annuals, short day plants, flowering, photoperiod, facultative short day plants

Introduction

Flowering is the end result of physiological processes, biochemical sequences, and gene action, with the whole system responding to the influence of environmental stimuli (photoperiod, temperature) and the passage of time (Zheng *et al.,* 2006). After attaining a certain size (completing the 'juvenile' phase), plants enter into the 'reproductive' phase (initiation and development of flowering). Evans (1969) referred to flowering as the inductive processes occurring in the leaf (O'Neil, 1992), mediated by the photoreceptor (phytochrome) that leads to the initiation of floral development (McDaniel *et al.,* 1992) at the meristem (evocation). It is also believed that flowering is induced by a stimulus (florigen), which is produced within the leaf (Chailakhyan, 1936) but this hormone has not yet been identified (Turck *et al.,* 2008). When the apical meristem of the plant is differentiated for flowering, its fate becomes irreversible (Bernier, 1988), although flower or inflorescence reversion to vegetative growth can also occur spontaneously in some species. This condition can be caused if plants are transferred to certain specific photoperiod or temperature regimes, which favour vegetative development (Battey and Lyndon, 1990).

Many flowering plants use a photoreceptor protein, such as phytochrome or cryptochrome, to sense seasonal changes in day length (photoperiod), which they take as signals to flower (Weller and Kendrick, 2008). The photoperiodic response of flowering is generally categorised into three main groups: short-day plants (SDPs) in which flowering is induced by longer nights; long-day plants (LDPs) where shorter nights promote flowering; and day-neutral plants (DNPs) in which flower are produced irrespective to day length. SDPs and LDPs can be further classified as qualitative or obligate (species that require a specific minimum or maximum photoperiod for flowering) and quantitative or facultative (flowering process is hastened by a specific minimum or maximum photoperiod). It is in fact, the night length rather than day length that controls flowering, so flowering in a long day (LD) plant is triggered by a short night (which, of course, also means a long day). Conversely, short day (SD) plants will flower when nights get longer than a critical length. This can be observed by using night breaks. For example, a short day plant (long night) will not flower if a pulse (5 minutes) of artificial light is shone on the plant during the middle of the night. This generally does not occur from natural light such as moonlight, lightning, fire flies, etc, since the light from these sources is not sufficiently strong to trigger the response (Thomas and Vince-Prue, 1997). Keeping in view the importance of photoperiod on flower induction an expeiment was desgined to determine the flowering response of six facultative SDPs to four photoperiods under the sub-tropical environmental conditions.

Materials and methods

The experiment was conducted in Agricultural Research Institute, Dera Ismail Khan, Pakistan, during the year 2005. Seeds of facultative SDPs such as zinnia (*Zinnia elegans* L.) cv. Lilliput, sunflower (*Helianthus annuus* L.) cv. Elf, French marigold (*Tagetes patula* L.) cv. Orange Gate, African marigold (*Tagetes erecta* L.) cv. Crush, cockscomb (*Celosia cristata* L.) cv. Bombay, cosmos (*Cosmos bipinnatus* Cav.) cv. Sonata Pink were sown on 1st of March 2005 into module trays containing locally prepared leaf mould compost. Seed trays were kept at room temperature at night and they were moved out during the day (08:00–16:00h) under partially shaded area. After 70% seed germination, six replicates of each cultivar were shifted to the respective photoperiod chamber. Plants remained outside the photoperiod chambers for 8h (from 08:00 to 16:00h) where they were exposed to natural daylight and temperature (Table 1). At 16:00h each day, all plants were moved into the photoperiod chambers where they remained until 08:00h the following morning. Photoperiod within each of the chambers was extended

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Table 1. Environmental details of the experiment

Growth Period	Diurnal temperature (°C)			Daily light integral 08:00-16:00 MJ m^{-2} d^{-1}
	Maximum	Minimum	Average	
March 2005	26.19	13.29	19.74	8.43
April 2005	32.87	15.73	24.30	9.45
May 2005	36.39	20.35	28.37	9.40
June 2005	42.27	30.70	36.48	9.99
July 2005	36.77	25.68	31.23	9.42

by two 60Watt tungsten light bulbs and one 18Watt warm white florescent long-life bulb (Philips, Holland) fixed above one metre height from the trolleys providing a light intensity (Photosynthetic Photon Flux Density, PPFD) of 7mmol m^{-2} s^{-1}. In all photoperiod chambers, the lamps were switched on automatically at 16:00h for a duration dependents on the day length required (8, 11, 14, 17 h d^{-1}). These chambers were continuously ventilated with the help of micro exhaust fan (Fan-0051, SUPERMICRO® USA) with an average air speed of 0.2 m s^{-1} over the plants when inside the chambers, to minimize any temperature increase due to heat from the lamps.

Temperature and solar radiation were measured in the weather station situated one kilometer away from the research venue. Temperature was recorded with the help of Hygrothermograph (NovaLynx Corporation, USA) while solar radiation was estimated using solarimeters (Casella Measurement, UK). Plants were potted into 9 cm pots containing leaf mould compost and river sand (3:1 v/v) after 6 leaves emerged. Plants were irrigated by hand and a nutrient solution [(Premium Liquid Plant Food and Fertilizer (NPK: 8-8-8); Nelson Products Inc. USA)] was applied twice a week.

Plants in each treatment were observed daily until flower opening (corolla fully opened). Number of days to flowering from emergence were recorded at harvest and the data were analysed using GenStat-8 (Lawes Agricultural Trust, Rothamsted Experimental Station, U.K. and VSN International Ltd. U.K.).

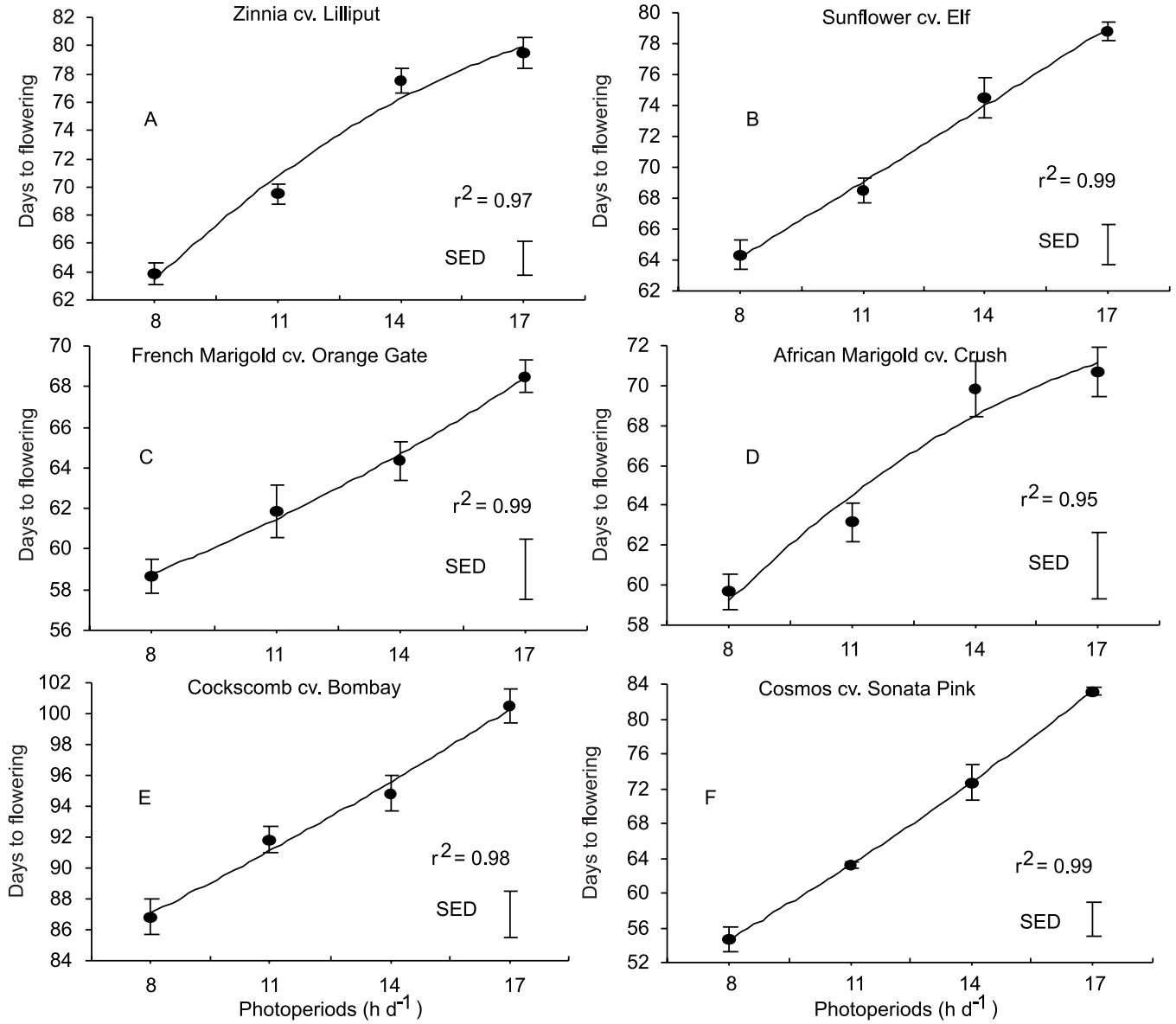

Fig. 1. Effect of different photoperiods on flowering time of (A) Zinnia cv. Lilliput, (B) Sunflower cv. Elf, (C) French marigold cv. Orange Gate, (D) African marigold cv. Crush, (E) Cockscomb cv. Bombay and (F) Cosmos cv. Sonata Pink. Each point represents the mean of 6 replicates. Vertical bars on data points (where larger than the points) represent the standard error within replicates whereas SED vertical bar showing standard error of difference among means.

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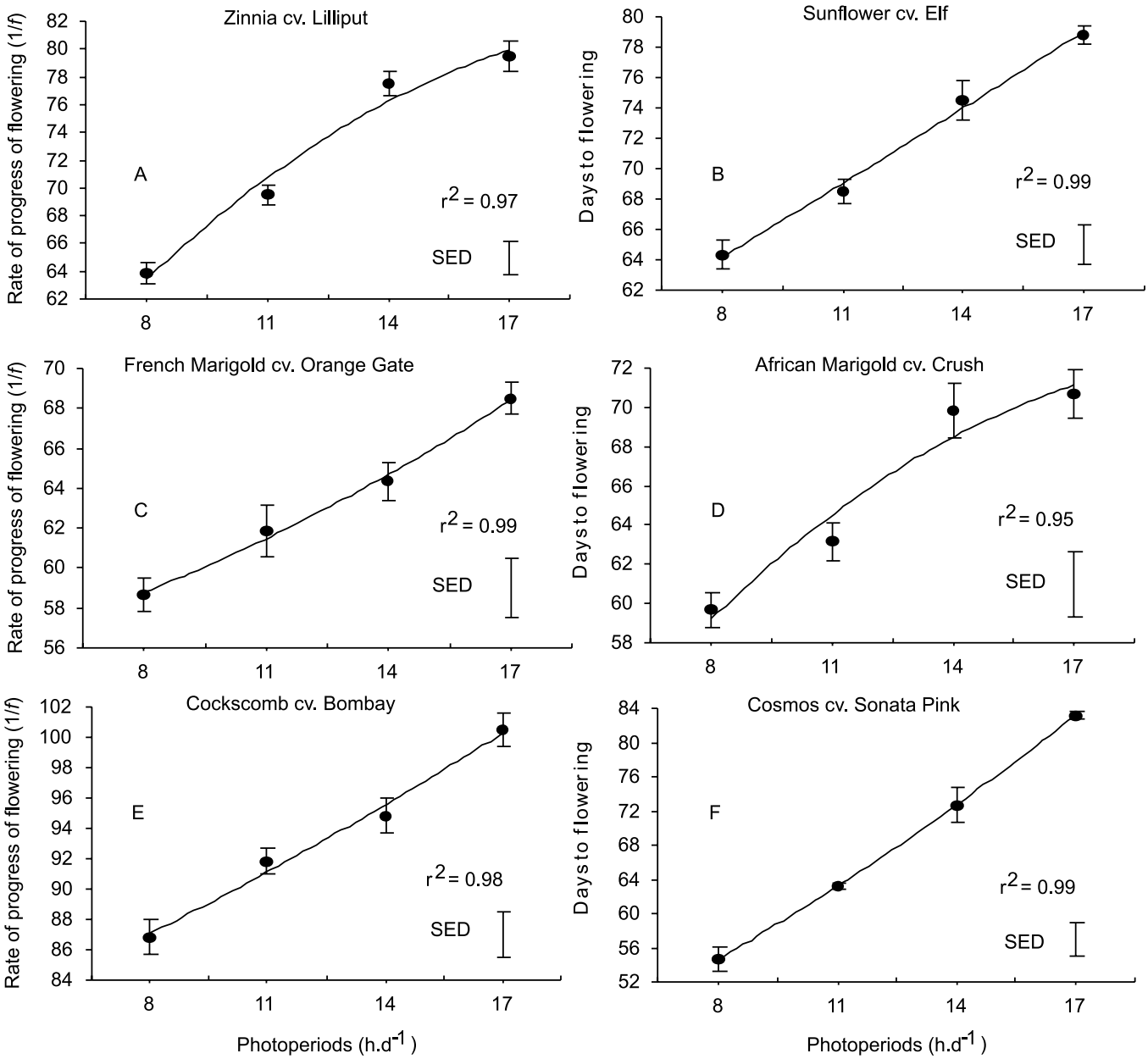

Fig. 2. Effect of different photoperiods on rate of progress to flowering (*1/f*) of (A) Zinnia cv. Lilliput, (B) Sunflower cv. Elf, (C) French marigold cv. Orange Gate, (D) African marigold cv. Crush, (E) Cockscomb cv. Bombay and (F) Cosmos cv. Sonata Pink. Each point represents the mean of 6 replicates. Vertical bars on data points (where larger than the points) represent the standard error within replicates.

Results

Time to flowering in SDPs such as zinnia cv. Lilliput, sunflower cv. Elf, French marigold cv. Orange Gate, African marigold cv. Crush, cockscomb cv. Bombay and cosmos cv. Sonata Pink increased significantly ($P<0.05$) with increase in photoperiod. Plants received maximum duration of light took maximum time to flower whereas it was decreased significantly under minimum photoperiod treatments.

It was observed that zinnia cv. Lilliput (Fig. 1A) flowered 16 days earlier under SD *i.e.* 8 h d^{-1} photoperiod (64 days) as compared to LD *i.e.* 17 h d^{-1} photoperiod (80 days) followed by 14 h d^{-1} photoperiod (78 days) and 11 h d^{-1} photoperiod (70 days). Similarly, sunflower cv. Elf (Fig. 1B) bloomed 15 days earlier under 8 h d^{-1} photoperiod (64 days) compared to 17 h d^{-1} photoperiod (79 days) while plants grown in 14 and 11 h d^{-1} photoperiod flowered after 69 and 75 days, respectively. French marigold cv. Orange Gate (Fig. 1C) flowered 10 days early under 8 h d^{-1} photoperiod (59 days) as compared to 17 h d^{-1} photoperiod (69 days) followed by 14 and 11 h d^{-1} photoperiod *i.e.* 64 and 62 days, respectively. Similarly, African marigold cv. Crush (Fig. 1D) grown under 8 h d^{-1} photoperiod (60 days) flowered 11 days earlier than the 17 h d^{-1} photoperiod (71 days). Plants of same cultivar took 70 and 63 days to flower when grown under 14 and 11 h d^{-1} photoperiod. Cockscomb cv. Bombay (Fig. 1E) flowered 14 days earlier when grown under 8 h d^{-1} photoperiod (87 days) as compared to 17 h d^{-1} photoperiod (101 days) followed by 95 days in 14 h d^{-1} photoperiod and 92 days in 11 h d^{-1} photoperiod. Similarly, cosmos cv. Sonata Pink (Fig. 1F) when grown under 8 h d^{-1} photoperiod flowered 29 days earlier (55 day) as compared to 17 h d^{-1} photoperiod (83 days) whereas plants grown under 14 and 11 h d^{-1} photoperiod bloomed after 73 and 63 days from emergence, respectively.

Data from facultative SDPs were analysed using the following

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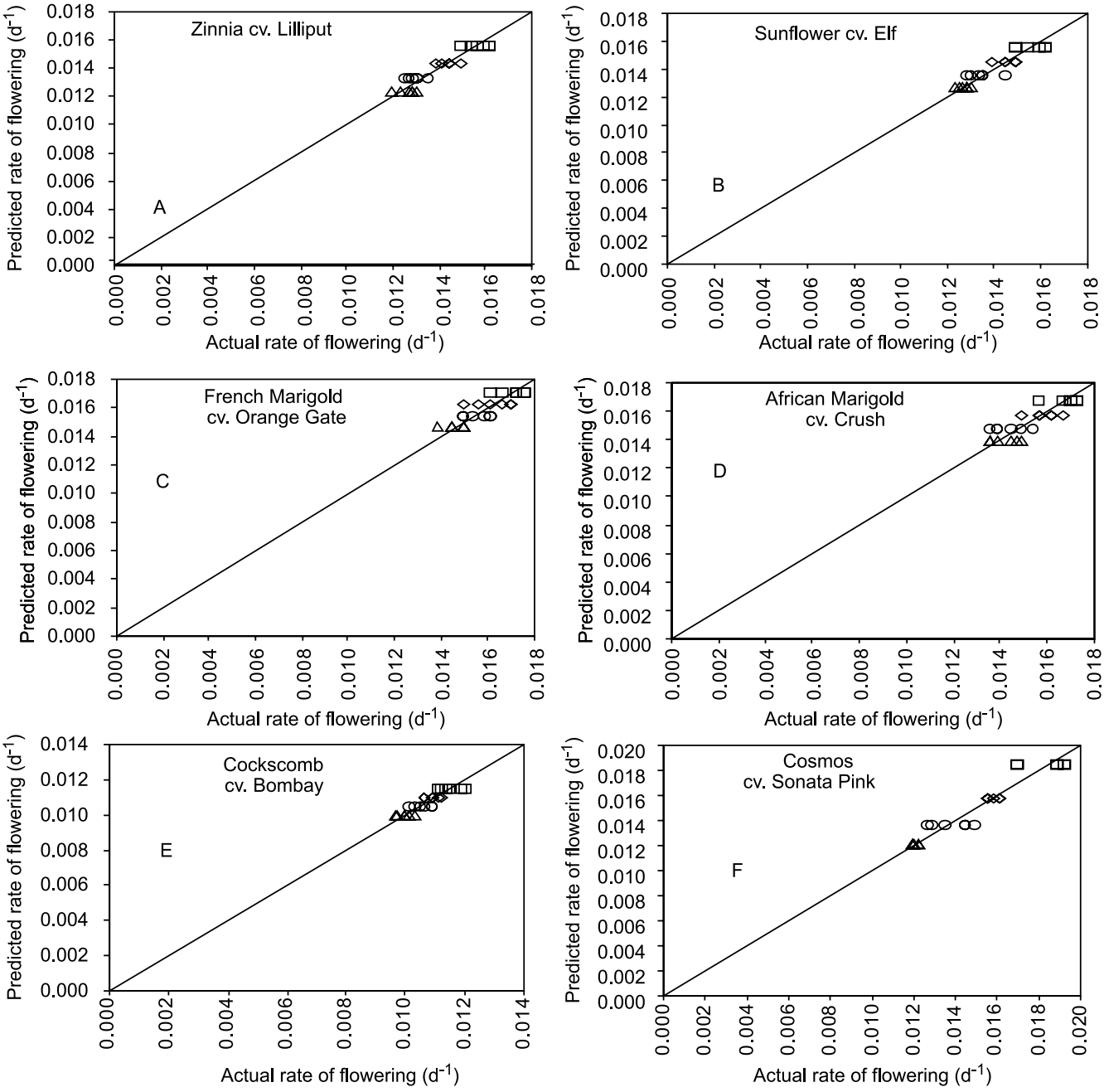

Fig. 3. The relationship between the actual rate of progress to flowering against those fitted by the flowering model ($1/f = a + bP$) for (A) Zinnia cv. Lilliput, (B) Sunflower cv. Elf, (C) French marigold cv. Orange Gate, (D) African marigold cv. Crush, (E) Cockscomb cv. Bombay and (F) Cosmos cv. Sonata Pink grown under 8 (□), 11 (◊), 14 (○) and 17 (Δ) h d^{-1} photoperiod. The sold line is the line of identity.

model:

$1/f = a + bP$

The best fitted model describing the effects of mean photoperiod (P) on the rate of progress to flowering ($1/f$) can be written as:

Zinnia cv. Lilliput (Fig. 2A) and (Fig. 3A):

$1/f = 70.67\ (\pm 1.92) + 1.88\ (\pm 0.15)\ P \quad (r^2 = 0.97, \text{d.f. } 23)$ Eq. 1

Sunflower cv. Elf (Fig. 2B) and (Fig. 3B):

$1/f = 71.92\ (\pm 1.77) + 1.65\ (\pm 0.14)\ P \quad (r^2 = 0.99, \text{d.f. } 23)$ Eq. 2

French marigold cv. Orange Gate (Fig. 2C) and (Fig. 3C):

$1/f = 50.00\ (\pm 1.84) + 1.07\ (\pm 0.14)\ P \quad (r^2 = 0.99, \text{d.f. } 23)$ Eq. 3

African marigold cv. Crush (Fig. 2D) and (Fig. 3D):

$1/f = 70.31\ (\pm 2.35) + 1.32\ (\pm 0.18)\ P \quad (r^2 = 0.95, \text{d.f. } 23)$ Eq. 4

Cockscomb cv. Bombay (Fig. 2E) and (Fig. 3E):

$1/f = 96.17\ (\pm 1.98) + 1.47\ (\pm 0.15)\ P \quad (r^2 = 0.98, \text{d.f. } 23)$ Eq. 5

Cosmos cv. Sonata Pink (Fig. 2F) and (Fig. 3F):

$1/f = 28.83\ (\pm 2.39) + 3.17\ (\pm 0.19)\ P \quad (r^2 = 0.99, \text{d.f. } 23)$ Eq. 6

Above equations are based on individual arithmetic means of respective factors, although all data were originally tested. The values in parenthesis show the standard errors of the regression coefficients. The outcome of this model indicated that photoperiod had significant effects on the rate of progress to flowering in all facultative SDPs studied. To validate the model, actual data of rate of progress to flowering were plotted against the predicted

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ones in order to develop a fitted relationship. Almost all values were successfully plotted near the line of identity which also showed that the photoperiod had a significant effect on the rate of progress to flowering.

Discussion

A facultative SD photoperiodic response of zinnia cv. Lilliput, sunflower cv. Elf, French marigold cv. Orange Gate, African marigold cv. Crush, cockscomb cv. Bombay and cosmos cv. Sonata Pink was observed during present investigation. These results are in line with the findings of Erwin and Warner (2002) who reported that flowering was hastened by SD photoperiod in SDPs. Present study indicated that flowering was hastened up to 16 (zinnia), 15 (sunflower), 10 days (French marigold), 11 (African marigold), 14 (cockscomb) and 29 days (cosmos) under SD environment (8 h d^{-1}). The response of SDPs observed in present study is supporting the fact that most SDPs are of tropical or sub-tropical origin (Summerfield *et al.*, 1997). Studies have been carried out previously to support this evidence in zinnia (Young *et al.*, 2003), sunflower (Young *et al.*, 2003; Yañez *et al.*, 2004), French and African marigold (Tsukamoto *et al.*, 1968, 1971), cockscomb (Kanellos and Pearson, 2000; Young *et al.*, 2003; Goto and Muraoka, 2008) and cosmos (Warner, 2006).

SDPs grown under inductive environment (8 h d^{-1} photoperiod) induced flowering earlier than those grown above this. The reason of early flowering under inductive environment is due to the stimulation of floral genes which are involved in the transition of flowering (phase change) that encode photoreceptors such as phytochrome (perceives red (660nm) and far-red (730nm) light) and the cryptochromes (perceives UV-A and blue light). It is reported in *Arabidopsis* that the phytochromes A and B in conjunction with the cryptochromes 1 and 2 are involved in the photoperiodic response (Mouradov *et al.,* 2002). Therefore, any ascending alteration in photoperiod (in SDPs) from the optimum one affects plants' perception of light and can delay phase change from juvenile to flowering. However, in general, far-red and blue light promote flowering in *Arabidopsis* whereas red light inhibits flowering (Lin, 2000).

The transduction of the light signals involves a complex web of interactions between photoreceptors and their corresponding interacting proteins. In term of floral induction, perception of photoperiod appears to be one of the most important transducers of the plant's environment. An important mechanism used by the plants phytochromes and cryptochromes is to communicate photoperiod activity which involves the entrainment of the circadian rhythms, a self-reinforcing endogenous clock that allows light/dark coordinated gene expression. Mizoguchi *et al.* (2005) reported that GIGANTEA (GI) gene regulates circadian rhythms and acts earlier in the hierarchy than CO and FT and suggested that GI acts between the circadian oscillator and CO to promote flowering by increasing CO and FT mRNA abundance.

These studies established an understanding that different genes control flowering process and these genes are evoked when a leaf is fated to respond to the inductive photoperiod, the leaf exports floral stimulus towards apex. In most cases, when the photoperiod becomes non-inductive (17 h d^{-1}, in present study), the leaf stops exporting signal. The important developmental event in leaf formation, as far as photoperiodic induction is concerned, appears to be the commitment of a leaf to develop the capacity to respond to the inductive photoperiod (McDaniel, 1996). In present study, it is revealed that after completing the juvenile phase (attaining a specific leaf numbers), the competent leaf (newly developed one) responded to the inductive photoperiod (short day-length) and induced floral signal toward apex to produce flower that is why an early flowering response was observed under inductive photoperiod environment in SDPs.

It can be concluded from the findings of present research that flowering time in zinnia, sunflower, French marigold, African marigold, cockscomb and cosmos can be prolonged under 17 h d^{-1} non-inductive environment to facilitate continuous supply of these plants in the market and to enhance their flower display period. However, these SDPs can be subjected to SD inductive environment (8 h d^{-1}) if an early flowering is required. These floweres can also be grown under non-inductive environment during juvenile phase to improve their quality for marketing viewpoint. The outcome of present study indicated a possibility of year-round production of these flowers, which will eventually increase the income of ornamental growers.

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Journal of Applied Horticulture, 12(1): 16-20, January-June, 2010

Efficacy and physical properties of ground, composted rice hulls as a component of soilless substrate for selected bedding plants

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Abstract

Ground, composted rice hulls were combined as a root substrate component with peat moss and coir at five rice hulls percentages (0, 25, 50, 75, and 100) in a factorial design. Seventy-five percent of the rice hull particles were 0.51 to 1.40 mm and 90% of the particles were 0.51 to 2.00 mm. In physical property evaluations, increasing the percentage of rice hulls in both the peat moss and coir series of substrates increased the dry bulk density and airspace at container capacity; however, as air space increased, container capacity and available water decreased. In the first two of three plant growth experiments, *Impatiens walleriana* Hook. f. 'Super Elfin White' was grown in 288 cell plug trays. In the third experiment, *Verbena ×hybrida* Voss. 'Romance Deep Rose' was grown in 48 cell bedding plant flats. Due to problems with high pH in the coir, only the peat moss growth results were reported. Overall, growth was best in 25% rice hulls plus 75% peat moss. Rice hulls increased substrate Ca^{2+} and Mg^{2+} in both the peat moss and coir. Adding rice hulls to the substrate increased K^+ in peat and decreased K^+ in coir. There was no effect of rice hulls on substrate NO_3^--N, NH_4^+-N, and PO_4-P in the substrate solution. Ground, composted rice hulls are a potential alternative component of soilless substrate for plugs and bedding plants.

Key words: Rice hulls, root substrate, soilless, root media, *Impatiens walleriana, Verbena ×hybrida,*

Introduction

Rice hulls were first investigated as a potential replacement for soil or sand in soil-based substrates (Einert, 1972; 1973), then as a potential replacement for perlite in soilless substrates (Evans and Gachukia, 2004, 2007; Papfotiou *et al.*, 2001). Evans and Gachukia (2007) reported that rice hulls had a greater porosity than perlite. Conversely, Papfotiou *et al.* (2001) found replacement of perlite with rice hulls in a substrate decreased total porosity.

Papfotiou *et al.* (2001) replaced 50 or 100% of the perlite with rice hulls in a 1 peat : 1 perlite (v/v) substrate resulting in similar or slightly reduced growth in several plant species. Marianthi (2006) successfully replaced perlite with fresh rice hulls in a 7 peat moss : 3 perlite (v/v) substrate for the production of *Pinus halepensis* M. seedlings.

Composted rice hulls have a higher dry bulk density and container capacity than fresh rice hulls (Kang *et al.*, 2004). Composting also eliminates plant growth inhibitory chemicals, such as organic acids (Lee *et al.*, 2000). Composted rice hulls have a higher pH, EC, N content, and available P content than fresh rice hulls (Kang *et al.*, 2004). Growth of petunia was better in the root substrate amended with 30 to 60% composted rice hulls than with 0, 15, or 75% composted rice hulls (Song *et al.*, 1996). Laiche and Nash (1990) reported that 50 or 100% substitution of pine bark with composted rice hulls in the root substrate of several woody landscape species provided favourable growth. However, the dolomitic lime amendment had to be reduced or eliminated to compensate for innate Ca^{2+} and Mg^{2+} levels in the rice hulls.

Grinding has also been used to decrease the total porosity and air-filled space and increase the container capacity of rice hulls (Choi *et al.*, 1999). Sambo *et al.* (2008) determined that fresh rice hulls ground to pass through 1 and 2 mm diameter screens were closest to peat moss in physical properties. However, the ground rice hulls had more total and easily available water and a heavier bulk density than peat moss.

In this study, rice hulls were composted to eliminate toxic substances and viable seed, and ground to develop a finer aeration component than whole rice hulls that would be appropriate for plug substrates. The effects of replacing either peat moss or coir with increasing levels of composted, ground rice hulls on substrate physical properties and plant growth were tested.

Materials and methods

On 23 August, 1 m^3 of rice hulls was combined in a Twister 1 Batch Mixer Model 12101 (Bouldin & Lawson, McMinnville, TN) with 2.5, 5, and 10 kg of Gorang commercial compost promoter (Biwang Co., Seoul, South Korea), urea, and wheat bran, respectively. The mixture was then saturated with water and covered with polyethylene film. After 10 d, when the material had an internal temperature of 70-75 °C, it was turned. After 10 more days, the material was turned again. The pile remained in the field under the polyethylene cover until removed as needed for each experiment.

Composted rice hulls moistened to 30% water content by weight were ground using a hammer mill (model 10 BLHM, The C.S.

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Bell Co., Tiffin, OH) to pass through a 6.35 mm screen. Three 100 g samples of the ground compost were sieved for 5 min in a Ro-Tap Testing Sieve Shaker Model B (Tyler Industrial Products, Mentor, OH). The sieve fractions of >2.00, 2.00-1.41, 1.40-1.01, 1.00-0.72, 0.71-0.51, 0.50-0.25, and <0.25 mm averaged 2, 15, 36, 28, 11, 6, and 2 %, respectively, by weight of the total samples.

Ten root substrate formulations were prepared for each experiment using the ground, composted rice hulls at 30% moisture by weight and either sphagnum peat moss or coir at 67% moisture. In the peat moss and coir treatment series, rice hulls replaced peat moss or coir at volume percentages of 0, 25, 50, 75, or 100.

Substrate physical properties: Columns, 15 cm in height, constructed from 7.7 cm i.d. polyvinylchloride (PVC) pipe were used to determine the physical properties of the ten substrates. Each column was attached to a flat acrylic base plate. A 9.0 mm hole was drilled in the center of each base to provide drainage. A porous, fiberglass mat was placed over the drainage hole of each column. The tare weight of each empty column, including fiberglass mat, was determined. To facilitate filling each column, an extension was made by taping an additional PVC ring 4 cm in height to the top of each column. Each column with extension was filled loosely with substrate and dropped on a laboratory bench from a height of 15 cm three times to compact the substrate. The extension was removed and the substrate remaining above the top of each 15-cm column was cut free with the sawing action of a fine chrome wire. Three replicated columns were filled with each substrate as described.

One complete replication of all ten substrate formulation treatments in this experiment (ten packed columns) was placed in an empty tank at a time. Water was added incrementally to the bottom of the tank over a two hour period until the water level reached the surface of the packed columns. As water entered the base of each column, air within the substrate was displaced upward and expelled from the top of the column. A rubber stopper was weighed and then inserted into the hole in the base of the column. The column of saturated substrate, always maintained in a vertical orientation, was removed from the water and weighed. After subtracting the weight of the stopper and empty column, the net weight of saturated substrate was recorded as weight A. The stopper was removed from the base, the top of the column was loosely covered with plastic tape to prevent evaporation, and gravitational water allowed to drain. The columns drained for 24 h; however, container capacity was reached within 1 h and drainage beyond 1 h was negligible. After 24 h, the column, with its substrate at container capacity, was weighed. The weight of the column was subtracted and the net weight of the substrate at container capacity was recorded as weight B. The substrate was then transferred quantitatively to a ring on the porous plate of a pressure outflow chamber (Klute 1986), rewetted, and a pressure head of -300 cm water (-30 kPa) applied. The water content of the latter sample after it drained to equilibrium in the pressure chamber was taken to be the lower limit of available water. The ring plus substrate was weighed, the weight of the ring was subtracted, and the net weight of substrate at -30kPa was recorded as weight C. The substrate was then quantitatively transferred to an oven, dried at 105 °C for 24 h, and weighed (weight D).

Equation 1: $D_b = D / (Ht \times 7.7 \times 7.7 \times \pi) / 4$

Where, D_b = dry bulk density ($g \cdot cm^{-3}$); D = weight of substrate after oven drying at 105 °C for 24 h (g); Ht = height of column (cm); and $\pi = 3.14$.

Equation 2: $CC = (100 \times D_b) [(B – D) / D]$
Where, CC = container capacity in a 15-cm tall column (% by volume); D_b = bulk density ($g \cdot cm^{-3}$); B = weight of substrate at container capacity (g); and D = weight of substrate after oven drying at 105 °C for 24 h (g).

Equation 3: $LLAW = (100 \times D_b) [(C – D) / D]$
Where, LLAW = lower limit of plant available water (% by volume); D_b = bulk density ($g \cdot cm^3$); C = weight of substrate at -30 kPa; and D = weight of substrate after oven drying at 105 °C for 24 h (g).

Equation 4: AW = (CC – LLAW)
Where, AW = Available water between CC and -30kPa (% by volume); CC = container capacity (% by volume); and LLAW = lower limit of plant available water (% by volume).

Equation 5: $AS = (100 X D_b) [(A – B) / D]$
Where, AS = Air space at CC (% by volume); D_b = bulk density ($g \cdot cm^{-3}$); A= Weight of saturated substrate (g); B = weight of substrate at CC (g); and D = weight of substrate after oven drying at 105 °C for 24 h (g).

Plant efficacy tests for the ten substrates occurred in three experiments. In these experiments, substrates were irrigated at each irrigation to approximately 20% leaching with tap water. The night/day greenhouse temperatures were set at 21/24 ˚C.

Expt. 1 – First plug experiment: All ten substrates were amended with dolomitic limestone at 7 g L^{-1}. Seeds of *I. walleriana* 'Super Elfin White' were planted into 288-cell square (2.0 x 2.0 cm x 3.5 cm deep) Landmark (Landmark Plastic Corp., Akron, OH) plug flats cut down to 100 cells on 16 Dec. Fertilizer applications of 20N:4.4P:16.6K at 50 mg L^{-1} N at each irrigation began when the cotyledons separated and increased to 100 mg L^{-1} N when the first true leaves appeared. A destructive harvest of plants was made on 20 January when plant height and shoot dry weight were recorded.

Expt. 2 – Second plug experiment: Substrates were amended with dolomitic limestone at 4.5 g L^{-1} in the 100% rice hull treatment and four treatments containing peat moss and 3.5 g L^{-1} in the four treatments containing coir to correct for the high pH levels observed in Expt. 1. Seeds of *I. walleriana* 'Super Elfin White' were planted into 288-cell square Landmark plug flats cut down to 100 cells on 1 February. The protocols for fertilizer application were the same as in Expt. 1. A destructive harvest of plants was made on 3 March. Plant height and shoot fresh weight were recorded.

Expt. 3 – Verbena growth experiment: Substrates were amended with dolomitic limestone at 4.5 g L^{-1} in the 100% rice hull treatment and three treatments containing peat moss and 3.5 g L^{-1} in the three treatments containing coir as in Expt. 2. Plug seedlings of *V.* ×*hybrida* 'Romance Deep Rose' from C. Raker & Sons, Inc. (Litchfield, MI) were transplanted on 31 January into 48-cell (4.4 x 6.7 x 5.7 cm deep) bedding plant flats. Weekly fertilizer applications of 20N:4.4P:16.6K at 200 mg L^{-1} N began on 3 February. The date when 75% of the plants in each flat were in

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flower was recorded. A destructive harvest of plants was made on 19 March. Plant height and shoot fresh weight were recorded.

Analytical methods: Substrate solution was extracted at each destructive harvest by squeezing the plug cells. The root substrate extracts were analyzed for pH, EC, NO_3^--N, NH_4^+-N, PO_4^{3-}-P, K^+, Ca^{2+}, and Mg^{2+} as follows: A pH/conductivity/TDS/temperature meter (Extech Instruments, Model 695, Waltham, MA) was used for pH and soluble salts (EC) determination. Colorimetric analysis was employed for NO_3^--N (Cataldo *et al.,* 1975), NH_4^+-N (Chaney and Marbach, 1962), PO_4^{3-}-P concentrations under 10 µg mL^{-1} (Murphy and Riley, 1962), and PO_4^{3-}-P concentrations above 10 µg mL^{-1} (Chapman and Pratt, 1961) in a Lambda 3 UV/VIS spectrophotometer (Perkin and Elmer, Norwalk, CT). Atomic absorption spectroscopy was used for K^+, Ca^{2+}, and Mg^{2+} analyses (AAnalyst 100 atomic absorption spectrometer, Perkin Elmer, Norwalk, CT)

Experimental design and statistical analysis: A factorial arrangement of two substrate series (peat moss and coir) and five rice hull rates (0, 25, 50, 75, and 100%) in a randomized complete block design with three blocks (30 experimental units in total) was employed for the physical properties determination and for the three plant experiments. In Expts. 1 and 2, experimental units consisted of one 100-cell flat of plants. In Expt. 3, experimental units consisted of one 48-cell flat of plants. Data were subjected to analysis of variance using SAS 9.1 (Statistical Analysis System, SAS Institute, Cary, NC). Means were separated using a protected LSD. Values for dependent variables with significant analysis of variances were regressed by substrate additives using the PROC GLM procedure to determine the best-fit linear and quadratic models. Terms of the model were based on a comparison of F values at $\alpha = 0.05$.

Results and discussion

Physical properties: Physical property data for the substrates are presented in Table 1. The main effect of rice hull percentage on D_b and AS was significant, but the interactive effects of substrate series by rice hull percentage were not significant. As rice hull proportion increased, D_b and AS increased linearly. This is not a desirable trait for D_b because increased substrate weight results in increased shipping costs for formulators and growers. The increased AS at 25% rice hulls (20.7% by volume) was desirable. Higher AS percentages would pose a problem because it is not needed and comes at the expense of plant available water. The main effect of series was not significant for D_b but was for AS, where the mean AS values were 32.7% for peat and 39.0% for coir. There were interactive effects of substrate series and rice hulls on CC and AW. Both components of water declined as the percentage of rice hulls increased and were lower in the coir series than in the peat moss series. Thus, the slope was more negative in the peat moss series.

Growers desire maximum available water to reduce frequency of watering, particularly in the market channel. Even though 75% of the ground rice hulls in this study were in the 0.51 to1.40 mm particle size range, their main impact on peat moss and coir was an increase in AS at the expense of CC and AW due to the addition of large particles. Unlike finely ground rice hulls, which have physical properties similar to peat moss (Sambo *et al.*, 2008), coarsely ground rice hulls increased aeration in the substrates similar to perlite.

Growth: Substrate pH levels in Expt. 1 were significantly affected by substrate series but not by rice hull percentage or the interaction of series by rice hulls. The mean pH levels were 6.7 for peat moss and 7.0 for coir. There was a significant interactive effect of series and rice hull percentage in Expts. 2 and 3 (Table 2). While the pH levels in the peat moss series in each experiment were acceptable, they were too high for the coir series, particularly in the 0 to 50% rice hull treatments in the latter two experiments. Since the high pH levels in the coir series would confound interpretation, only the peat moss series was used for growth evaluation.

Shoot dry weight in Expt.1 was greatest in the 25 and 50% rice hulls treatments while height was highest in the 0, 25, and 50% rice hulls treatments (Table 3). Overall, the 25 and 50% rice hulls rates resulted in maximum Impatiens growth. In Expt. 2, shoot fresh weight was maximized in the 0 and 25% rice hulls treatments

Table 1. Effect of the volume proportion of rice hulls in a peat moss and a coir replacement series of treatments on mean bulk density, container capacity, plant available water and air space in a 15-cm tall column

Rice hulls	Bulk density	Container capacity (% by volume)		Plant available water (% by volume)		Air space
(%)	(g cm^{-3})	Peat	Coir	Peat	Coir	(% by volume)
0	0.079	76.30	67.68	56.89	46.24	7.7
25	0.102	68.75	58.27	48.97	39.38	20.7
50	0.115	54.55	46.97	38.31	31.11	40.1
75	0.135	40.57	36.94	28.17	23.98	50.8
100	0.134	24.45	24.45	15.68	15.68	59.9
$LSD_{0.05}$ [z]	0.010	3.85		3.85		7.6
Linear[y]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Quadratic	0.0044	0.0126	0.3157	0.1069	0.7135	0.1353
a	0.0847	81.4	68.4	59.2	46.6	8.97
b_0x	0.000568	-0.556	-0.431	-0.426	-0.306	0.538
b_1x^2	-	-	-	-	-	-
r2	0.8	0.98	0.98	0.98	0.97	0.88
CV	9	5	5	5	7	20

[z]LSD values for bulk density and air space are for comparing between rice hull contents. LSD values for container capacity and plant available water are for comparing substrate series for a given rice hull content or rice hull contents for a given substrate series.

[y]The equation coefficients for either the linear ($y = a + b_0x$) or quadratic ($y = a + b_0x + b_1x^2$) regression models are provided for whichever model had the lowest significant Pr>F. The coefficient of determination (r^2) and coefficient of variation (CV) were calculated for the best fit model (n = 3). The regression models for bulk density and air space combined data from both substrate series (coir or sphagnum peat moss) since the substrate series x rice hull rate interaction was not significant for these two dependent variables.

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Table 2. Effect of rice hull volume proportion in a peat moss and a coir replacement series of treatments on mean pH and electrical conductivity (EC) of substrate solutions of *Impatiens walleriana* 'Super Elfin White' in Expt. 1 and 2 and *Verbena×hybrida* 'Romance Deep Rose' in Expt. 3

Rice	Expt. 2 (pH)		Expt. 3 (pH)		Expt. 1 (EC mS·cm^{-1})		Expt. 3 (EC mS·cm^{-1})	
hulls(%)	Peat	Coir	Peat	Coir	Peat	Coir	Peat	Coir
0	5.4	7.2	5.3	7.3	0.23	0.42	0.30	0.85
25	5.8	7.2	5.9	7.3	0.32	0.40	0.29	0.67
50	6.3	7.0	6.6	7.3	0.39	0.39	0.29	0.40
75	6.5	6.3	6.7	6.9	0.38	0.31	0.37	0.31
100	6.5	6.5	6.9	6.9	0.37	0.37	0.50	0.50
$LSD_{0.05}$[z]	0.2		0.2		0.08		0.13	
Linear[y]	<0.0001	<0.00001	<0.0001	0.0003	0.0150	0.0663	0.0095	0.0016
Quadratic	0.0003	0.8791	0.0002	0.4167	0.0660	0.4280	0.0501	0.0005
a	5.54	7.31	5.50	7.39	0.270	-	0.256	0.892
b_0x	0.0114	-0.00955	0.0157	-0.00535	0.00136	-	0.00189	-0.0148
b_1x^2	-	-	-	-	-	-	-	0.000105
r^2	0.81	0.82	0.88	0.64	0.38	-	0.42	0.84
CV	3	2	4	2	20	-	24	17

[z]LSD values are for comparing substrate series for a given rice hull content or rice hull contents for a given substrate series.
[y]The equation coefficients for either the linear ($y = a + b_0x$) or quadratic ($y = a + b_0x + b_1x^2$) regression models are provided for whichever model had the lowest significant Pr>F. The coefficient of determination (r^2) and coefficient of variation (CV) were calculated for the best fit model (n = 3).

Table 3. Effect of rice hull volume proportion in a peat moss replacement series of treatments on mean shoot dry weight and plant height of *Impatiens walleriana* 'Super Elfin White' in Expt. 1, mean shoot fresh weight and plant height of *Impatiens walleriana* 'Super Elfin White' in Expt. 2, and mean shoot fresh weight, plant height, and days to flowering (DTF) of *Verbena ×hybrida* 'Romance Deep Rose' in Expt. 3

Rice hulls (%)	Expt. 1 Dry wt. (mg)	Expt. 2 Fresh wt. (g)	Expt. 3 Fresh wt. (g)	Expt. 1 Height (cm)	Expt. 2 Height (cm)	Expt. 3 Height (cm)	Expt. 3 DTF (d)
0	35	0.70	3.44	5.1	4.3	6.2	48.0
25	39	0.70	3.82	5.1	5.1	6.9	47.0
50	41	0.64	3.65	5.1	4.5	7.8	47.0
75	31	0.48	3.46	3.8	3.5	7.8	48.0
100	28	0.38	2.66	3.6	2.6	7.1	48.3
$LSD_{0.05}$[z]	2	0.04	0.36	0.2	0.2	0.3	0.5
Linear[y]	0.0178	<0.0001	0.0092	<0.0001	0.0003	0.0119	0.1448
Quadratic	0.0011	0.0006	<0.0001	0.0153	<0.0001	<0.0001	<0.0001
a	35.3	0.754	3.45	5.44	4.45	6.08	48.5
b_0x	0.213	0.00347	0.0193	-0.0179	0.0247	0.0531	-0.0486
b_1x^2	-0.00299	-	-0.000270	-	-0.000450	-0.000423	0.000419
r^2	0.75	0.88	0.86	0.79	0.93	0.93	0.70
CV	8	8	5	8	6	2	0.77

[z]LSD values are for comparing between rice hull contents.
[y]The equation coefficients for either the linear ($y = a + b_0x$) or quadratic ($y = a + b_0x + b_1x^2$) regression models are provided for whichever model had the lowest significant Pr>F. The coefficient of determination (r^2) and coefficient of variation (CV) were calculated for the best fit model (n = 3).

while height was highest in the 25% rice hulls treatment. Overall maximum Impatiens growth occurred at 25% rice hulls. In Expt. 3, the shortest number of days to flowering for verbena occurred in the 25 and 50% rice hull treatments (Table 3). Shoot fresh weight was greatest in the 25 and 50% rice hulls treatments and height was maximized in the 50 and 75% treatments. The best rice hulls rate for maximizing growth of verbena was the 50%. However, more compact plants could be produced at 25% rice hulls, where weight was maximized but plants were shorter. Taking into account all three experiments, the best rice hulls rate for plant growth was 25% when blended with peat moss. This was lower than the 30 to 60% composted rice hulls for the best growth of petunia reported by Song *et al.* (1996).

Available nutrients: In Expt. 2 only the main effect of series had a significant effect on substrate electrical conductivity (EC). The average EC values for the peat and coir series were 0.51 and 0.58, respectively. The series by rice hull percentage interaction was significant in Expts. 1 and 3 (Table 2). Substrate electrical conductivity (EC) levels were within the acceptable range in all treatments and experiments. Addition of rice hulls to peat moss resulted in increased EC in Expts. 1 and 3, while addition to coir caused a decrease. This indicated that rice hulls were a source of salts, but not as great a source as coir.

There was a significant interactive effect of series by rice hull percentage on substrate K^+ concentration in all experiments (Table 4). Available K^+ in the root substrate in the three experiments was highest in 100% coir and diminished linearly with increased substitution of coir by rice hulls. Conversely, K^+ increased linearly in the peat moss series when peat was substituted by rice hulls in the three experiments. Rice hulls were a source of K^+, but a much lower source than coir. Substrate K^+ levels did not become excessive in any treatments.

Only the rice hull percentage main effect had a significant effect on substrate Ca^{2+} levels in Expts. 1 and 3 (Table 4). Substrate Ca^{2+} increased with increasing rice hulls in these experiments. There were interactive effects of series by rice hull percentage on substrate Ca^{2+} levels in Expt. 2 and on substrate Mg^{2+} levels in all three experiments. There was a trend for available levels of Ca^{2+} and Mg^{2+} to increase with increasing substitution of coir or peat moss by rice hulls. This is consistent with the report of Laiche and Nash (1990) which indicated that composted rice hulls contributed Ca^{2+}, Mg^{2+} and K^+ to root substrate. There were no consistent effects of rice hulls on available substrate levels of PO_4-P, NH_4^+-N, or NO_3^--N in the peat moss and coir series.

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Table 4. Effect of rice hull volume proportion in a peat moss and a coir replacement series of treatments on mean K^+, Ca^{2+} and Mg^{2+} concentrations of substrate solutions of *Impatiens walleriana* 'Super Elfin White' in Expts. 1 and 2 and *Verbena* ×*hybrida* 'Romance Deep Rose' in Expt. 3

Rice hulls	Expt. 1 K^+ mg L^{-1}		Expt. 2 K^+ mg L^{-1}		Expt. 3 K^+ mg L^{-1}		Expt. 1 Ca^{2+} mg L^{-1}		Expt. 2 Ca^{2+} mg L^{-1}		Expt. 3 Ca^{2+} mg L^{-1}	Expt. 1 Mg^{2+} mg L^{-1}		Expt. 2 Mg^{2+} mg L^{-1}		Expt. 3 Mg^{2+} mg L^{-1}
(%)	Peat	Coir	Peat	Coir	Peat	Coir	Peat	Coir	Peat	Coir		Peat	Coir	Peat	Coir	Peat	Coir
0	21.3	72.0	33.7	102.0	9.3	151.0	10.3		12.0	4.7	11.5	8.0	7.0	12.0	4.3	15.7	6.3
25	31.0	66.0	41.3	100.3	16.0	117.7	11.0		11.7	6.7	11.0	11.3	7.0	11.3	6.7	10.7	7.3
50	44.3	58.3	37.3	68.0	16.0	73.7	14.5		15.0	11.0	9.3	12.7	9.0	14.3	10.0	11.3	6.3
75	38.7	42.7	40.3	58.7	31.7	41.3	15.3		17.0	18.7	12.2	12.7	8.3	14.3	15.0	12.0	9.7
100	45.0	45.0	47.3	47.3	25.7	25.7	20.7		23.3	23.3	16.7	10.7	10.7	14.3	14.3	11.0	11.0
$LSD_{0.05}$ [z]	15.5	-	14.6	-	22.7	-	6.4		1.6	-	3.9	2.8	-	1.4	-	3.9	
Linear[y]	0.0095	0.0015	0.0297	<0.0001	0.0491	<0.0001	0.0003		<0.0001	<0.0001	0.0309	0.1539	0.0018	0.0019	<0.0001	0.1427	0.0044
Quadratic	0.2451	0.7202	0.6750	0.8807	0.7293	0.1687	0.3654		<0.0001	0.0111	0.0125	0.0139	0.4668	0.4991	0.1649	0.1985	0.2264
a	25.1	72.3	34.7	105	10.1	147	9.37		10.2	3.00	11.9	8.02	6.67	11.7	4.40	-	5.80
b_0x	0.220	-0.309	0.105	-0.604	0.193	-1.31	0.100		0.112	0.197	-0.120	0.164	0.0347	0.0307	0.113	-	0.0467
b_1x^2	-	-	-	-	-	-	-		-	-	0.00166	-0.00137	-	-	-	-	-
r^2	0.42	0.55	0.31	0.82	0.26	0.91	0.37		0.84	0.95	0.33	0.50	0.54	0.54	0.90	-	0.48
CV	27	19	15	14	62	19	33		12	13	29	17	14	8	14	-	23

[z]LSD values for Ca^{2+} in Expts. 1 and 3 are for comparing between rice hull contents. LSD values for all other dependent variables are for comparing substrate series for a given rice hull content or rice hull contents for a given substrate series.

[y]The equation coefficients for either the linear ($y = a + b_0x$) or quadratic ($y = a + b_0x + b_1x^2$) regression models are provided for whichever model had the lowest significant Pr>F. The coefficient of determination (r^2) and coefficient of variation (CV) were calculated for the best fit model (n = 3). The regression models for Ca^{2+} concentration in Expts. 1 and 3 combined data from both substrate series (coir or sphagnum peat moss) since the substrate series x rice hull rate interaction was not significant for these two dependent variables.

Ground, composted rice hulls are a potential alternative component of plug and bedding plant substrates. When ground so that 75% of the particles were 0.51 to 1.40 mm and 90% of the particles were 0.51 to 2.00 mm, composted rice hulls provided AS to the substrate as would be expected from a coarse aggregate such as perlite. The ground rice hulls did not serve as an acceptible replacement for peat moss or coir since CC and AW declined with increasing addition of rice hulls. Rice hulls also contributed Ca^{2+}, Mg^{2+}, and K^+ to root substrate. Overall, the best growth was obtained with 25% rice hulls plus 75% peat moss.

Acknowledgements

We thank Hunter Edwards for assistance with the grinding procedures for rice hulls, Ellis Edwards for the physical properties measurements, and Nancy Mingis for chemical analyses. Use of trade names does not imply endorsement by the NCARS of products named or criticism of similar ones not mentioned.

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Journal of Applied Horticulture, 12(1): 21-25, January-June, 2010

Diallel analysis for fruit traits among tomato recombinant inbred lines derived from an interspecific cross *Solanum lycopersicum* x *S. pimpinellifolium*

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Abstract

Five recombinant inbred lines, generated from a single interspecific cross *S. lycopersicum* x *S. pimpinellifolium,* were crossed in a complete diallel combination without reciprocal. Fruit quality traits were analyzed according to Griffing (1956), method 2, model 1 (fixed effects). Significant general and specific combining ability (GCA and SCA) effects were found for all traits. Weight, reflectance percentage, chroma index, firmness, soluble solids content, pH and titratable acidity presented SCA values greater than GCA values, indicating nonadditive effects. Both additive and nonadditive effects were significant in determining diameter and shape. Positive unidirectional dominance was found for shape, shelf life and chroma index, while negative unidirectional dominance was involved in the expression of weight, diameter, height, reflectance percentage and firmness. Bidirectional dominance was found for soluble solids content, pH and titratable acidity. In spite of being a genetic pool generated from a single interspecific cross, high levels of genotypic and phenotypic variability was found among the fifteen genotypes for important agronomic traits. Both additive and nonadditive effects were important in the genetic determination of these traits.

Key words: Plant breeding, diallel analysis, combining ability, *Solanum lycopersicum*, recombinant inbred lines

Introduction

In most of tomato (*Solanum lycopersicum*) breeding programs, emphasis is given to increase yield relegating important traits as fruit quality (size, shape, color, and flavour) and postharvest attributes (firmness and shelf life). Quality is given by organoleptic characteristics, those that can be perceived by all senses (flavour, aroma, colour and texture). The genetic uniformity of the cultivated species is due to the high selection pressure applied to achieve the desired phenotype, decreasing the possibilities of a successful breeding program. In this way, a plateau has been reached that limits the increase of the fruit quality and postharvest characters (Kannenberg and Falk, 1993).

The interest for the wild species has increased in order to extend the genetic variability, in view of the fact that they present organoleptic characteristics demanded by the international market. By interespecific crossing (hybridization), it is possible to transfers this characteristics to cultivated species and generate a genetic variability owed to the heterotics effect caused by the interactions among divergent genotypes (Rick, 1976). Lecomte *et al.* (2004); Monforte *et al.* (2001); Rousseaux *et al.* (2005) pointed out that wild germplasm contributes to an increase in internal fruit quality but they reduce fruit weight.

Despite the advances of "molecular breeding", classical quantitative genetics remains useful in practical tomato improvement. Thus, estimation of genetic parameters such as heterosis and combining ability gives inferences about the predominant action of the genes, indicates the appropriate selection strategy to be applied in the breeding program and allows the identification of the best parents. Diallel mating designs provide useful genetic information, such as general combining ability (GCA) and specific combining ability (SCA), to devise appropriate breeding and selection strategies. Griffing method (1956) partitions the genetic variability into additive component (estimated by GCA) and non additive component (estimated by SCA). The diallel analysis helps specifically to choose the most promising segregant populations for selection.

Pratta *et al.* (1996) evaluating postharvest characteristics in cultivated and wild genotypes, selected the wild accession LA722 of *S. pimpinellifolium* as the high postharvest value. This wild species presents fruits with small size and low weight, but with high nutritional quality and, in natural conditions, preserves organoleptic qualities for a larger period of time than the commercial ones. Pratta *et al.* (2003) analyzed productive traits such as fruit weight, diameter, height and shelf life in a diallel design among domestic, exotic and mutant germplasms. They found that the most promising hybrid for breeding all traits simultaneously was the F_1 between the cultivar Caimanta and the accession LA722 of *S. pimpinellifolium*, since it had the largest differences in allele frequencies and the greatest genetic divergence. Based on these results, Rodriguez *et al.* (2006a) carried out a selection for fruit weight and shelf life in a F_2 generation from a cross between the cultivar Caimanta and the accession LA722 obtaining 17 RILs (Recombinant Inbred

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Lines). They observed transgressive segregation for shelf life, obtaining superior genotypes with higher values of this trait than the progenitors. Rodriguez *et al.* (2006b) evaluating the 17 RILs have found divergence values of fruit weight and shelf life, also they found divergence of quality traits. It is possible to select high-performing RILs from a particular heterotic cross and, by intercrossing them, to produce a second cycle hybrid (SCH) that perform even better than the original. Crossing the best RILs will ensure that a maximum number of superior SCHs actually be produced (Kearsey and Pooni, 1996). Segregant F_2 populations can be generated from these superior SCHs to begin a new breeding program.

The present study was carried out using a diallel cross system among five RILs to assess the general and specific combining abilities and discriminate the superior parents and second cycle hybrid combinations as a step in developing a new selection process in the tomato breeding program.

Materials and methods

The experiment was carried out at the field station "José F. Villarino" (Facultad de Ciencias Agrarias-Universidad Nacional Rosario, Zavalla, Argentina, 33°S 61°W).

Plant material: Five RILs (ToUNR1, ToUNR8, ToUNR9, ToUNR15 and ToUNR18) obtained from an interspecific cross between cv. Caimanta of *S. lycopersicum* and the accession LA722 of *S. pimpinellifolium* were selected based on their agronomic performance (Rodríguez *et al.*, 2006a) and crossed in a complete diallel combination without reciprocal crosses (Griffing, 1956). Fifteen seeds of the five parents and from each hybrid H (1x8), H (1x9), H (1x15), H (18x1), H (9x8), H (15x8), H (18x8), H (15x9), H (18x9) and H (18x15) were grown in seedling trays and transplanted to greenhouse in a complete randomised design.

Traits evaluated: A minimum of ten breaker stage tomatoes per plant of the five RILs and the hybrids (fifteen genotypes) were harvested and evaluated for the following traits: weight (W, in g), height (H, in cm), diameter (D, in cm), shape index (S, ratio H/D), shelf life (SL, measured as the number of days elapsed from harvesting to the first symptoms of deterioration in the fruits, stored at 25±3°C, according to Schuelter *et al.*, 2002), firmness (FR, determined with a fruit pressure tester -12.5 N- type Shore A with a tip of 0.10 in a 0–100 scale), soluble solids content (SS, in ºBrix, determined with a hand refractometer in the homogenised juice from the pericarp tissue), pH, colour (a/b, determined with a chromameter CR 300 measuring through the chroma index by calculating the a/b ratio, where a and b are the absorbencies at wavelength of 540 and 675 nm, respectively). L, reflectance percentage, indicates darkness or lightness of colour and ranges from black (0) to white (100) and titratable acidity (TA, g of citric acid/100 g of homogenized juice).

Data analysis: The normal distribution was verified according to Shapiro-Wilk test (1965) for all fruit traits. Mean values were compared by one-way ANOVA. The Diallel crosses were analyzed following Griffing (1956) method 2, model 1 (fixed effects):

$$Y_{ijk} = \mu + g_i + g_j + s_{ij} + e_{ijk}$$

Where, Y_{ijk} is the mean phenotypic value, μ is the general mean, g_i y g_j are the GCA effect of each parent, s_{ij} is the SCA effect of the hybrid and e_{ijk} is the experimental error. The greater the value of the GCA effect the greater the number of genes that increase the trait expression and the greater the number of positive differences between the gene frequency and the average frequency in the diallel parents. SCA value of hybrids is an indicator of the hybrid's divergence relative to the parents. The higher the SCA value of hybrids, the greater the differences between the expected performance of the hybrid assuming additivity and the observed performance due to non additive effects. Similar information is given by the mid-parent heterosis (Hm), which was also calculated for all traits. In respect to SCA in parents, positive values indicate that there is unidirectional dominance of lowest values, while negative values indicates unidirectional dominance of greatest values. If positive and negative SCA values are found among parents for a given traits, then bidirectional dominance is present (Soriano Viana, 2000).

Results

All traits showed a normal distribution, with W values greater than 0.80. Mean values and standard errors of all fruit quality traits in the fifteen genotypes are shown in Table 1. Highly significant differences were detected among genotypes for all traits. An interesting discrepancy was found for the phenotypic mean values of ToUNR1 and ToUNR8. ToUNR1 had high values of height, diameter, weight, shelf life and pH, whereas ToUNR8 ranked towards the low values of the same traits. An opposite situation was found for soluble solids content and titratable acidity, where ToUNR1 had the lowest values and ToUNR8, the higher ones.

Highly significant GCA and SCA effects were found for all traits (Fig. 1 and 2 and Table 2). GCA effects were greater than SCA effects for height and shelf life. Instead, weight, L, a/b, firmness, soluble solids content, pH, and titratable acidity presented SCA values greater than GCA values. Diameter and shape showed similar values of both combining abilities. ToUNR1 showed positive GCA values for diameter, height, weight, shelf life, and firmness, and ToUNR18 for shape, weight, shelf life, and firmness. Negative or near to zero values were found for these lines in soluble solids content, pH, and titratable acidity. Another line, ToUNR8, presented high GCA values for L, titratable acidity and soluble solids contents, and near to zero values for shape, a/b, and pH.

The SCA values for all traits of each hybrid were verified by the mid-parent heterosis (Table 2). Most hybrids had negative mid-parent heterosis for weight. The exception was H (18x8), which presented a value of 29.80. Positive values of mid-parent heterosis were found for shelf life, and the hybrids H (1x8) and H (15x9) had outstanding values of 23.96 and 29.40, respectively. In general, the mid-parent heterosis was negative for fruit firmness, the values of H (15x9) and H (18x15) being noticeable (-19.58 and -17.86, respectively). For a/b, the remarkable genotype H (15x8) presented a value of 23.01. Finally, most hybrids had negative SCA values for soluble solids content. Exceptions were H (1x8) and H (1x9), for which SCA was 11.32 and 12.71, respectively.

Discussion

The fruit quality traits analysis suggest that in spite of being a genetic pool generated from a single interspecific cross *S.*

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Table 1. Mean value and standard error of fruit quality traits in tomato

Genotype	D	H	S	W	SL	L	a/b	FR	SS	pH	TA
ToUNR1	3.55 ± 0.06	2.85 ± 0.09	0.81 ± 0.02	21.66 ± 1.35	24.79 ± 1.18	39.81 ± 1.68	1.04 ± 0.15	54.17 ± 7.06	5.54 ± 0.25	4.96 ± 0.05	0.32 ± 0.02
ToUNR8	1.58 ± 0.06	1.42 ± 0.04	0.90 ± 0.01	2.34 ± 0.21	17.98 ± 0.62	40.42 ± 1.37	1.14 ± 0.11	54.55 ± 4.68	9.30 ± 0.66	4.51 ± 0.09	0.65 ± 0.02
ToUNR9	1.83 ± 0.06	1.63 ± 0.04	0.89 ± 0.01	3.81 ± 0.32	18.70 ± 0.43	37.09 ± 0.40	1.37 ± 0.05	53.39 ± 3.38	7.04 ± 0.18	4.35 ± 0.05	0.79 ± 0.04
ToUNR15	2.84 ± 0.09	2.22 ± 0.07	0.79 ± 0.02	11.59 ± 1.04	14.41 ± 1.49	38.81 ± 1.66	1.12 ± 0.16	54.32 ± 4.52	7.91 ± 0.34	4.61 ± 0.11	0.44 ± 0.03
ToUNR18	2.83 ± 0.06	2.77 ± 0.07	0.97 ± 0.02	13.72 ± 0.72	21.11 ± 1.20	36.54 ± 1.54	1.15 ± 0.10	52.47 ± 4.51	7.69 ± 0.26	4.72 ± 0.07	0.37 ± 0.03
H (1x8)	2.21 ± 0.05	1.90 ± 0.03	0.88 ± 0.01	5.80 ± 0.23	26.51 ± 1.24	35.39 ± 0.94	1.18 ± 0.06	47.15 ± 4.68	8.26 ± 0.24	4.50 ± 0.07	0.59 ± 0.05
H (1x9)	2.17 ± 0.03	2.02 ± 0.02	0.94 ± 0.02	5.81 ± 0.02	24.60 ± 1.27	37.82 ± 1.04	1.19 ± 0.07	55.71 ± 6.88	7.09 ± 0.21	4.65 ± 0.11	0.39 ± 0.05
H (1x15)	2.40 ± 0.07	2.09 ± 0.07	0.89 ± 0.01	7.31 ± 0.05	21.93 ± 1.40	37.96 ± 1.14	1.25 ± 0.09	45.53 ± 7.40	5.49 ± 0.44	4.49 ± 0.09	0.42 ± 0.06
H (18x1)	2.59 ± 0.11	2.59 ± 0.09	1.02 ± 0.02	10.62 ± 0.99	23.08 ± 1.80	37.11 ± 1.00	1.15 ± 0.12	54.85 ± 7.72	6.48 ± 0.34	4.54 ± 0.08	0.44 ± 0.07
H (9x8)	1.8 ± 0.04	1.67 ± 0.07	0.93 ± 0.02	3.36 ± 0.26	18.77 ± 0.93	38.09 ± 1.04	1.27 ± 0.08	48.89 ± 6.27	8.09 ± 0.87	4.42 ± 0.08	0.55 ± 0.08
H (15x8)	1.84 ± 0.04	1.64 ± 0.04	0.89 ± 0.01	3.50 ± 0.22	15.47 ± 0.75	37.46 ± 0.58	1.39 ± 0.07	52.39 ± 5.12	6.89 ± 0.36	4.62 ± 0.11	0.55 ± 0.04
H (18x8)	2.57 ± 0.05	2.47 ± 0.06	0.97 ± 0.02	10.42 ± 0.66	20.58 ± 1.06	36.99 ± 0.68	1.23 ± 0.11	44.87 ± 7.07	7.31 ± 0.27	4.74 ± 0.10	0.33 ± 0.02
H (15x9)	2.36 ± 0.04	2.11 ± 0.03	0.91 ± 0.01	7.37 ± 0.32	21.42 ± 0.84	36.29 ± 0.99	1.24 ± 0.12	43.31 ± 8.33	7.20 ± 0.22	4.50 ± 0.09	0.55 ± 0.04
H (18x9)	1.96 ± 0.05	1.89 ± 0.05	0.98 ± 0.01	4.62 ± 0.31	20.67 ± 0.98	36.86 ± 1.13	1.26 ± 0.10	49.00 ± 7.13	7.23 ± 1.52	4.62 ± 0.08	0.40 ± 0.07
H (18x15)	2.63 ± 0.08	2.41 ± 0.09	0.92 ± 0.03	10.64 ± 0.95	14.68 ± 0.88	37.39 ± 1.27	1.19 ± 0.17	43.86 ± 8.44	7.35 ± 0.53	4.65 ± 0.18	0.46 ± 0.03
F	70.80***	50.17***	14.52***	61.90***	10.78***	37.13***	21.14***	14.35***	28.51***	23.90***	79.11***

***$P<0.0001$

lycopersicum x *S. pimpinellifolium* ('Caimanta' x LA722), there exist high levels of genetic variability among the fifteen genotypes. Recombination and/or genomic rearrangements occurring during the selfing for obtaining and stabilizing the lines could be the causes of such a genetic variability. Rodríguez *et al.* (2006b) analyzed 17 RILs (that included the five ones evaluated in this work) and also found high genetic variability, suggesting that these genotypes constitute a new source of variability for use in breeding programs.

High significant values of GCA and SCA indicate that both additive and nonadditive gene actions affect the expression of the traits. Additive gene action was important for determining height and shelf life since GCA effects were greater than SCA effects. Instead, weight, L, a/b, firmness, soluble solids content, pH and titratable acidity presented SCA values greater than GCA values, indicating that nonadditive effects have the greatest contribution in controlling these traits. Garg *et al.* (2008) found the similar genetic control for firmness, pH, and titratable acidity in different tomato genotypes. Dhatt *et al.* (2004) have also reported the importance of nonadditive effects in the genetic control of fruit firmness. In contrast, Chadha *et al.* (2002), Das *et al* (1988) and Rodriguez *et al.* (2004) reported additive effects in the expression of fruit weight. These discrepancies could be explained by differences in gene frequencies among the populations studied in each research. In the present one, original experimental lines obtained in a novel breeding program were analyzed whereas in the others, plant material included more divergent germplasm such as mutant and exotic genotypes. Pratta *et al.* (2003) and Zorzoli *et al.* (2000) also found predominance of additive effects in the genetic control of weight and nonadditive effects for shelf life, when analyzing the parents of the single cross from which the RILs were derived. These results agree with the above mentioned authors but not with the present results. Hence differences in genetic frequencies could not be proposed to explain these discrepancies, so that the previously mentioned recombination and/or genomic rearrangements could account for them. Finally, both additive and nonadditive effects were important in determining the expression of diameter and shape.

Table 2. Mid-parent heterosis (Hm) and specific combinatory ability (SCA) of fruit quality traits in tomato hybrids

Genotype	D		H		S		W		SL		L		a/b		FR		SS		pH		TA	
	Hm	SCA	Hm	SCA	Hm	SCA	Hm	SCA	Hm	SCA	Hm	SCA	Hm	SCA	Hm	SCA	Hm	SCA	Hm	SCA	Hm	SCA
H (1x8)	-13.80	-0.12	-11.00	-0.14	2.92	-0.01	-51.70	-2.74	23.96	3.40	-11.78	-2.99	8.26	0.02	-13.27	-4.63	11.32	0.94	-4.96	-0.13	21.65	0.11
H (1x9)	-19.30	-0.22	-9.80	-0.08	10.60	0.04	-54.40	-2.87	13.13	0.74	-1.64	0.23	-1.24	-0.02	3.59	3.74	12.71	0.55	-0.11	0.07	-29.73	-0.11
H (1x15)	-24.90	-0.41	-17.50	-0.26	11.25	0.04	-56.00	-4.69	11.90	1.00	-3.43	-0.13	15.74	0.09	-16.07	-5.02	-18.36	-0.92	-6.16	-0.17	10.53	0.00
H (18x1)	-18.80	-0.29	-7.80	-0.07	14.60	0.08	-39.90	-3.06	0.57	-0.54	-2.79	-0.22	5.02	0.02	2.87	3.76	-2.04	-0.07	-6.20	-0.19	27.54	0.09
H (9x8)	5.50	0.11	9.50	0.11	3.91	0.00	9.30	1.10	2.34	-1.03	-1.72	0.38	1.19	-0.01	-9.41	-1.77	-0.98	0.01	-0.22	-0.04	-23.61	-0.07
H (15x8)	-16.70	-0.27	-9.90	-0.17	5.32	0.02	-49.70	-2.06	-4.48	-1.39	-5.44	-0.75	23.01	0.16	-3.76	3.15	-19.93	-1.05	1.32	0.07	0.92	0.01
H (18x8)	16.50	0.39	17.90	0.35	3.74	0.01	29.80	3.20	5.30	1.01	-3.87	-0.47	7.40	0.30	-16.15	-4.91	-13.95	-0.78	2.71	0.12	-35.29	-0.14
H (15x9)	1.10	0.20	9.60	0.24	8.30	0.03	-4.28	1.70	29.40	3.76	-4.37	-1.13	-4.00	-0.04	-19.58	-6.11	-3.68	0.04	0.45	0.01	-10.57	-0.01
H (18x9)	-15.90	-0.27	-14.10	-29.00	5.38	0.01	-47.30	-2.76	3.84	0.31	0.12	0.19	0.00	0.00	-7.43	-0.96	-1.83	-0.08	1.87	0.06	-31.03	-0.09
H (18x15)	-7.20	-0.03	-3.40	-0.02	4.54	0.00	-15.90	-0.06	-17.34	-2.75	-0.76	0.23	4.85	0.00	-17.86	-4.68	-5.77	0.18	-0.32	0.00	13.58	0.06

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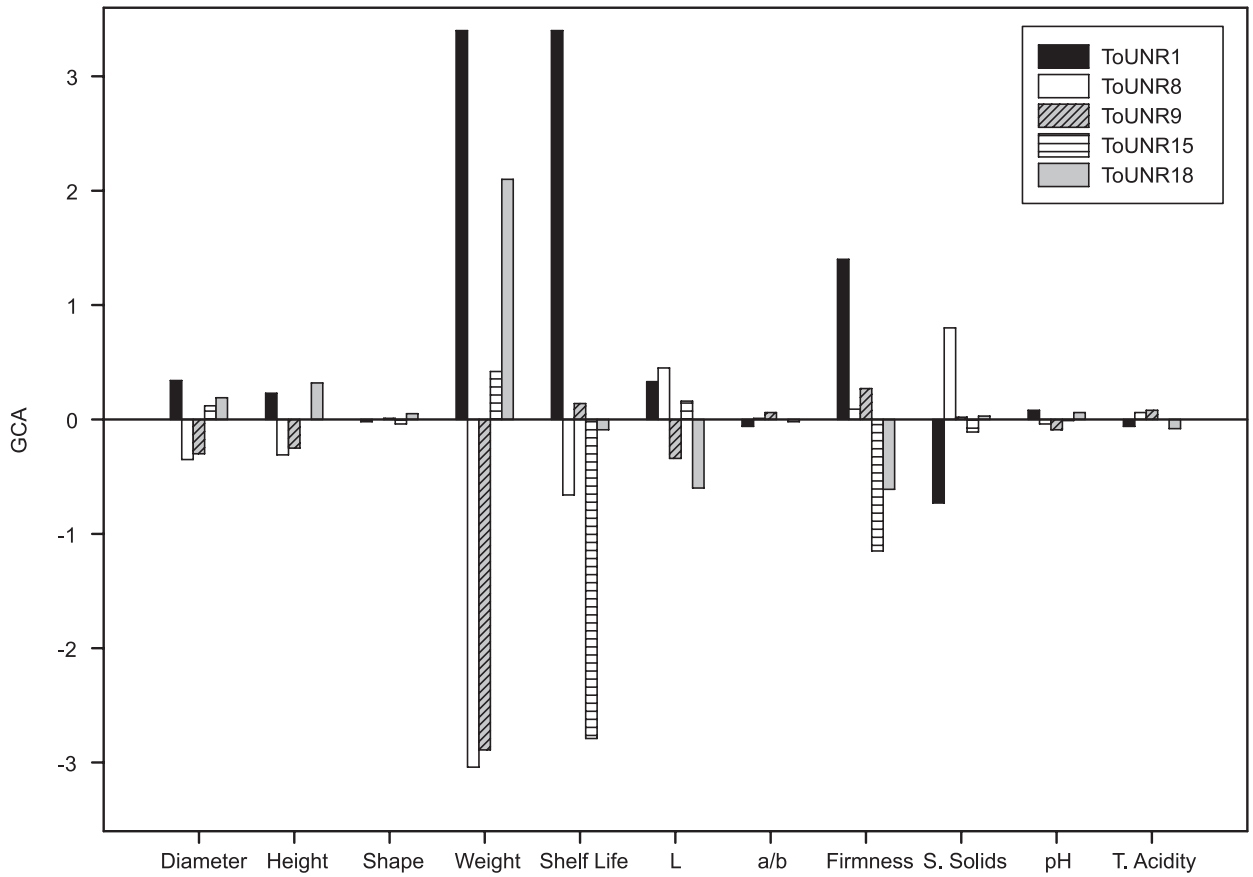

Fig. 1. General combinatory ability (GCA) of fruit quality traits in parents

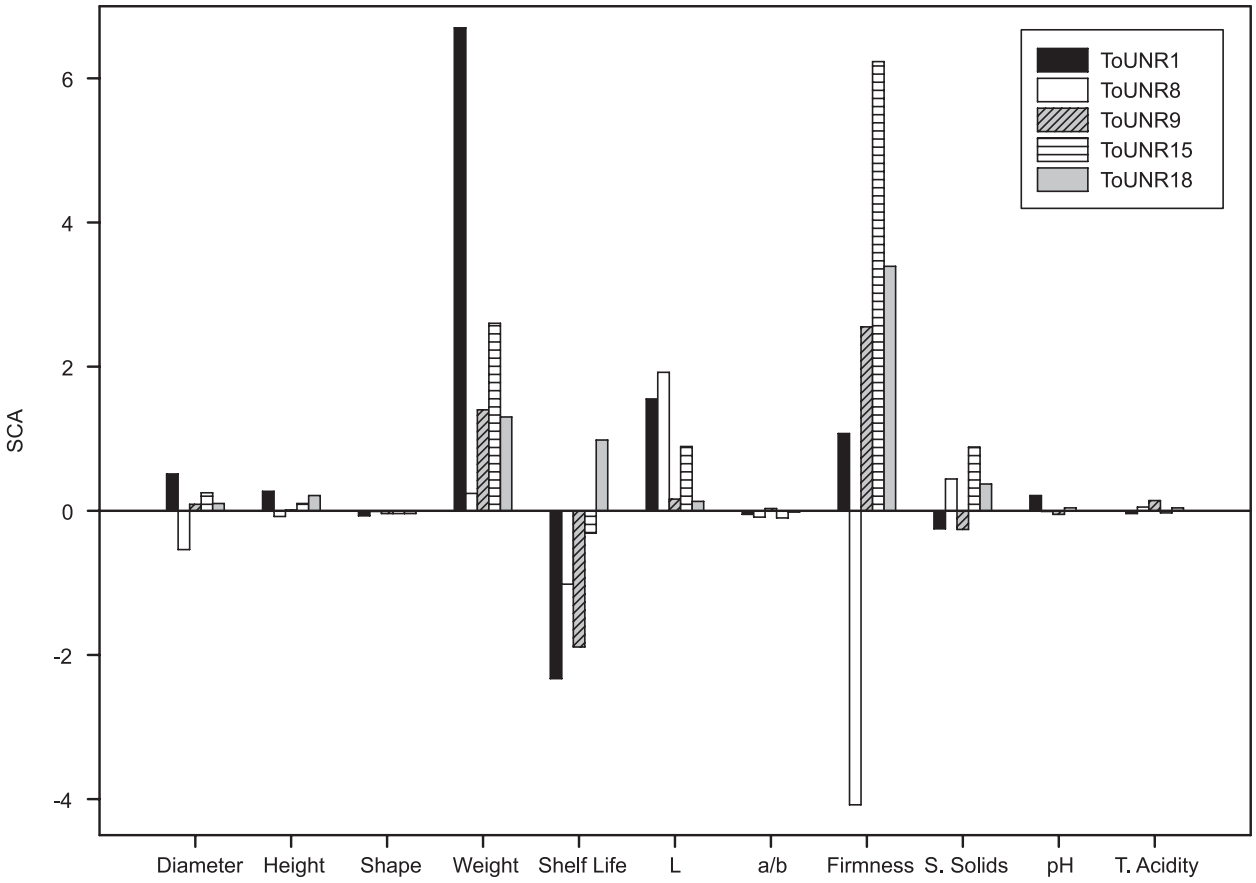

Fig. 2. Specific combinatory ability (SCA) of fruit quality traits in parents

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Most parents had negative SCA values for shape, shelf life and a/b (Fig. 2), indicating that unidirectional dominance of the highest values was involved in the expression of these traits (Griffing, 1956). Instead, most parents had positive SCA values for weight, diameter, height, L and firmness, corresponding to dominance effects of the lowest values. These results partially agree with Pratta *et al.* (2003), who found negative unidirectional dominance for both weight and shelf life. Again, variations in gene frequency and chromosomal recombination or genomic rearrangements could explain these differences. Finally, parents presented both positives and negatives SCA values for soluble solids content, pH and titratable acidity. This fact indicated that bidirectional dominance is involved in the expression of these traits, *i.e.*, for a given trait, the highest values were dominant in some crosses but recessive in others.

One of the advantages of the diallel analysis in breeding programs is to allow choosing the best combinations of promissory parents to generate segregant populations for continuing the traits improvement. In this research, the positive GCA values presented by ToUNR1, ToUNR18 and ToUNR8 would indicate that the three lines are potential parents to improve agronomically important traits such as weight, shelf life, colour, soluble solids content and firmness. On the other hand, the hybrid H (15x9) presented high SCA values for weight and shelf life, traits that were the targets of the selection practiced in the interspecific cross 'Caimanta' x LA722 to obtain the RILs used as parents in this experiment. Therefore, H (15x9) is an interesting second cycle hybrid to generate a segregant population for continuing the improvement of both traits. On the other hand, the SCA values of H (1x8) indicate that it is an promising genotypic combination for improving shelf life, soluble solids content, pH and titratable acidity. Also interesting, was that both parental lines had extreme phenotypic mean values for these agronomically important traits. It is a well known fact that crossing phenotypically divergent materials is of importance for broadening the genetic variability. H (1x8) appears as another promissory second cycle hybrid to generate a basal segregant population for beginning a new breeding program.

In conclusion, though the five recombinant lines were generated from an original single interspecific cross, high level of phenotypic and genotypic variability was found among them in important agronomic traits. Both additive and nonadditive effects were important to the genetic determination of these traits. Recombinant lines ToUNR1, ToUNR8 and ToUNR18 are promissory parents, while H (15x9) and H (1x8) are suitable second cycle hybrids, to develop new basal populations for continuing the breeding program.

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Journal of Applied Horticulture, 12(1): 26-29, January-June, 2010

Pepper (*Capsicum annuum* L.) responses to surface and drip irrigation in southern Tunisia

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Abstract

Field experiments were performed to study the impact of two different irrigation systems (surface drip and surface) on water use efficiency and yield components of a pepper crop (*Capsicum annum.* L). Irrigation scheduling was carried out based on estimated crop evapotranspiration (ETc) using crop coefficients for pepper and reference evapotranspiration ETo calculated using the Penman-Monteith equation (Allen *et al.*, 1998). The crop received total water needs computed according to Veirmeiren and Jobling (1983) procedure for surface drip irrigation. Border irrigation was scheduled by Cropwat model (Smith, 1992). Experimental plots were irrigated simultaneously during the appropriate duration for each one and received the same nutrients (N, P, and K) ratio. Comparison was made on fruit number per plant, fruit weight, fruit weight by harvest and yield per unit surface. The results showed that compared with surface irrigation, drip irrigation presented a significant difference in total fruit yield and water use during cropping season (May to September). With drip irrigation, average yield was 19.73 kg m^{-2} which was 68% greater than that irrigated with surface irrigation (11.90 kg m^{-2}). Applied water volume by unit production (m^3/kg) was 0.38 for drip and 1.05 for border, respectively. Drip irrigation increased fresh pepper fruit yield with a reduction of 60% in water use compared to traditional surface irrigation.

Key words: Arid, drip irrigation, border irrigation, yield, *Capsicum annum.*L, water use.

Introduction

In near future, the main constraint to agricultural development of arid and semi arid areas will be the availability of water resources rather than soils. Under these conditions of limited water resources, crop production must be maintained at expense of minimum inputs but aiming at achieving maximum incomes. In order to achieve that goal, improvement of irrigation water use efficiency is necessary which has increased the search for technology with improved water use efficiency.

In Tunisia, where rainfall is erratic and water demand increase steadily, a national water saving strategy for an efficient management of available water resource was undertaken with following objectives: safeguarding water resources to meet necessary needs for different uses; minimizing water losses; improving water use efficiency especially in agriculture which use 80% of total resources.

In the arid southern part of the country, where water resources are finite, irrigated agriculture is dominated by traditional methods of surface irrigation (Thabet *et al.,* 1999) which causes large percolation losses and restrains the increase in production due to soil frequent drought at irrigation intervals and poor irrigation management. In these conditions, drip irrigation, in which water is applied directly to the roots zone of plants by different ways (orifices, emitters, porous tubing, or perforate pipe) and operated under low pressure (Spellman, 2008). This can help in conserving water by reducing evaporation and deep percolation, if well managed (Tanji and Hanson, 1990). Advantage of surface drip irrigation are the ease of installation, inspection, changing and cleaning emitters. It also permits the possibility of checking soil surface wetting patterns and measuring individual dripper discharge rates.

In order to conserve precious water resources and maximize plant performance, farmers are incited to use this method for a subsidy which can reach 60 % of irrigation materials cost.

The main objective of this study was to evaluate the effects of surface drip and surface irrigation on yield components of pepper which is a widely cropped vegetable in summer and used as condiment in North Africa countries. Major studied factors during irrigation season were gas exchange, water consumption, crop yield and soil salinity.

Materials and methods

The experiments were carried out in the experimental field of Aridoculture and Oases Laboratory in the Institute of Arid Regions (33°3′ N, 10°3′E). Climate is typically Mediterranean with dry and hot summers and precipitations irregularly distributed throughout the year. The soil at the study site is loamy sand and almost flat. Major soil characteristics of trial plots are summarised in Table 1. One month old seedlings of a local green pepper cultivar were transplanted in 60x50 cm spacing for border irrigation and in 100x50 cm for surface trickle irrigation. Borders were 2 m wide and 8 m in length, while the drip lines length was 20 m where drippers were spaced out at 50 cm (Fig.1).

Irrigation water characteristics are given in Table 2. It was applied from a well by a pump in drip irrigated plots and by gravity from the basin for borders. A drip irrigation system was used, with 4L/h PVC emitters. Irrigation frequency was three days for each trial. It was chosen to be the nearest of farmer's practices.

Soil water content data were collected from each experimental plot, once a week one day after irrigation. It was calculated by gravimetric method for surface irrigation where three samples

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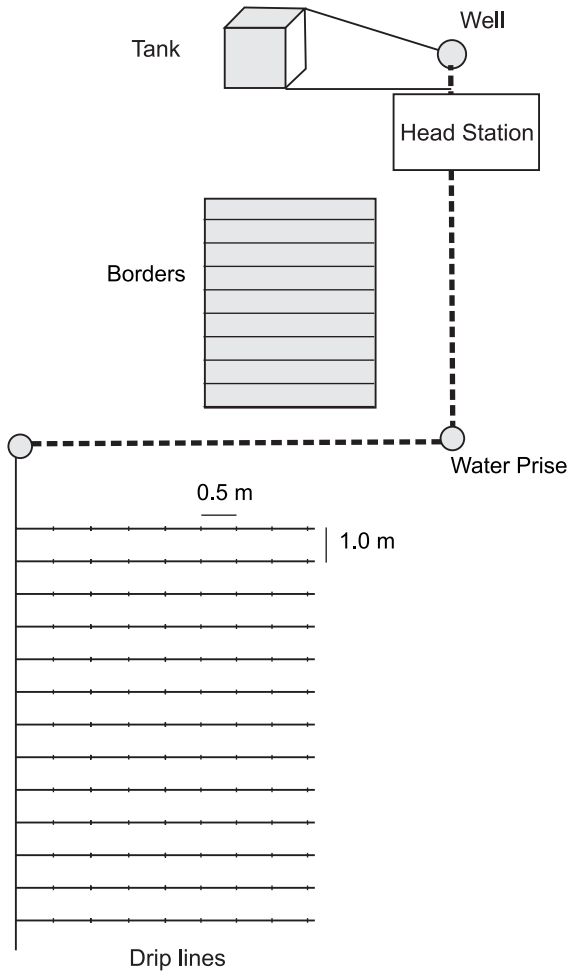

Fig. 1. Experimental layout

were taken at the head, the middle and the end of the border with a step of 20 cm until 60 cm considered as a maximum root depth for pepper (Dirks and Tan, 1988; Gough, 2001). Gravimetric method was also used to determine soil moisture at the point of middle distance between drippers. Near the dripper, soil water content was measured by mean of 4 densitometers placed around the dripper at a 10 cm circumference at 15, 30, 45 and 60 cm depths.

The total available water (TAW) which is water that soil can hold between field capacity and permanent wilting point for a given depth is calculated as:

$$TAW = Z_r * \frac{(\theta_{fc} - \theta_{pwp})}{100} \qquad (1)$$

Where:

TAW: total available water (mm)
θ_{fc}: volumetric soil moisture at field capacity [%];
θ_{pwp}: volumetric soil moisture at permanent wilting point [%] ;
Z_r : root zone depth [mm].

When soil moisture is less then field capacity, the available water (AW) stored in the root zone is computed as:

$$AW = Z_r * \frac{(\theta_v - \theta_{pwp})}{100} \qquad (2)$$

Where:

θ_v: measured volumetric soil moisture [%];
θ_{pwp}: volumetric soil moisture at permanent wilting point [%] ;
Zr : root zone depth [mm].

These two variables were used to calculate the percent of total available water stored in the root zone depth which equals:

$$SW = 100 * \frac{AW}{TAW} \qquad (3)$$

As pepper is considered one of the most susceptible crops to water stress (Smittle *et al.*, 1994; Delfine *et al.*, 2001; Antony and Singandhupe, 2004; Sezen *et al.*, 2006), the critical value of stored water (SW_c) was taken 50% and equation (3) was used to calculate water stress factor (S_f) computed as :

$$S_f = 100 * \frac{SW}{SW_c} \text{ for } SW < SW_c \qquad (4)$$
$$= 1 \quad \text{if } SW \geq SW_c$$

Gas exchange measurements were conducted weekly at 10 plants per treatment one day after irrigation using a portable LCi Ultra Compact Photosynthesis System (ADC BioSientific Ltd, UK). These measurements were conducted in leaves of the same physiological stage (two well-developed leaves per plant) at the same time of the day (9-11a.m.). Measured parameters were:

transpiration rate: E [mmol $m^{-2}s^{-1}$] ;
stomatal conductance : g_s [mol $m^{-2}s^{-1}$] ;
photosynthetic rate : A [µmol $m^{-2}s^{-1}$] ;
substomatal CO_2 : C_i [vpm].

During irrigation season, all plots received the same amount of fertilizer (130-35-75). Yield was determined by hand harvesting of each plot depending on physiological maturity of plants.

Results and discussion

Soil water content: Fig. 2 and 4 illustrates the time course of soil available water content (AW) calculated weekly, 24 hours

Table 1. Soil particle size and water retention properties

Soil depth (cm)	Particle size distribution (%)			Bulk density (g cm^{-3})	Volumetric moisture content (cm^3 cm^{-3})	
	Clay	Silt	Sand		θ_{fc}	θ_{pwp}
0-20	7.33	6.78	84.11	1.48	18.13	5.11
20-40	9.75	10.25	78.25	1.53	16.5	8.92
40-60	11.38	12.13	75.38	1.49	19.8	13.08
60-80	12.04	13.41	72.34	1.46	27.5	15.28

Table 2. Irrigation water characteristics

Cations (méq L^{-1})			Anions (méq L^{-1})				Rs (g L^{-1})	EC (ds m^{-1})	SAR	pH
Na^+	K^+	$Ca^{++}+Mg^{++}$	SO_4^{--}	HCO_3^-	CO_3^-	Cl^-				
31.52	0.41	17.16	20.67	0.63	0.1	35.1	2.94	4.28	10.76	7.9

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after irrigation. For surface irrigation, soil available water was evaluated from three gravimetric measurements of soil moisture at three points as cited in ″materials and methods″. During the cropping season, soil moisture ranged from 8 to 14 % with an average of 11.2%.

For drip irrigated plots, soil moisture was measured at two different points: one at 10 cm from the dripper and the other at 25 cm (middle point between two successive drippers). Near the dripper, soil moisture ranged from 14.4 % to 16.6 % with an average of 15.25 %. According to formula (4), Figs. 3 and 5 showed a water stress factor equal to 1 for the point near the dripper during all the cropping season however at 25 cm from dripper, its average was 0.97. In surface irrigation, the average of this water stress factor was 0.61. These results show that the nearest the point from dripper the more the soil moisture. It also show that even at 25 cm from dripper, the average soil moisture was more than that in surface irrigation. This behaviour may be attributed to evaporation losses caused by important evaporative area in surface irrigation added to percolation losses due to land levelling and distribution uniformity, however the reduced area of the slowly applied water in the case of surface drip irrigation decreased water losses by evaporation and deep percolation and so increased soil water content.

Fruit number and yield: Different measured parameters for each irrigation treatment are summarized in Table 3. Graphics and curve fitting were performed using Microsoft Excel 2000 software (Microsoft Corporation).

Analysis of variance (ANOVA test) indicated that irrigation technique did not affect average fruit weight factor, while fruit number was significantly affected by irrigation system. The highest fruit number was obtained at dripped plants with a significant difference so they had higher total mass of fresh fruit than those surface irrigated.

Table 3. Yield parameters for different irrigation techniques

Parameters	Drip	Surface
Total yield per plant (g)	986.42a	396.86b
Number of fruits per plant	57.43a	28.61b
Average fruit weight (g)	17.18a	13.87a
Yield by square meter (g)	1972.84a	1190.58b
Irrigation water amount (m^3 m^{-2})	0.75a	1.25b

(a), (b): For each row, values with the same letter were not significantly different ($P < 0.05$)

Table 4. Correlation coefficients between net CO_2 assimilation rate (A) and stomatal conductance (g_s) under drip and surface irrigation.

Type of irrigation	Stage 2	Stage 3	Stage 4
Surface drip	0.57	0.66	0.64
Surface	0.60	0.49	0.58

Gas exchange: Transpiration rate (E), net CO_2 assimilation rate (A) and stomatal conductance (g_s) were measured in order to compare the effect of soil water availability difference between the two irrigation systems during different cropping stages (Katerji *et al.,* 1993). Analysis of variance (ANOVA) of collected data was performed with Microsoft Excel 2000 software considering irrigation system and crop stages factors. The first conclusion was the high correlation between net CO_2 assimilation rate (A) and stomatal conductance (g_s) especially for drip irrigation. During different crop stages, analysis of variance (ANOVA) indicated that irrigation technique affect stomatal conductance (g_s), photosynthetic rate (A), substomatal CO_2 (C_i) and water use efficiency defined as A/g_s.

Spatial changes in soil salinity: Salinity is an environmental stress which limits plants growth and development. Plants response to excess NaCl is complex and involves changes in their morphology, physiology and metabolism (Hilal *et al.*, 1998). Pepper (*Capsicum annuum* L.) is generally considered salt sensitive (Maas and Hoffman, 1977; Navarro *et al.*, 2002). As any irrigation water supplies contain a substantial amount of salt, soil electrical conductivity was measured at the beginning and the end of the trial in order to evaluate salt accumulation in the root zone for each irrigation system. There are two processes

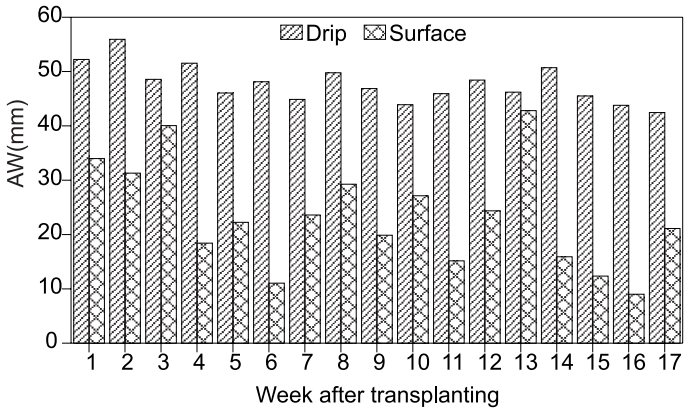

Fig. 2. Available water in 0-60 cm soil depth for surface and at 10 cm from dripper

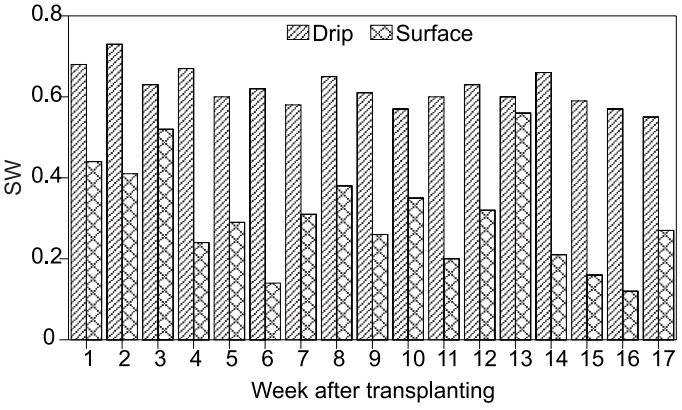

Fig. 3. Total available water ratio in 0-60 cm for surface and at 10 cm from dripper

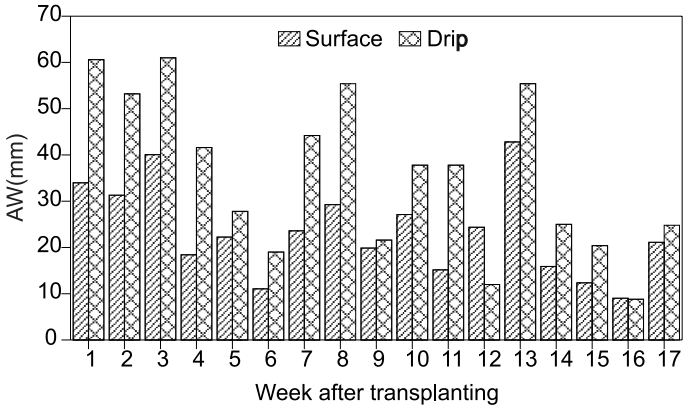

Fig. 4. Available water in 0-60 cm soil depth for surface and at 25 cm from dripper

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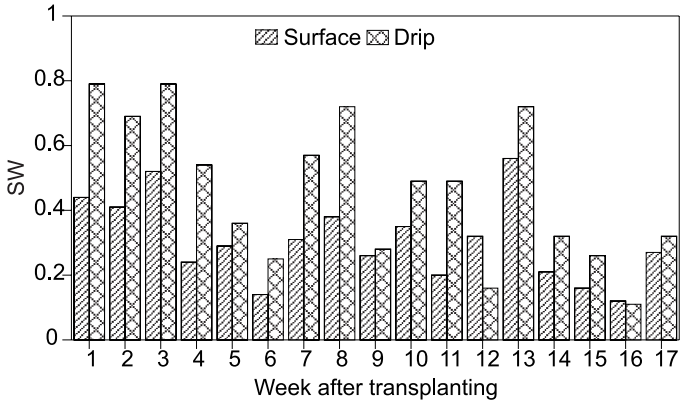

Fig. 5. Total available water ratio in 0-60 cm for surface and at 25 cm from dripper

by which salt accumulates in the root zone. The first one is the upward movement of a shallow saline-water table and the second is salts left in the soil due to insufficient leaching which is the process of applying more water to the field than can be held by the soil in the crop root zone such that the excess water drains below the root system, carrying salts with it**.** In the present case salinity was due to leaching efficiency only. Soil salinity was evaluated by the electrical conductivity of its saturated paste taken in a 10 cm grid along surface and depth. Results showed that high salt concentration was at the bulb periphery and decreased towards the middle. For surface irrigation, as water was distributed over more surface, average soil electrical conductivity values where less than in wetted bulb.

Application of water by drip irrigation system increased fresh pepper fruit yield compared to traditional surface irrigation. Yield data analysis indicated a significant difference between surface drip and traditional surface irrigation systems. One kg of fresh pepper fruit was obtained with 0.38 m^3 for drip and 1.05 m^3 for surface irrigated water and the average water use efficiency along cropping season was 59 and 46%, respectively.

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Journal of Applied Horticulture, 12(1): 30-34, January-June, 2010

In vitro flowering and shoot multiplication of *Gentiana triflora* in air-lift bioreactor cultures

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Abstract

Hormonal control of flower induction *in vitro* was investigated in Gentian. The effect of PBZ concentrations on flowering was studied in plantlets cultured in MS medium containing 30 g L^{-1}sucrose. Paclobutrazol (PBZ) concentration at 1.0 mg L^{-1} induced the highest flowering in terms of flowering percentage (91.5%), number of flowers, days to first flowering, flower length and flower diameter. PBZ did not trigger flowering but it rather stimulated flowering and its role seemed to be additive but not essential for flowering. Comparison between solid and bioreactor cultures (continuous immersion with a net) revealed that shoot multiplication and growth were more efficient using bioreactor culture. The highest shoot number per explant (29.9) was obtained in bioreactor culture. Regenerated shoots were cultured microponically for 6 weeks. Hundred percent of plants rooted and were acclimatized successfully in growing media containing perlite: vermiculite (1:1).

Key words: Bioreactor, *in vitro* flowering, Gentian, mass propagation, microponics

Introduction

Gentians (*Gentiana* spp.) are herbaceous perennial plants that are highly valued in the world market particularly in Japan as cut flowers. Flowering season of cut gentians lasts for only three months a year. *In vitro* flowers can, therefore, shorten the breeding period (Kumar *et al.*, 1995) and aid basic studies of the flowers throughout the year without the usual limitation of the season in the field. *In vitro* flowering of gentian has been reported and flowers were found to be similar to those of garden-grown plants in many aspects (Zhang and Leung 2000; 2002). Tissue culture techniques have important applications in flowering studies because a single parameter can be assayed *in vitro* to study its effect on flowering. Now, it is widely accepted that flowering results from subtle interactions of various promoters and inhibitors (Taiz and Zeiger, 2002). Growth retardants, among other plant hormones, are thought to play an essential role in the floral development of plants (Teixeira da Silva and Tan Nhut, 2003; Sudhakaran *et al.*, 2006; Taylor and van Staden, 2006). Exogenously applied paclobutrazol induce flowering in many plant species *e.g.*, *Euphorbia millii* (Dewir *et al.*, 2006a), Dendrobium (Wang *et al.*, 2006) and *Saposhnikovia divaricata* (Qiao *et al.*, 2008). It has been shown that flower-inducing or flower-inhibiting effect of paclobutrazol depends on its concentration (Dewir *et al.*, 2006a).

In vitro plant propagation methods have been developed for some *Gentiana* species (Ivana *et al.*, 1997; Lamproye *et al.*, 1987; Morgan *et al.*, 1997; Sharma *et al.*, 1993; Viola and Franz, 1989; Yamada *et al.*, 1991). Previous report on micropropagation of *Gentiana triflora* investigated the influences of several factors including the position of the explant, requirements for sucrose, cytokinin or GA_3, variations of pH and photosynthetic photon flux density (PPFD) on *in vitro* shoot development (Zhang and Leung, 2002). Bioreactor culture is a promising and efficient method for plant propagation. As compared to conventional tissue culture techniques using solid or semi-solid medium, bioreactors require fewer culture vessels, less labour, utilities and space. The aim of bioreactor application is to achieve either maximum yield, high quality of propagules, or to keep the production costs as low as possible by integrating automated facilities and simple low cost devices. The use of the bioreactor for micropropagation was first reported in 1981 for begonia propagation (Takayama and Misawa, 1981). Since then it has proved applicable to many species including shoot, bulbs, microtubers, corms and somatic embryos (Paek *et al.*, 2005).

In the present study, we investigated the effects of paclobutrazol (PBZ) as a growth retardant on flower induction of Gentian. We also applied the bioreactor culture system and microponics for the mass propagation of this important ornamental plant.

Materials and methods

Plant material: *In vitro* shoots of Gentian (*Gentiana triflora* Pall. var. axillariflora Akita Blue) were multiplied on MS (Murashige and Skoog, 1962) semisolid medium (30 g L^{-1}sucrose + 8.0 g L^{-1} agar supplemented with 1.0 mg L^{-1}BA) at 25 °C under a 16 h photoperiod (35 µmol m^{-2} s^{-1} PPFD). These cultures were sub-cultured and maintained on the same medium for one year. For the current experiments, shoots were individually separated and cultured on MS medium without growth regulators for 2 weeks and these shoots (3-4 cm in length at the 5-7 leaf-stage) were used as a plant material.

***In vitro* flowering in solid culture**: Young shoots of Gentian were cultured on MS medium supplemented with 3% (w/v) sucrose and different concentrations of PBZ at 0, 0.5, 1.0, 2.0, 4.0 and 6.0 mg L^{-1}. All media were solidified with 0.8% (w/v) Sigma agar-agar. The pH of the medium was adjusted to 5.7 (using Beckman 340 pH/Temp. Meter, USA) before autoclaving at 121°C (1.2 kg cm^{-2})

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for 30 min. Each treatment contained five replicates and each replicate was represented by a cylindrical culture vessel (120 mL capacity) containing 25 mL MS medium with 4 shoots rendering a group of 20 shoots per treatment. MS medium supplemented with only 30 g L^{-1}sucrose was used as a control (non-flowering medium). The cultures were incubated under a 16 h photoperiod (35 µmol m^{-2} s^{-1} PPFD from cool-white fluorescent lamps) at 25 ± 2 °C. Observations on flowering, opened flowers, days to first flowering, number of flowers per explants, flower length and diameter, number of shoots per explants, shoot length and fresh and dry weights were recorded after 12 weeks of culture.

Shoot multiplication in solid/bioreactor culture: For shoot multiplication, double strength WPM medium (Lloyd and McCown, 1980) was supplemented with 0.5 mg L^{-1}BA and 30 g L^{-1}sucrose as optimal conditions (Zhang and Leung, 2002). For solid culture, Gentian shoots (4 per culture bottle) were inoculated to cylindrical culture bottle (500 mL capacity) containing 150 mL double strength WPM medium supplemented with 0.5 mg L^{-1}BA, 3% sucrose and solidified with 0.8% (w/v) Sigma agar-agar. The experiment contained fifteen replicates and each replicate was represented by a cylindrical culture bottle with 4 shoots rendering a group of 60 shoots. The pH of the medium was adjusted to 5.7 (using Beckman 340 pH/ Temp. Meter, USA) before autoclaving at 121 °C (1.2 kg cm^{-2}) for 30 min. The cultures were incubated under a 16 h photoperiod (40 µmol m^{-2} s^{-1} PPFD from cool-white fluorescent lamps) at 25 ± 2 °C. For bioreactor culture, a continuous immersion bioreactor culture system was used. A supporting net was used to hold the plant material in order to avoid the complete submersion of explants in the liquid medium. The volume of input air was adjusted to 0.1 vvm (volume per volume per min) using air-flow meter. Sixty shoots were transferred to 5 L balloon-type bubble bioreactor (BTBB) with 2 L of double strength WPM liquid medium supplemented with 0.5 mg L^{-1}BA and 3% sucrose. The layout of BTBB is shown in Fig. 1. The bioreactors were incubated under the same culture conditions as solid culture. After 6 weeks of culture, 20 explants were randomly selected and observation on the number of shoots, shoot height and fresh weight were recorded. Dry weight was measured after drying the shoots for 48 h at 60°C. The remaining shoot clumps from bioreactor culture were served as plant material for rooting experiments.

Microponics culture: Shoots (3-5 cm) at the 6-8 leaf stage, after being harvested from the bioreactor, were transplanted into plug trays {3.3 cm × 4.1 cm; 20 cells per tray (15.2 cm × 22.5 cm)} filled with a mixture of perlite and vermiculite (1:1, v/v). Cultured trays were placed in an individual container to supply the plants with nutrient solution. The nutrient solution contained half strength of MS (Murashige and Skoog, 1962) basal salts. The pH and the EC of the nutrient solution were adjusted to 5.8 and 1.2 dS m^{-1} using a pH and EC controller (HM-20E, and CM-20E, TOA, Tokyo, Japan). The nutrient solutions were drained off weekly and replaced with fresh solutions. Air was supplied through air pump to the nutrient solution at 0 and 0.1 vvm (volume per volume per min) using air-flow meter. Each individual container was covered with a clear plastic lid for the first 10 days thereafter the lid was removed. The environments in the growth chamber were adjusted to a 25 ± 2 °C air temperature, 40-50% relative humidity and a 100 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) with a 16 h photoperiod using cool-white fluorescent lamps. Observation on the rooting percentage, number of roots, shoot height and fresh weight were recorded after 4 weeks of culture and dry weight was measured after drying the shoots for 48 h at 60°C.

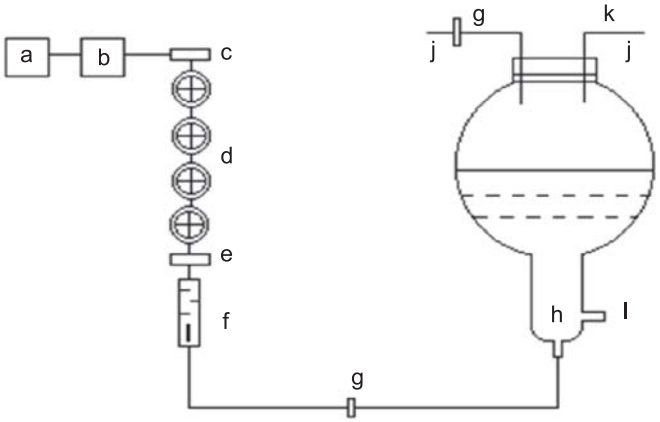

Fig. 1. Schematic diagram of an airlift bioreactor. a: air compressor, b: air reservoir, c: air cooling device, d: air filter system, e: air dryer, f: air flow meter, g: membrane filter, h: glass sparger, i: medium feeding port, j: vent, k: pre filter.

Statistical analysis: Each experiment was carried out under a completely randomized design with three replications and repeated twice. The data were subjected to ANOVA, LSD using SAS program (Version 6.12, SAS Institute Inc., Cary, USA) and Student's unpaired *t*-test, and the treatment mean values were compared at $P \leq 0.05 - 0.001$.

Results and discussion

Effects of PBZ on flowering and growth: PBZ had a florigenic effect on the apical meristems of Gentian plantlets in tissue culture (Fig. 2 A, B). Table 1 shows that PBZ concentration at 1.0 mg L^{-1}induced the highest flowering in terms of flowering percentage (91.5%), number of flowers, days to first flowering, flower length

Table 1. Effect of paclobutrazol on flower bud formation and vegetative growth of Gentian after 12 weeks in agar-solidified culture

PBZ (mg L^{-1})	Flowering (%)	Opened flowers %	No. of Flowers/ explant	Days to first flowering	Flower length (cm)	Flower diameter (cm^2)	No. of shoots/ explant	Shoot length (cm)	Fresh weight (mg)/ explant	Dry weight (mg)/ explant
0.0	58.3 ± 1.51	20.8 ± 1.85	1.0 ± 0.00	56 ± 0.34	1.7 ± 0.08	1.0 ± 0.02	3.6 ± 0.14	11.9 ± 0.46	1734 ± 123	284 ± 17
0.5	74.5 ± 2.52	26.5 ± 1.26	1.8 ± 0.24	37 ± 0.67	3.8 ± 0.22	1.1 ± 0.07	2.7 ± 0.18	7.5 ± 0.09	754 ± 44	128 ± 5
1.0	91.5 ± 1.49	91.5 ± 1.49	1.5 ± 0.14	28 ± 1.00	5.0 ± 0.20	2.0 ± 0.14	1.6 ± 0.08	6.7 ± 0.14	968 ± 14	155 ± 2
2.0	91.5 ± 1.49	33.2 ± 1.02	1.0 ± 0.00	35 ± 1.30	3.5 ± 0.20	1.2 ±0.03	1.4 ± 0.11	5.6 ± 0.19	746 ± 42	110 ± 5
4.0	33.0 ± 2.04	0.0 ± 0.00	1.0 ± 0.00	42 ± 0.67	1.7 ± 0.12	0.0 ± 0.00	1.0 ± 0.00	4.0 ± 0.12	446 ± 30	69 ± 2
6.0	8.3 ± 1.41	0.0 ± 0.00	0.3 ± 0.13	63 ± 2.00	0.6 ± 0.28	0.0 ± 0.00	1.0 ± 0.00	2.9 ± 0.09	282 ± 11	53 ± 3
LSD	6.83	5.99	0.60	4.79	0.97	0.33	0.89	1.12	336	46
Significance	**	**	**	***	***	***	***	***	***	***

Data presented are means ± SE. ** and *** = significantly different at $P \leq 0.01$ and $P \leq 0.001$, respectively

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Table 2. Effect of culture type on shoot multiplication and vegetative growth of Gentian after 6 weeks in culture

Shoot multiplication and growth characteristics	Culture type	
	Solid culture	Bioreactor culture
Number of shoot/ explant	5.1 ± 0.28	29.9 ± 1.79***
Length of main shoot (cm)/explant	3.3 ± 0.05	4.5 ± 0.09**
Fresh weight (g) / explant	0.523 ± 0.04	2.528 ± 0.12***
Dry weight (g) / explants	0.055 ± 0.01	0.282 ± 0.01***

Data presented are means ± SE. ** and *** = significantly different at $P \leq 0.01$ and $P \leq 0.001$, respectively, according to Student's unpaired *t*-test.

Table 3. Effect of air supply on rooting and vegetative growth of Gentian after 6 weeks in microponics culture

Rooting and growth characteristics	Air supply (vvm)	
	0.0	0.1
Rooting (%)	100 ± 0.00	88 ± 0.94**
Number of root/ plantlet	24.3 ± 0.93	9.3 ± 0.38 **
Length of main root (cm) / plantlet	1.25 ± 0.05	0.95 ± 0.03 NS
Length of main shoot (cm) / plantlet	4.9 ± 0.16	3.9 ± 0.17 NS
Fresh weight (g) / plantlet	1.523 ± 0.07	0.437 ± 0.06**
Dry weight (g) / plantlet	0.520 ± 0.04	0.103 ± 0.01**

Data presented are means ± SE. NS= non significant and ** = significantly different at $P \leq 0.01$ according to Student's unpaired *t*-test.

and flower diameter. The percentage of opened flowers and flower size were influenced by PBZ concentration. PBZ at 1.0 mg L^{-1} was optimal for flowering since the highest percentage of opened flowers (91.5%) was obtained at this concerntations. Higher concentrations of PBZ (>2.0 mg L^{-1}) reduced flowering and were associated with abnormal growth. The transition to flowering was earlier and faster in PBZ treated plantlets. However, there was a trend towards decreasing shoot length and fresh and dry weight with increasing PBZ concentration. Plantlets grown in PBZ free medium formed flower buds (58.3%) but large number of these flowers failed to open (Fig. 2 C). The above results indicate that Gentian plantlets were able to form flower buds without PBZ treatment. This indicates that PBZ does not trigger flowering but it rather stimulate flowering and its role seems to be additive but not essential for flowering. It is well established that during the change from the vegetative to the flowering state, the growth correlations within the apical meristem of a shoot are changed, which leads to loss of apical dominance (Pidkowich *et al.*, 1999). It is also reported that the mature state of the plantlets is usually a prerequisite for *in vitro* flowering and juvenility does not allow for flower production due to inability to produce a flowering factor or the inability of active meristems to respond to a flowering factor (Hackett, 1985). Very few studies have been conducted to investigate the effects of PBZ on *in vitro* flowering. PBZ induced *in vitro* flowering of *Euphorbia millii* and the response of flowering depended on the concentration of PBZ in the culture medium (Dewir *et al.*, 2006). PBZ also promoted *in vitro* flowering in *Dendrobium* (Wang *et al.*, 2006) and in *Cymbidium* (Zheng and Pang, 2006). In *Saposhnikovia divaricata*, low concentration of PBZ or ethephon promoted *in vitro* flowering of up to 26 or 21%, respectively (Qiao *et al.*, 2008). On the other hand, it has been reported that application of PBZ with N^6-benzyladenine (BA) totally abolished the promotive effect of cytokinin in flower induction of *Cymbidium niveo-marginatum* orchid *in vitro* (Kostenyuk *et al.*, 1999). PBZ, a widely used plant growth retardant in potted ornamental crops, provides height control in several floriculture crops (Prusakova *et al.*, 2004). *In vitro* studies showed that PBZ which prevents gibberellin biosynthesis, stimulate production of reproductive part at the expense of vegetative plant growth.

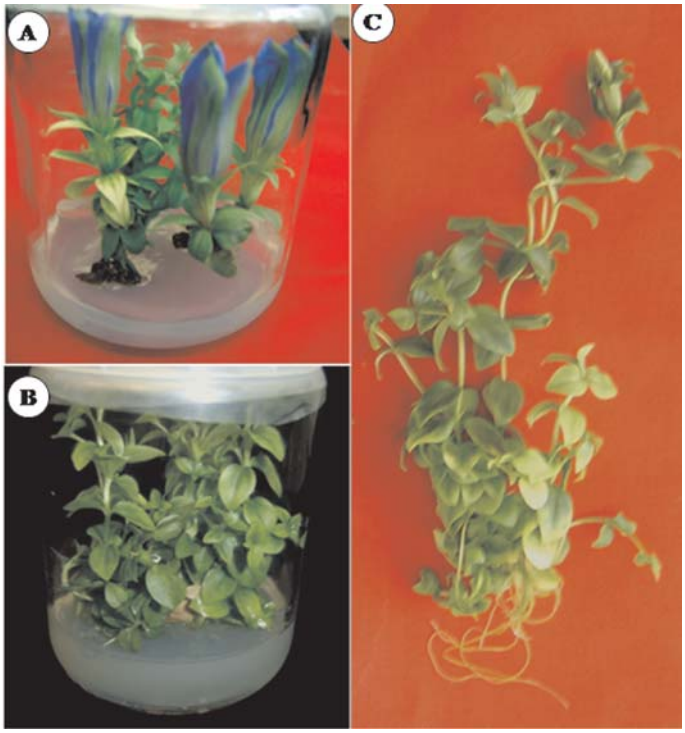

Fig. 2. *In vitro* flowering of Gentian plantlets. A and B) Flowering and non-flowering plantlets after 12 weeks' culture. C) Plantlets forming flower buds but failed to open after 12 week's culture.

Shoot multiplication and growth in solid/bioreactor culture: Comparative studies between solid and bioreactor culture (continuous immersion with net) revealed that shoot multiplication was more efficient in bioreactor culture (Table 2). Number of shoots, length of main shoot and fresh and dry weight significantly increased in bioreactor culture. The highest shoot

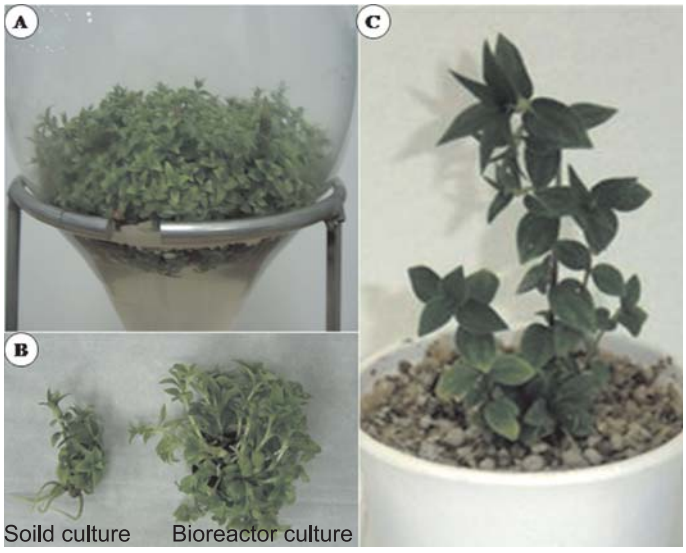

Fig. 3. Shoot multiplication and acclimatization of Gentian plantlets. A) Shoot multiplication after 6 weeks' culture in an air-lift bioreactor. B) Shoot clump developed in solid and bioreactor cultures. C) Acclimatized plantlet.

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number per explant (29.9) was obtained in bioreactor indicating that bioreactor culture clearly stimulated shoot proliferation of Gentian (Fig. 3 A, B). In the case of immersion system with net, the basal area of shoots was continuously in contact with the medium, thus enabling a constant supply of nutrients as well as constant aeration to explants, leading to plantlet growth. Micropropagation by axillary or shoot meristem proliferation is typically labour-intensive methods of producing elite clones, but recently the adaptation of air-lift BTBB immersion bioreactors for propagation of shoots and bud-clusters has provided a workable technique for large-scale transplant production. Large-scale propagation using bioreactors has been applied to several horticultural and medicinal plants (Dewir *et al.*, 2005b, 2006b; Paek *et al.*, 2005; Jo *et al.*, 2008)

Rooting and acclimatization in microponics: After harvest from the bioreactor, shoots (3-5 cm) at the 6-8 leaf stage were microponically cultured for 30 d and 100% of plantlets rooted and acclimatized successfully (Fig. 3C). Plantlets grown in microponics without air supply had the highest rooting percentage, root number, and fresh and dry weights (Table 3). Air supplement in microponics usually improves rooting providing suitable aeration to the root system. However, in the present study, air supplement had a deleterious effect on Gentian rooting. The use of perlite and vermiculite as rooting substrate provided suitable aeration and more air supplement was unnecessary. Recently, because of the small dimensions of cultivated plants, a microponic culture system combining micropropagation with hydroponics has been applied to a number of horticultural plants (Chakrabarty *et al.*, 2003; Dewir *et al.*, 2005a, 2005b). The enhanced plantlet growth, rooting and *ex vitro* acclimatization rates prove that microponic culturing is a feasible system for efficient transition from *in vitro* to *ex vitro* acclimatization.

The immersion-type bioreactor (with net) system is a valuable option for Gentian micropropagation. Rapid and efficient multiplication rate, *ex vitro* rooting in microponics and successful transfer of plantlets to the greenhouse makes this protocol suitable for large-scale propagation of this important floricultural crop.

Acknowledgments

The University of KwaZulu-Natal and the National Research Foundation (NRF) are thanked for financial support.

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Journal of Applied Horticulture, 12(1): 35-41, January-June, 2010

Diversity and efficiency of wild pollinators of watermelon (*Citrullus lanatus* (Thunb.) Mansf.) at Yatta (Kenya)

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Abstract

Citrullus lanatus (Thunb.) Mansf. (Watermelon) is an important crop plant in Kenya. Being monoecious, watermelon is entirely dependent upon pollination services usually by insects for production. Although the centre of origin for this plant is thought to be tropical Africa, essentially not much has been studied of its pollination requirements in this region. The current study investigated the identity of the wild pollinators of watermelon, their behaviour and relative pollination efficiencies at Yatta, a farm near Thika (Eastern Province). The main pollinator for this crop was found to be the honey bee, *Apis mellifera* but three wild species of *Lasioglossum* were found as important pollinators. These wild bees have a significantly higher ($P< 0.0001$) pollen deposition on stigmas of watermelon than honeybees. One of the *Lasioglossum* (*Ctenonomia*) sp. 4 deposited on average three times as much pollen as the honeybee. At about the time of stigmatic receptivity, the number of visits by this species to female flowers increases until it equals visits to male flowers irrespective of number of flowers per plot. This behavioural pattern coupled with the high pollen deposition potential makes *Lasioglossum* (*Ctenonomia*) sp. 4 a superior candidate as an alternatively managed pollinator for watermelon. Knowing that visitation occurs mostly in the morning, and that flowers last only for one day, spraying can be done in the later hours of the day when the pollinators have virtually stopped foraging on the flowers. In view of the reported pollinator decline globally, the wild pollinator species reported in this study warrant further investigation on their nesting biology and potential for domestication.

Key words: Native pollinators, watermelon, visitation patterns, pollen deposition

Introduction

The global research, conservation and agricultural communities are increasingly recognising that pollinator diversity and effectiveness is critical to the health of agricultural ecosystems. The contribution made to plants' reproductive success by pollination is determined in part by the pollinators foraging behaviour. Many insect visitors forage on the floral resources presented to them by the plant but only a few are good pollinators. Information on actual or potential role of individual pollinator species is necessary to compare the potential value of different pollinator species as candidates for introduction as managed pollinators (Corbet *et al.*, 1991). The most efficient pollinator will carry and deposit plenty of pollen on stigmas as it moves from flower to flower (Kearns and Inuoye, 1993).

The consequences of pollinators visiting a plant can be expressed by either direct or indirect measure of pollinator effectiveness. The indirect methods include visitation rates and time of visitation while pollen load deposition by a single visit to virgin stigmas is a direct measure among others (Spears, 1983; Inouye and Pyke, 1988; Stubbs and Drummond, 1999; Dag and Kammer, 2001).

Pollinator effectiveness will of course be related to the timing of visits. If an insect arrives at a flower to take nectar before pollen is presented, or forages after pollen is shed or stigmas have ceased to be receptive, they will not be contributing to reproductive success.

Thus for a pollinator dependent plant such as the watermelon, the temporal pattern of pollinator visitation becomes significant in determining important or effective pollinators. Although the visitation patterns of honeybee on watermelon have been studied (Njoroge *et al.*, 2004; Gikungu, 2006; Kasina, 2007; Karanja, 2010), it is important to compare these visitation rates with those of other important indigenous pollinators as an indicator of relative importance.

For crops that are dependent on insect vectors such as pollinators, managed honey bee colonies are often used to provide pollination services in developed countries or those with a strong apiculture development. In developing countries such as Kenya, however, where most horticultural crops, until recently, have been grown in biodiverse smallholder systems, there is no tradition of providing pollination services, and pollination deficits have not been recognised as a constraint in productivity. This changing scenerio in horticultural sector is intensifying and expanding, resulting in greater uniformity in crop fields, greater fragmentation of natural habitats, and greater use of agrochemicals. The challenge, for agroecosystems under development in Kenya, is to identify means of conserving and managing natural pollination services, as a much needed basis for pollination needs. Supplementation by managed honeybee populations may effectively be combined with the natural system, with both providing an effective insurance against risks of disease, agricultural chemicals, and inclement weather impacts on diverse pollinator species.

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Tropical Africa is endowed with great diversity of both fauna and flora. It has been estimated that East Africa has about 240 species of bees in 7 families but the diversity and taxonomy is poorly known (Gikungu, 2002). *Ctenonomia*, for example is a major group of *Lasioglossium* bees in Africa. Although the group has many species, it is poorly known in Africa (Mischener, 2000). Systematic characterisation of bees acting as pollinators of plants critical for human livelihoods and ecological sustainability is not only relevant but urgent.

In Kenya, studies in pollination biology have been focused mainly on the ecology and taxonomy of the honeybees (Kigatiira, 1974, 1976, 1984; Mbaya, 1981; Kahenya and Gathuru, 1985; Ng'ethe, 1985). Recently some studies have been done on pollination biology targeting both natural and agroecosystems in Kenya (Gemmill and Ochieng, 1999; Gikungu, 2002; Siro, 2004; Morimoto *et al.*, 2004; Njoroge *et al.,* 2004; Gikungu, 2006; Kasina, 2007; Karanja, 2010). There is need to document more extensively important plant-pollinator interactions so that conservation measures can be put in place.

The importance of pollination in watermelon: It is believed that *Citrullus lanatus* (watermelon) is native of tropical Africa (Cobley, 1965; Masefield *et al.,* 1969; Kirkbrid, 1993). Its cultivation began in ancient Egypt and Sudan then spread from there to other countries via the Mediterranean, Near East and Asia. From immuno-chemical data the crop is thought to have originated from its semi cultivated variety *Cordophanus* found in Sudan (Fursa and Gavrilyuk, 1990). The long association of *Citrullus,* and many other cucurbits crops with human settlements has indicated that there is no clear demarcation between wild relatives and crop varieties (Heiser, 1969).

Watermelon is now cultivated in many parts of the tropics (Cobley, 1965; Masefield *et al.,* 1969). The fruit is an important source of vitamins, minerals, carotenes and proteins. It is also a rich source of natural lycopene, a carotenoid of great importance because of its antioxidant property (Denton, 2004). Highly prized oil is now being extracted from seeds of watermelon. This oil is used for cooking, cosmetic purposes and is of importance in pharmaceutical industry (Denton, 2004).

Global production of watermelon has increased from 30 million tons (2.1 million ha.) in 1992 to 81 million tons (3.2 million ha.) in 2002 (Denton, 2004). In Kenya, the growing of watermelon is increasing, especially in the arid and semi arid areas under irrigation.

This study aimed at investigating the time of the day when watermelon has most visitations by the main pollinators so as to furnish farmers and pollination managers with data that can be applied in integrated pest management for pollinator safety and conservation. Daily visitation patterns are also useful in assessing the effective pollinators for a given plant and region. Further, an investigation was sought to establish the full cast of pollinators of watermelon at Yatta (Eastern Province, Kenya) and the efficacies of the four main pollinators with regard to pollen deposition on virgin stigmas.

Materials and methods

Growing conditions of the crop: Plants were sown in nursery beds at Yatta National Youth Service farm near Thika and transplanted on ridges with planting distance of 3 × 2m. A total of 1000 plants were sown in early December so that flowering and fruiting could coincide with the dry months of January and February. The plants were top-dressed with calcium ammonium nitrate (C.A.N.) fertiliser when the plants began to spread and again just before flowering (IRACC, 2000). Drip irrigation was used every second day.

Observation on anthesis: The flowers were observed to indicate time of opening and closing for both male and female flowers. To estimate the approximate time of the day when anthers are dehisced, the anthers were stroked gently with a small paintbrush. At every stroke the brush was examined for the presence of any pollen grains adhering on it. From 6.30 a.m. the anthers were stroked every 30 minutes until some pollen grains were observed on the paintbrush. At the same time the stigmas were observed for receptivity every 30 minutes until a glassy stigmatic secretion was observed (Owens, 1992).

Diversity in insect visitors: In documentation of flower visitors special attention was paid to discriminate between pollinators and mere floral visitors. Those insects that evidently carried watermelon pollen and had contacts with anthers and stigmas were recorded as pollinators. Representatives of pollinators were captured and killed in a jar using ethyl acetate fumes. Voucher specimens were prepared, insects identified and then preserved in insect boxes and deposited at Jomo Kenyatta University, Botany Department Laboratories.

Pollinator behaviour: Initially observations for any visitors to the flowers began at 6.30 a.m. but it was noted that no visitor arrived before 7.30 a.m. Consequently, recording of insect foraging behaviour and visitation patterns began at about 7.30 a.m. and ended at about 4.00 p.m. when all the flowers closed. Observations were standardized by counting the number of flowers on 1 m^2 plots of the flowering crop and the number and types of pollinators coming to the flowers recorded over 10-minute periods of time (Inouye and Pyke, 1988; Motten, 1986; Wolf *et al.,* 1999).

Measurement of pollinator efficiency: The number of pollen grains deposited on the stigma by a single pollinator visit is usually used to estimate relative pollinator efficiency (Yeboah and Woodell, 1987). Female flowers were bagged while in bud stage to prevent any contact of stigmas with pollinators. When the flowers were open, receptive stigmas were held in a long rod and presented to the pollinators. The pollinator was followed to ensure it has consistently visited male watermelon flowers before the virgin female flowers were presented (Kearns and Inouye, 1993; Stanghellini *et al.,* 2002). When the targeted pollinator made one visit, the stigmas were picked and crushed on a microscope slide and kept in a cool box in the field. The crushed stigmas were then observed in the laboratory under a microscope and the number of pollen grains deposited counted. Ten stigmas were presented to each of the four main pollinators of watermelon. The mean number of pollen grains deposited was calculated and compared between the four pollinators by t-test. Genstart software was used for all the data analysis.

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Results and discussion

Anthesis in watermelon: The male flowers began to open at about 7.15 a.m. and began to close at 1 p.m. On the other hand the female flowers began to open at about 7.20 a.m. and closed much earlier at about 12 p.m. Sometimes when the temperatures were high some female flowers begin to close at 11.30 a.m. The anthers dehisced from 8.30 a.m. while the stigmas became receptive from 10.30 a.m.

Pollinator diversity in watermelon: The main pollinator of watermelon in this region was found to be the honeybee (*Apis mellifera* L.). Three wild bee species, *Lasioglossum* (*Ctenonomia*) *scobe, Lasioglossum* (*Ctenonomia*) sp. 4 and *Lasioglossum* (*Ipomalictus*) *nairobiense* were observed to be regular pollinators of watermelon though not as regular as the honeybee. The first two, make use of ground conditions below the watermelon crop to nest.

Other insects however, were also flower visitors of this crop. The following may not be important pollinators of watermelon in the study area but benefited by obtaining nectar and/or pollen: *Danaus chrysippus, Eurema brigitta, Neocoenyra gregorii, Junonia hierta, Aphthona marshalii, Leptaulaca fissicollis, Xylocopa lataritia, Anthophora bipartite, Braunsapis* sp., *Nomia* sp. 1, *Macrogalea* sp. 1, *Trinomia cirrita, Lipotriches hylaeoides, Chrysomya* sp., *Cosmina* sp. *and Phytomia* sp.

Although previous studies on watermelon in Kenya (Njoroge *et al.*, 2004) and in other regions (Rao and Suryanarayana, 1988; Sanford, 1992; Hodges and Baxendale, 1995; Stanghellini *et al.*, 1998) have shown that honey bee is the main pollinator of watermelon, this study however, identified three wild *Lasioglossium* species, which have not been reported before, as pollinators of watermelon. In the past, most efforts to conserve and sustainably use pollinators for agricultural production have been focused on the honeybee (Cane, 1997). Wild pollinators however are an important reservoir of pollination capacity with potential for application in agricultural production. The wild bees identified in this study are a valuable reservoir, which can be considered for management in improving watermelon production in the area.

Daily pollinator visitation patterns in watermelon: The daily visitation patterns of the three most common bee species that evidently had contact with anthers and stigmas of watermelon and were seen to have substantial pollen as they left the male flowers (*Lasioglossum* (*Ctenonomia*) *scobe, Ctenonomia* sp. 4 and *Lasioglossum* (*Ipomalictus*) *nairobiense*) were compared with honey bee patterns (Fig. 1a-d). The honeybee was the first visitor at about 7.30 a.m. followed by *Lasioglossum* (*Ctenonomia*) *scobe and Ctenonomia* sp. 4 at about 7.40 a.m. The *Lasioglossum* (*Ipomalictus*) *nairobiense* arrived only after 8.30 a.m., This visitor was rather more sporadic than the others and seemed to avoid the female flowers but had evident watermelon pollen on its body. All the four species made more visits to male flowers than female flowers, except *Ctenonomia* sp. 4 (Fig. 1c), which at about 11.30 a.m. made equal number of visits in male and female flowers irrespective of the number of flowers of each sex in the 1 m^2 plots.

In the case of honeybees, visits were few before 8.00 a.m. but there was increase in number of visits to both male and female flowers gaining a peak at about 10.40 a.m. (Fig.1a). The highest

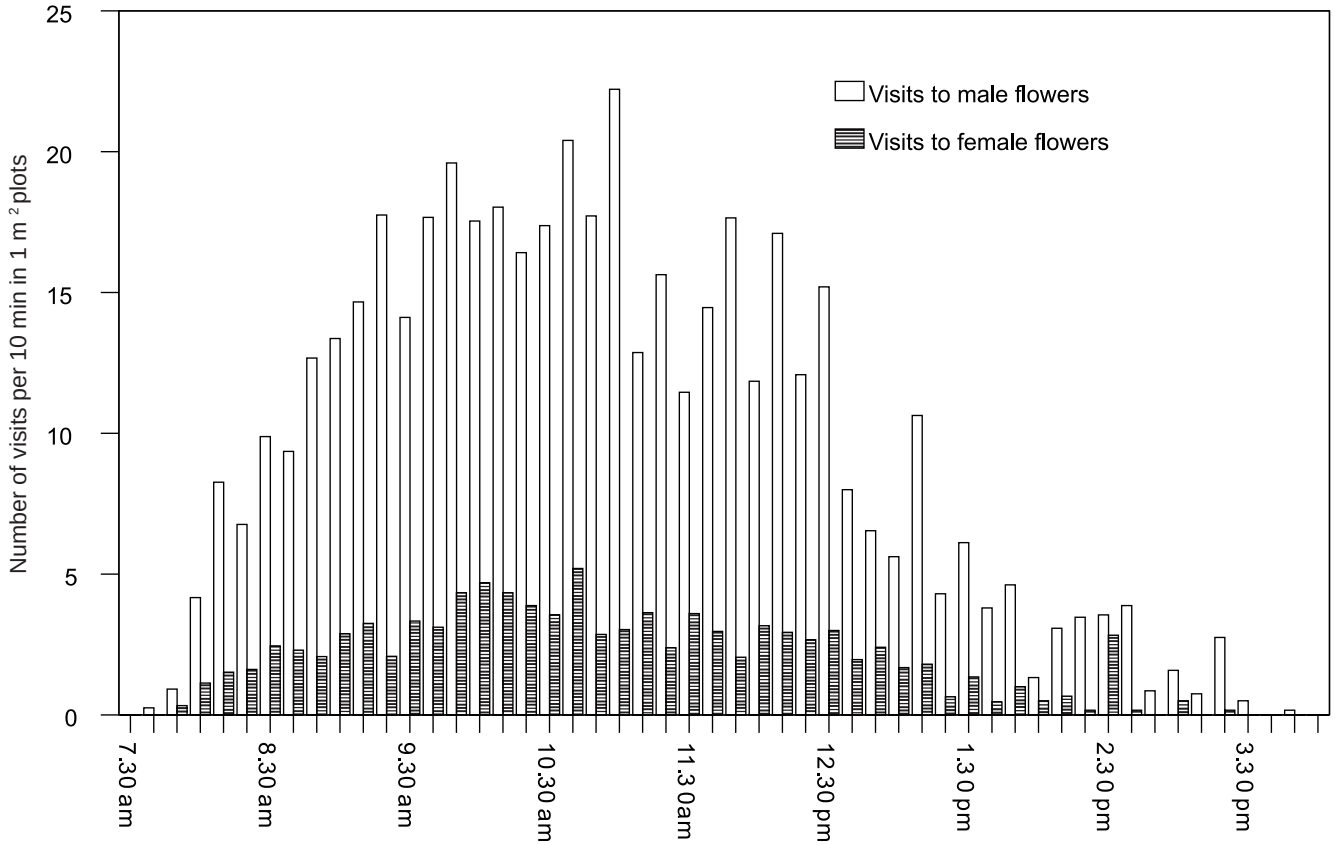

Fig. 1a. Honeybee daily visitation rates on watermelon flowers every 10 min in 1 m ^{2} plots

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mean visits to male flowers were observed at about 11.00 a.m. while in female flowers this was observed to be at about 10.40 a.m. After 12 p.m. the visits decreased gradually but more so in female flowers as most of them began to close.

Lasioglossum (*Ctenonomia*) *scobe* began appearing on watermelon flowers at about 7.40 a.m. (Fig.1b) visiting male flowers only up to 8.30 a.m. when it began to visit female flowers. This pollinator visited the male flowers up to 12 p.m. but the female flowers up to 11.30 a.m. Most visits to female flowers were made between 10.00 a.m. and 10.40 a.m., a time when the female flowers had become receptive.

Ctenonomia sp. 4 began foraging on watermelon male flowers at about 7.50 a.m. with maximum visitation occurring at about 8.30 a.m. (Fig.1c). This species foraged on female flowers between

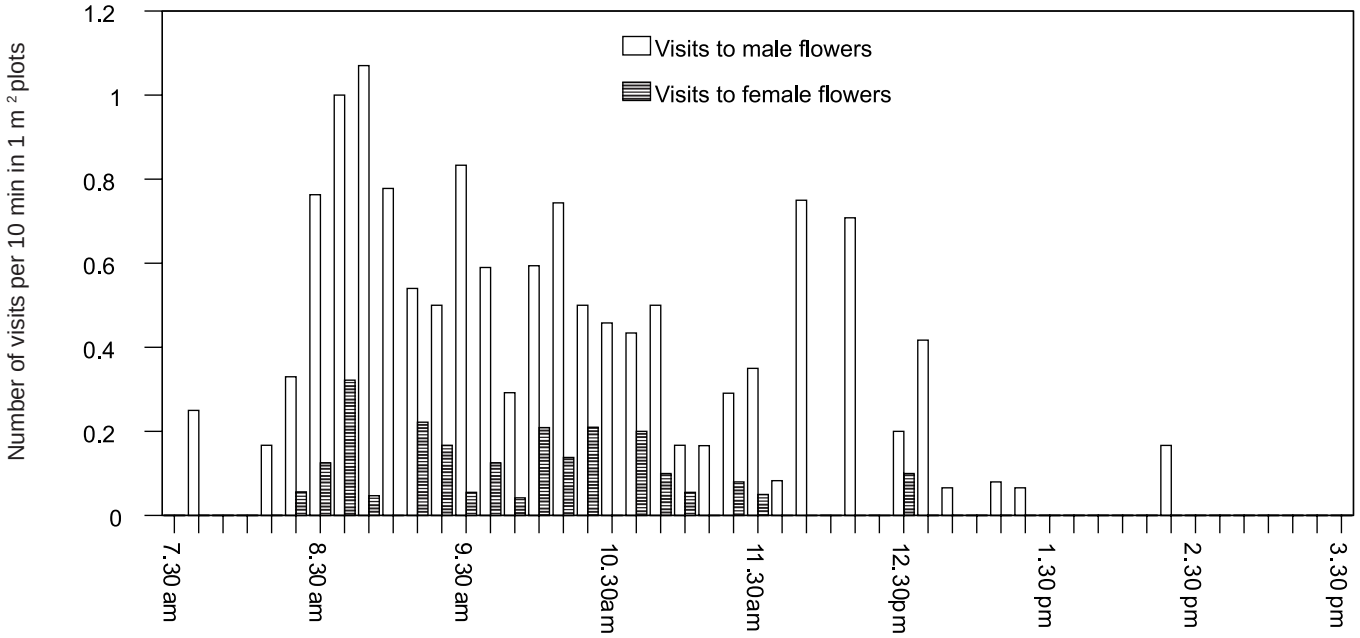

Fig. 1b. *Lasioglossium* (*Ctenonomia*) *scobe* daily visitation rates on watermelon flowers every 10 min in 1 m ^{2} plots

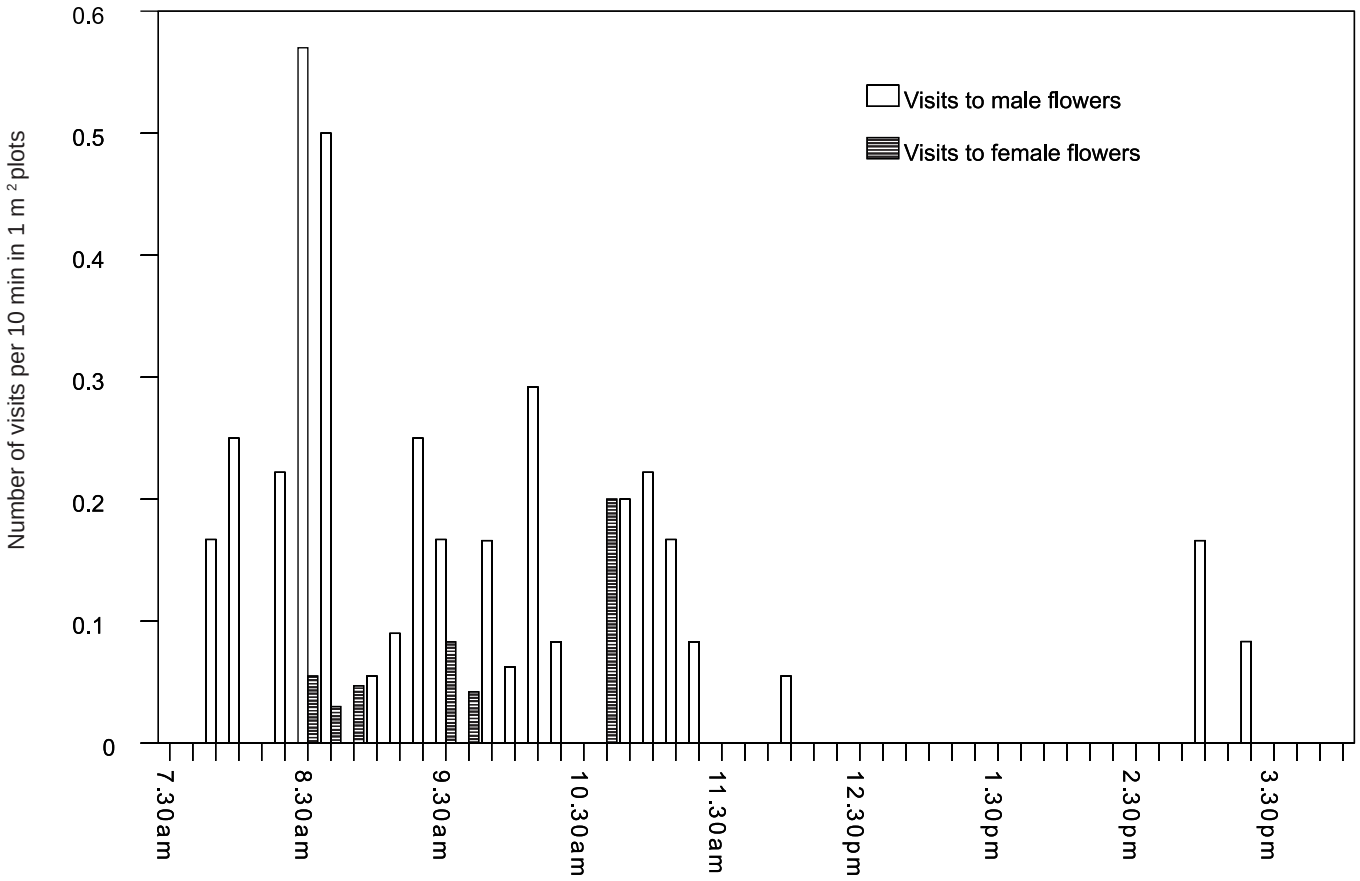

Fig. 1c. *Lasioglossium* sp. (*Ctenonomia*) sp. 4 diurnal visitation rates on watermelon flowers every 10 min in 1 m ^{2} plots

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8.30 a.m. and 10.40 a.m. Maximum visitations to female flowers occured at 10.40 a.m. when the female flowers were receptive. *Lasioglossum* (*Ipomalictus*) *nairobiense* appeared on the crop at about 8.40 a.m. (Fig. 1d). It regularly visited male flowers between 8.40 a.m. and 9.20 a.m., then again at about 12.00 p.m. and did not come back to the crop for the rest of the day.

It was observed that only a few honeybees, *Lasioglossum* (*Ctenonomia*) *scobe* and *Ctenonomia* sp. 4 made occasional appearances after 1.00 p.m mostly to male flowers. No pollination would be possible after this time as the female flowers began to close at about 12.00 p.m. Where bees visits were observed in female flowers after 12.00 p.m., they were forcing their way into already closing female flowers.

Daily visitation patterns to male and female flowers are particularly useful in unisexual plants as they help establish if the main pollinators are discriminating either of the sex of flowers during foraging. The absolute number of visits to female and male flowers in this study showed that the latter were visited more than the former. But when the total visits were compared with the proportion of male and female flowers in $1m^2$ of the crop the bees tended to show a preference for female flowers. These results are contrary to those obtained in a study of the *Antennaria parvifolia* Nutt. (Asteraceae) where pollinators have been shown to increase the cost of sex by avoiding female flowers (Bierzychudek, 1987). There are several plant families where studies have shown pollinators discriminate against female flowers. These include Saliacaeae (van der Werf, 1983), Caricaceae (Baker, 1976) and Sapindaceae (Bawa, 1977) to name only a few.

Such observations have led to the general conclusion that visits to female flowers may be made only by mistake. It has therefore been predicted that discrimination against female flowers is a common phenomenon among dioecious and monoecious species. In the case of watermelon (monoecious) the three species of pollinators: honeybee, *Lasioglossum* (*Ctenonomia*) *scobe* and *Ctenonomia* sp.4 are all found to decrease the cost of sex by seeking out for female flowers in their foraging. The current study did not confirm the general prediction that pollinators of unisexual plants increase the cost of sex. Since the family Cucurbitaceae in Kenya has many unisexual species, pollinator visitation patterns should be investigated in the other species including wild species.

Pollinator daily visitation patterns have been found to have implications on the pollinators' effectiveness (Spears, 1983; Stanhellini *et al.*, 2002). In this study, the honeybee was found to be the most abundant and regular pollinator to watermelon flowers. The maximum visitation of *Lasioglossum* (*Ctenonomia*) *scobe* and *Ctenonomia* sp.4 to female flowers coincided with stigmatic receptivity while in male flowers it corresponded to time of anther dehiscence in this crop. This synchronisation makes them effective pollinators as they first make visits to male flowers, collecting loads of pollen and then make visits to female flowers facilitating dislodgement of the pollen on the already receptive stigmas.

The daily visitation patterns also find application in pest management. A primary concern for beekeepers, when involved in contracting their bees for pollination, is the use of pesticides by the growers (Sanford, 1992). From these results, it is recommended that pesticides be applied when these bees have left the flowers for example late in the evening. Since the flowers for that day will have closed and new flowers will emerge the following morning, the bees stand some chance of escaping poisoning. The fresh flowers and nectar produced in the following morning will have less direct contact with the pesticide sprayed the previous day, than the flowers sprayed in the morning when they are open. Applying

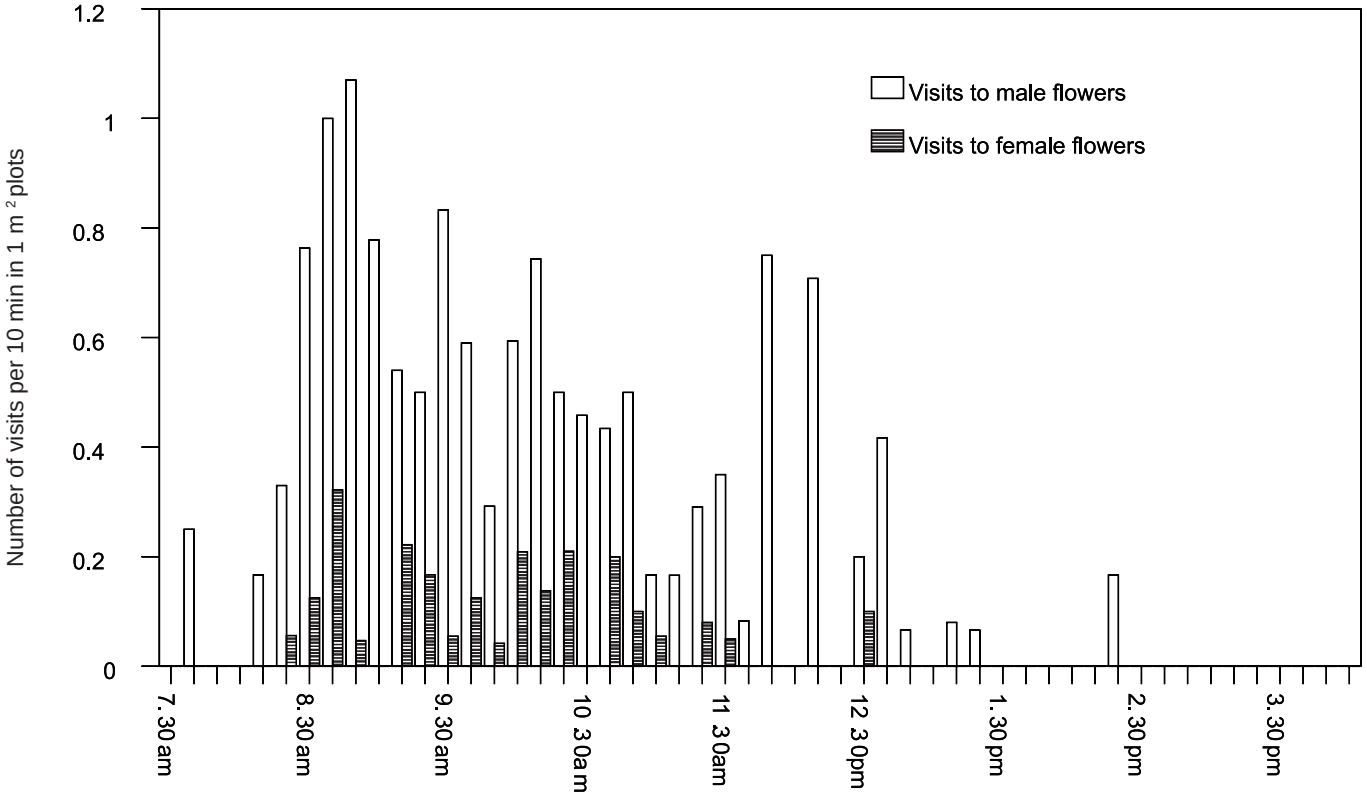

Fig. 1d. *Lasioglossum* (*Ipomalictus*) *nairobiense* diurnal visitation rates on watermelon flowers every 10 min in 1 m^2 plots

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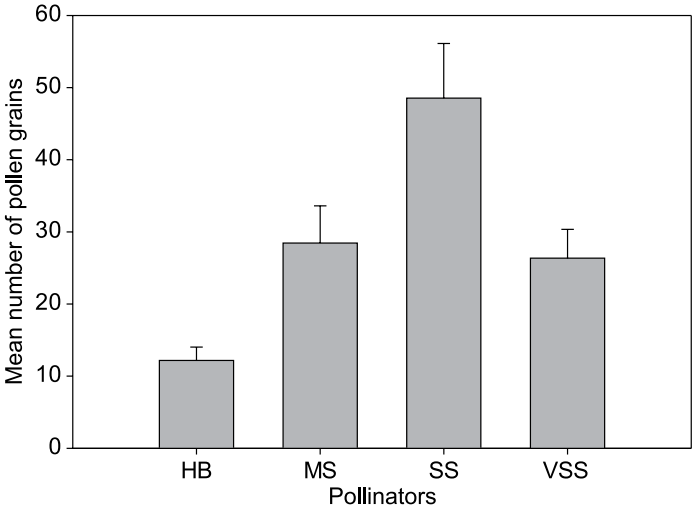

Fig. 2. Comparison of pollen deposits between honeybee and the three wild pollinators of watermelon. (HB= honeybee; MS= *Lasioglossum* (*Ipomalictus*) *nairobiense*; SS= *Lasioglossum* (*Ctenonomia*) sp 4; VSS= *Lasioglossum* (*Ctenonomia*) *scobe*.

pesticides in the latter parts of the day will also be important in ensuring pollen germination as some fungicides have been found to reduce *in vitro* pollen germination and the germ tube elongation (Abbot *et al.*, 1991).

Pollinator efficiency: Pollen load analysis revealed that the four insect species have significantly different capacity for depositing pollen ($P< 0.0001$). The highest potential for pollen deposition was found in *Ctenonomia* sp. 4 followed by *Lasioglossum* (*Ipomalictus*) *nairobiense* and *Lasioglossum* (*Ctenonomia*) *scobe* while the honeybee had the least (Fig. 2). Consequently, the three species *Lasioglossum* (*Ctenonomia*) *scobe, Ctenonomia* sp. 4 and *Lasioglossum* (*Ipomalictus*) *nairobiense* showed higher pollination efficiency than the abundant honeybee.

This study revealed for the first time in this region the pollinators for watermelon superior to honeybees. Although bees have been pollinating flowering plants for more than 100 million years, only about ten out of the available 20,000 non-parasitic bee species have been managed for agricultural pollination (Cane, 1997). Loss of feral and managed bees (particularly honeybees) has become a limiting factor in pollination of many bee-pollinated crops (Corbet *et al.*, 1991; Stanghellini *et al.*, 1998) leading to the current search in different parts of the world for alternative agricultural pollinators. The current study provides biologists and agriculturists with important watermelon wild pollinators that can be targeted for development and management as alternative pollinators considering their higher pollination efficiencies.

These results support the hypothesis that local pollinators are important in the pollination of watermelon crop that has been bred from the indigenous African *Citrullus lanatus*. Other than the popular honeybees, wild *Lasioglossium* bee species were identified to be important pollinators with a unique relationship with the crop as some of them nest in the soils where watermelon is growing. These bees also forage the male flowers soon after the anthers dehisce and the female flowers soon after they have become receptive. The indigenous pollinators of a crop are thought to be significant especially in continents where the crop's close relatives are found (Cane, 1997). Since watermelon has its origin in Tropical Africa where wild relatives are found (Cobley, 1965; Kirkbrid, 1993; Nayar and More, 1998) these indigenous pollinators and the pollination biology of wild watermelon as well as other wild cucurbits need to be studied further. Conservation status of these wild pollinators needs to be assessed so that strategies can be worked out to ensure their continued survival and hence more benefits to watermelon production and other plants in the ecosystem.

Limited information on the importance of wild pollinators and their interaction with crops in the past has been a hindrance to the development of appropriate strategies for pollinator conservation and sustainable use. This study will significantly contribute to the understanding of the interaction between watermelon and the diversity of pollinators particularly the wild pollinators. Data from this study will contribute to the local and global understanding of ecosystem service that pollinators provide. This is especially an important key since there is now an established International Pollinator Initiative (IPI), which is a centralised entity to facilitate information exchange on pollinator conservation and sustainable use.

Acknowledgement

The financial support given by the Plant Protection Research of South Africa's Agricultural Research Council, BIOTA (East Africa) and ICIPE, Nairobi is gratefully acknowledged. The authors are also grateful to Laban Njoroge who assisted in field collection of the pollinators. Our sincere appreciation to Alain Pauly for identifying the bees collected in this study.

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Journal of Applied Horticulture, 12(1): 42-45, January-June, 2010

In vitro mass propagation of Sikkim Himalayan Rhododendron (*R. dalhousiae* Hook. f.) from nodal segment

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Abstract

The first successful micropropagation protocol was developed for an important Sikkim Himalayan Rhododendron*, R. dalhousiae* Hook. f. also known as Lahare Chimal in Sikkim. *In vitro* raised shoot tip explants from *R. dalhousiae* were used to produce multiple shoots on a medium containing various concentrations of growth regulators. Among the combinations used, Murashige and Skoog (MS) medium containing 5 mg L^{-1} 2-isopentenyladenine (2iP) along with additives such as, 100 mg L^{-1} polyvinyl pyrrolidone (PVP), 100 mg L^{-1} ascorbic acid, 10 mg L^{-1} citric acid was found to be best for induction of multiple shoots within 12 weeks of culture. The combination of 5 mg L^{-1} 2iP +1 mg L^{-1} indole-3-acetic acid (IAA) resulted in further multiple shoot production than using them alone. Rooting of shoots *in vitro* was achieved on MS medium containing 0.2 mg L^{-1} indolebutyric acid (IBA). Rooted plantlets were transferred to small polythene bags containing autoclaved fresh peat moss and soil (1:3) and maintained with a high humidity for acclimation. These *in vitro*-raised plants grew normally in greenhouse and natural habitat (arboretum of the Institute) without showing any morphological variation. The protocol developed from the present study could be used for large scale multiplication of *R. dalhousiae* in a limited time.

Key words: Micropropagation, shoot formation, rooting, acclimatization, *Rhododendron dalhousiae,* Sikkim Himalaya

Introduction

Creamy white flowered Rhododendron (*R. dalhousiae* Hook. f., local Name -Lahare Chimal) is found on steep banks, rocks and trees of *Michelia* and *Quercus,* growing mostly epiphytically in association with ferns, orchids, etc. and distributed in Sikkim Himalaya at an elevation of 1500 to 2500m amsl. The genus *Rhododendron*, having about 50 species in India, is mainly distributed in the Himalayan region (Gamble, 1936; Pradhan and Lachungpa, 1990; Singh, *et al.,* 2003) and is one of the most neglected groups of Indian plants in terms of scientific research. The major threats to Rhododendrons are deforestation and unsustainable extraction for firewood and incense by local people. The regeneration status in the form of available seedlings/ saplings is very poor due to the above situation for many of the Rhododendrons (Kumar, *et al.*, 2005; Paul, *et al.*, 2005; Singh, *et al.,* 2008a).

In vitro propagation from meristematic tissues is a suitable method for production of Rhododendrons and Azaleas; their propagation by conventional methods (seeds, cuttings, etc.) is either difficult or ineffective (Almeida, *et al.,* 2005; Vejsadova, 2008; Singh *et al.,* 2008b). All the species of Sikkim Himalayan Rhododendrons have the potential of commercial value. Various reports are available on the Rhododendron research and development internationally (Rotherham, 1983; Krishna, *et al.,* 2002; Alper and Rotherham, 2006) with regards to it's growing condition, reproductive methods, taxonomy, breeding etc. (Hsia and Korban, 1977), but actual study on conservation issue of these plants in the Sikkim Himalaya is almost non existent. Therefore, considering the importance of the *Rhododendron* spp., the present experiment was initiated to study the *in vitro* response of *R. dalhousiae* and attempt was made to regenerate plants from nodal segment explants.

Materials and methods

Plant material, sterilization and culture conditions: Seeds were collected in the month of October-November from Rate-chu, East Sikkim and were washed for 30 min in running tap water followed by detergent (Tween-80; 1.0%, v/v; 10 min). Surface sterilization was performed with freshly prepared aqueous solution of mercuric chloride (0.05%; w/v; 3 min). Each treatment was followed by repeated washing (4 times) with sterile distilled water under aseptic conditions. The seeds were placed aseptically in test tubes (10 seeds per culture test tube, 25 mm dia, 15 mL medium) onto Murashige and Skoog medium (MS) (1962) containing 3% sucrose and 0.8% (w/v) agar. The medium was adjusted to pH 5.6 before autoclaving at 121°C for 15 minutes at 1.05 kg/cm^2 pressure. All the cultures were maintained at 23 ± 1 °C temperature and 80% relative humidity under 16 hr/8 hr (light/dark) photoperiod and a light intensity of 60 µmol m^{-2} s^{-1} photon flux provided by cool-white fluorescent lamps.

Selection of best medium for mass propagation: In order to induce shoot proliferation *in vitro*, following culture media were tested: Murashige and Skoog medium (MS) (1962), half-strength MS (half amount of inorganic constituents, full amounts of organic and other constituents per litre of MS medium) (½ MS), Anderson medium (AM) (1975) and Woody Plant medium (WPM) (Lloyd and McCown, 1981). The medium gelled with 0.8% Agar supplemented with 5 mg L^{-1} 2iP along with additives (100 mg L^{-1} PVP, 100 mg L^{-1} ascorbic acid and 10 mg L^{-1} citric acid) and 3% sucrose was used for multiplication of shoots.

Nodal segment (10-15 cm) were excised from six weeks old *in vitro* seedlings and directly planted onto conical flask (250 mL) containing MS medium supplemented with 2iP, Zeatin and TDZ at various concentration along with auxin as shown in tables.

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MS medium lacking growth regulators served as control. After 12 weeks of culture, percentage of initiating shoots, average number of shoots and the average length of shoots per explant was recorded. All plant growth regulators (PGRs) used were from Sigma Chem. Company, USA.

***In vitro* rooting and acclimatization**: Well developed shoots obtained from MS medium fortified with 5 mg L^{-1} 2iP and 1 mg L^{-1} IAA were transferred to MS-liquid nutrient medium containing different concentrations of IBA, NAA and IAA on filter paper bridge. After 9 weeks of culture, percentage of rooted shoots, mean number of roots per shoot and root length were recorded. Rooted plants were washed with sterile distilled water then dipped in systemic fungicides (Bavestin, 0.15% , w/v; 20 min) and then planted in plastic pots (125 mL) containing autoclaved fresh peat moss and soil (1:3). The plants were hardened under high relative humidity (80%) in the mist chamber of greenhouse (25°C) of the Institute at the Pangthang, East Sikkim (Longitude 27°4'46" to 28°7'48" North, and Latitude 88°55' to 88°55'25" East with an elevation 2087 m amsl). Well developed surviving plants were transferred to pots containing normal garden soil and maintained in greenhouse. Later, the plants were established in the field site (arboretum of the Institute).

Statistical analysis: In all experiments, each treatment consisted of 20 replicates and each experiment was repeated twice. Standard error of the mean was calculated. Least significance difference (LSD) at $P<0.05$ level was calculated following the method of Snedecor and Cochran (1967).

Results and discussion

R. dalhousiae seeds germinated within 15-20 days of inoculation on hormone-free MS medium. On MS medium containing 3% sucrose and 0.8% (w/v) agar, germination was first recorded on the 15th day of inoculation and after 30 days of inoculation an average germination of 67% was observed (Fig. 1A). The nodal segments that were obtained after six weeks old aseptic seedlings were used as the source of explants for the multiple shoot induction. The response of multiple shoots cultured on various media type, *i.e.*, MS, ½, MS, AM and WPM, each supplemented with 5 mg L^{-1} 2iP was compared. Of the four different medium tested, the frequency of multiple shoot was highest (71%) on MS medium and the lowest (18%) on WPM (Table 1). Thus MS medium was the best basal media among the four media attempted. Later, it was selected for supplementing various plant growth regulators for promoting shoot growth. Nodal segments cultured on different medium without any growth regulator did not show any response.

Nodal segment (1.0-1.5 cm in length) were placed onto MS medium containing various concentrations of 2iP, Zeatin and TDZ. The explants cultured on MS basal medium without growth regulators failed to produce shoots even after 6 weeks of culture. While comparing the effect of cytokinin type (2iP, Zeatin and TDZ) on shoot formation, the best response was achieved in MS medium supplemented with 2iP (Fig. 1B). It was found that mean number of shoots per explant was highest on media containing 2iP, and increased as the concentration of 2iP increased from 1 to 5 mg L^{-1}. Maximum percent of multiple shoots (68 %) and maximum mean number of shoots (9.0 ±0.6) were recorded at 5 mg L^{-1} 2ip after 12 weeks of culture (Table 2). Kumar *et al.* (2004) had also observed that 2iP was effective for shoot multiplication of *R. maddeni*. In the MS medium, the number of shoots induced from each nodal segment increased till 5 mg L^{-1} 2iP and decreased thereafter (Table 2). It was observed that the type and concentration of the cytokinin had a significant effect on the frequency of shoot regeneration, shoot number and shoot length as also seen in *R. maddeni* (Kumar *et al.,* 2004). When higher

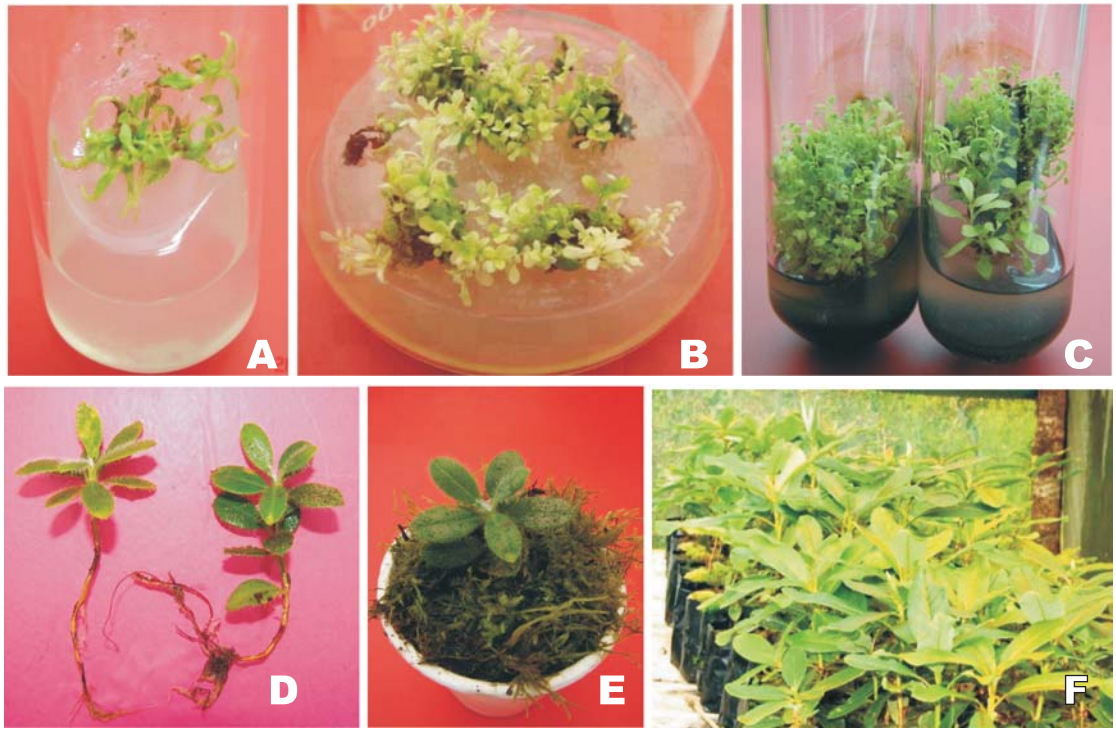

Fig. 1. *In vitro* propagation of *R. dalhousiae*. (A) Germinating seeds in MS medium. (B) Multiplication of shoots on MS medium with 2iP (5 mg L^{-1}). (C) Established shoots derived from nodal segment grown on MS medium supplemented with 2iP (5 mg L^{-1}) and IAA (1 mg L^{-1}). (D) Rooted microshoots. (E) Hardened *in vitro* raised plants after transfer to fresh peat moss and soil in green house. (F) Hardened plants in the greenhouse.

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Table 1. Effects of different media/substrates supplemented with 5 mg L^{-1} 2iP on shoot proliferation from nodal explant of *R. dalhousiae*

Media/ substrate	Percent of explant producing shoots	Mean number of shoots/ explant	Mean shoot length (cm)
MS	71 ± 2.0	8.0 ± 0.6	2.4 ± 0.2
½ MS	31 ± 2.0	3.3 ± 0.3	1.47 ± 0.2
AM	65 ± 2.2	6.3 ± 0.9	2.16 ± 0.2
WPM	18 ± 2.0	2.0 ± 0.6	0.73 ± 0.7
LSD (P=0.05)	12.7	2.11	0.23

Nodal segments cultured on different medium without growth regulators did not show any response. Values represent means ± standard error. Each treatment consisted of 20 replicates and the experiment was repeated twice. Data were recorded 12 weeks after transfer to culture medium.

Table 2. Effects of plant growth regulators on induction of multiple shoots from nodal explant of *R. dalhousiae* on MS medium

Plant growth regulators	Concentration (mg L^{-1})	Percent of explant producing shoots	Mean number of shoots/ explant	Mean shoot length (cm)
Control	0	0	0	0
2iP	1	37 ± 2.7	2.3 ± 0.7	0.6 ± 0.1
	3	58 ± 3.2	6.3 ± 0.3	1.0 ± 0.1
	5	68 ± 3.1	9.0 ± 0.6	3.4 ± 0.2
	7	63 ± 2.9	7.7± 0.3	1.8 ± 0.1
	9	53 ± 4.1	4.7 ± 1.2	0.8 ± 0.2
Zeatin	1	8 ± 1.2	3.0 ± 0.6	0.9 ± 0.1
	1.5	16 ± 1.2	4.7 ± 1.2	0.9 ± 0.1
	3	20 ± 0.9	4.0 ± 1.2	0.8 ± 0.1
	4	16 ± 0.6	4.0 ± 1.2	0.6 ± 0.1
	5	11 ± 1.8	3.3 ± 1.5	0.5 ± 0.6
TDZ	1	13 ± 2.4	2.0 ± 0.6	0.6 ± 0.1
	1.5	22 ± 1.7	4.7 ± 0.58	0.7 ± 0.1
	3	28 ± 1.2	3.7 ± 0.3	0.7 ± 0.1
	4	15 ± 2.3	4.0 ± 0.6	0.7 ± 0.1
	5	13 ± 2.5	3.3 ± 0.6	0.6 ± 0.1
LSD (P=0.05)		13.38	2.85	0.45

Nodal segments cultured on MS medium without growth regulators did not show any response. Values represent means ± standard error. Each treatment consisted of 20 replicates and the experiment was repeated twice. Data were recorded 12 weeks after transfer to MS medium.

concentration of TDZ was used, the number of shoots decreased or the treatment was less effective in inducing multiple shoots. Zeatin was found to be the least effective of all cytokinins used (Table 2) although TDZ and Zeatin have been successfully used during shoot proliferation of several *Rhododendron* species and hybrids (Fordham *et al.,* 1982; Mertens *et al.,* 1996; Almeida *et al.,* 2005). Presence of IAA in medium also proved to be beneficial for shoot elongation. Of all the treatments used individually or in combination, a medium supplemented with 5 mg L^{-1} 2iP + 1 mg L^{-1} IAA induced highest numbers of multiple shoots per explant (10.7 ± 0.6) (Table 3, Fig. 1C). Regenerated shoots were subcultured every three weeks onto the freshly prepared same medium that was subjected to produce the highest proportional shooting response. Highest number of shoot regeneration was recorded at fourth subculture. The superiority of 2iP over all growth regulators for multiple shoot induction has been reported in many rhododendron species (Meyer, 1982; Anderson, 1984; Douglas, 1984) and related species such as azalea (Fordham *et al.,* 1982).

In vitro grown individual shoots were separated from the shoots of the primary culture and placed in MS-liquid medium containing different concentrations of auxins (NAA, IAA and IBA). Rooting was induced within 3 weeks of transfer. In the presence of auxins, the shoots produced root in all three concentrations, but IBA was found more suitable for root induction than IAA and NAA. A maximum frequency of root formation (86%) and the highest number of roots (5.3 ±0.9) with maximum root length (1.6 ±0.2 cm) was achieved on MS medium with 0.2 mg L^{-1} IBA after 9 weeks (Table 4, Fig. 1D). At higher concentration (0.5 mg L^{-1} IBA), a small amount of callus formed at the base. According to Ludvig-Muller (2000) transport velocity of IBA was markedly slower as compared to other growth regulators. Higher concentration of auxin lowered the rooting percentage as well as root number. Control shoots that did not receive auxin treatment did not root. Similar observations were made in other plants (Anderson, 1978; Wong, 1981; Kumar *et al.,* 2004; Singh, 2008).

Plantlets with fully expanded leaves and well developed roots were washed with sterile distilled water then dipped with systemic fungicides (Bavistin, 0.15%, w/v; 20 min) and planted in plastic pots (125 mL) containing autoclaved fresh peat moss and soil (1:3) (Fig. 1E). After two months these were planted in polythene bag containing normal garden soil, and placed for hardening under high relative humidity (80%) in the mist chamber of a greenhouse (Fig. 1F). After 180 days of transplantation 93% of the plants were found to survive with progressive growth in the field site.

The *in vitro* regeneration of shoots, rooting and soil establishment protocol in this study suggests that there is possibility of adapting tissue culture technique for mass propagation of *R. dalhousiae.*

Table 3. Effects of cytokinin combined with IAA on shoots proliferation from nodal explant of *R. dalhousiae* on MS medium

Plant growth regulators (mg L^{-1})		Percent of explants producing shoots	Mean number of shoots/ explant	Shoots length (cm)	Nature of shoots
2iP	IAA				
3	-	54.3 ± 2.2	6.3 ± 0.9	0.7 ± 0.1	Yellow, defoliated
3	1.0	55.0 ± 1.8	6.7 ± 0.7	1.2 ± 1.8	Greenish yellow, defoliated
5	-	66.0 ± 3.1	8.7 ±0.9	2.8 ± 0.48	Greenish branched
5	1.0	70.0 ± 1.2	10.7 ± 0.6	3.6 ± 0.2	Green healthy, branched
7	-	57.7 ± 1.5	8.3 ± 1.2	1.7 ± 0.3	Greenish yellow, clusters
7	1.0	56.3 ± 1.2	8.3 ±3.5	2.1 ± 0.4	Green healthy, clusters
LSD (P=0.05)		11.38	3.84	0.84	

Values represent means ± standard error. Each treatment consisted of 20 replicates and the experiment was repeated twice. Data were recorded 12 weeks after transfer to MS medium.

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Table 4. Effect of different auxins incorporated in MS medium on rooting of the shoots of *R. dalhousiae*

Auxins (mg L^{-1})	Rooting frequency (%)	Mean number of roots per shoot	Mean root length (cm)
IBA			
0.1	24	4.0 ± 0.7	1.2 ± 0.1
0.2	86	5.3 ± 0.9	1.6 ± 0.2
0.3	58	3.3 ± 0.3	1.2 ± 0.1
0.5	56	2.7 ± 1.2	1.3 ± 0.1
IAA			
0.1	27	3.3 ± 1.5	1.2 ± 0.2
0.2	39	3.7 ± 1.3	1.4 ± 0.2
0.3	26	3.0 ± 1.5	1.1 ± 0.1
0.5	20	3.3 ± 1.3	1.0 ± 0.1
NAA			
0.1	26	3.7 ± 0.7	1.1 ± 0.1
0.2	45	5.0 ± 0.6	1.2 ± 0.2
0.3	56	4.3 ± 1.2	1.2 ± 0.1
0.5	28	3.3 ± 0.3	1.2 ± 0.1
LSD (P=0.01)	17	1.4	0.98

Values represent means ± standard error. Each treatment consisted of 20 replicates and the experiment was repeated twice. Data were recorded 9 weeks after transfer to liquid-MS medium.

This is the first report for *in vitro* regeneration of *R. dalhousiae* where large number of plant have been successfully produced and transferred to the field.

Acknowledgements

Authors are highly grateful to Dr. L.M.S. Palni, Director, G.B. Pant Institute of Himalayan Environment and Development, Almora, for all kinds of laboratory and institutional facilities. Miss Shanty Rai and Mr. Mohan Thapa are thanked for their assistance.

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Specimen Copy: Not for sale

Journal of Applied Horticulture, 12(1): 46-49, January-June, 2010

Induction of multiple shoots in *Amomum hypoleucum* Thwaites – A threatened wild relative of large cardamom

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Abstract

An efficient and repeatable micropropagation protocol has been established for *Amomum hypoleucum,* a lesser known threatened medicinal plant of the family Zingiberaceae. Eighty percent of the rhizome nodes from greenhouse grown plants, cultured on MS medium supplemented with 1 mg L^{-1} BA and 0.5 mg L^{-1} IAA, showed axillary bud break in 8-10 days. Multiple shoots proliferated from such shoot explants when transplanted to medium with 3 mg L^{-1} BA and 1 mg L^{-1} TDZ. An average of 9.2 shoots could be recovered in two months and about 65-70% of the shoots showed simultaneous rooting. Isolated shoots were also rooted in medium fortified with 0.5 mg L^{-1} NAA. Plantlets, transferred to the field after acclimatization in greenhouse conditions, showed 85% survival.

Key words: *Amomum hypoleucum*, micropropagation, tissue culture, Zingiberaceae

Introduction

Several of the members of the family Zingiberaceae have direct influence on India's agri-produce export trade. Cardamom (*Elettaria cardamomum*), ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), large cardamom (*Amomum subulatum*) etc. are prominent spice members of international trade importance. These domesticated members of the spice family are well focused in research, while their wild relatives have received less attention in propagation, biology, ecology etc. *A. hypoleucum* is a wild relative of large cardamom. Its distribution is restricted to evergreen hilly forests of South India (Kerala and Karnataka) and Sri Lanka (Sabu, 2006).

The genus *Amomum* is represented by 90 species worldwide, out of which 22 are reported from India. *A. hypoleucum* Thwaites which occurs in small scattered populations has been included in the red list of threatened vascular plants (Rao *et al.,* 2003). This wild ginger is a perennial herb with inflorescence having 2-3 small white flowers borne distinctly on long creeping underground rhizomes. Flowering and fruit set are very rarely observed. Recent studies showed that fresh rhizomes and leaves contain 0.03 and 0.04% essential oils, respectively. The rhizome oil constitutes cryptone (15.4%), β-pinene (11.9%), caryophyllene oxide (15.4%) etc, whereas the leaf oil mostly contains (E)-nerolidol (26.5%), α-fenchyl acetate (15%), β- caryophyllene (8.4%) etc. The essential oils have pleasant aroma as well as anti-bacterial activity against *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli* (Sabulal *et al.,* 2007).

Apart from the rare fruit setting in *A. hypoleucum*, most of the half-ripe fruits are eaten by rodents in their natural habitat which are the major factors for its rarity in the wild. In lieu of lack of seed set, it is necessary to establish propagation protocols for this rare plant. There are no reports on the micropropagation of *A. hypoleucum* and therefore, the present work was undertaken to develop and establish non-conventional propagation system for increasing the population as well as for possible exploitation of its antimicrobial properties.

Materials and methods

Amomum hypoleucum (Zingiberaceae) plants, collected from Palode Forest Ranges (Thiruvananthapuram, Kerala, India) and maintained in the Tropical Botanic Garden and Research Institute (TBGRI), Palode, Thiruvananthapuram, served as the source of explants. Herbarium specimens were also processed and kept in the TBGRI Herbarium for reference (No. TBGT - 47720). Nodal segments isolated from young rhizomes were washed thoroughly in running tap water for 10 min, followed by treatment in a mixture of 0.2% labolene (Qualigens, Mumbai) and 2% commercial bleach for 60 min and rinsed thrice in distilled water.

The explants were surface sterilised with 15% bleach for 15 min and then in 0.1% $HgCl_2$ for 8 min. They were washed thoroughly in sterile distilled water thrice, trimmed to 1.5-2.0 cm size by keeping the node at the middle. The explants were inoculated onto MS basal medium (Murashige and Skoog, 1962) containing 3% sucrose. Plant growth regulators (PGRs) such as N^6-benzyladenine (BA), thidiazuron (TDZ) and kinetin (KN) at various concentrations (0.5, 1.0 and 2.0 mg L^{-1}) were added either singly or in combination with indole-3-acetic acid (IAA; 0.1, 0.5, 1.0 mg L^{-1}) to the medium for evoking responses. The developed shoots (2-3 cm) were subcultured as decapitated and non-decapitated explants to test their influence. After standardizing the PGR regime for optimum shoot multiplication, effect of mineral salt formulations of MS, B5 (Gamborg *et al.,* 1968), Nitsch (Nitsch, 1969) and WPM (Lloyd and McCown, 1981) were also tested. *In vitro* rooting of micro shoots was studied in MS medium supplemented with auxins – α-Naphthylacetic acid (NAA) and indole-3-butyric acid (IBA).

The cultures were incubated at 25±2^{0}C under 16 h light period provided by cool white fluorescent tubes (1000 lux). All well rooted *in vitro* plantlets were deflasked and washed thoroughly in tap water. Plantlets free of agar were dipped in 3% commercial fungicide (Indofil M-45) for 5 min prior to planting in clay pots containing river sand and coarse charcoal (3:1). All the plantlets

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were maintained in semi shade (50% shade) and high humid (80-90% RH) net houses and sprinkled with water 3-4 times a day. Well established plants were potted and kept in the same net house conditions till field transfer.

All the experiments were set up in completely randomized block design. Each treatment was replicated 8 times. The data were analysed by single factor analysis of variance and the means were compared using Duncan's Multiple Range Test (DMRT) at P=0.05.

Results and discussion

Culture initiation: Nodal explants from underground rhizome of *A. hypoleucum* were initially established with 70 percent survival. The initial response of the primary explants varied according to the PGRs present in the medium. A combination of BA (1 mg L^{-1}) and IAA (0.5 mg L^{-1}) favoured the best culture initiation and showed 80 percent bud break within 10 days (Fig. 1A), while only 30-60 % bud break was observed in combinations of BA (0.5 - 2 mg L^{-1}) + IAA (0.1-1 mg L^{-1}) and BA (0.5- 2 .0 mg L^{-1}) used alone. KN was least effective of all the cytokinins tested. The superiority of BA over KN for multiple shoot formation was also demonstrated in *Jetropha integerrima* (Sujatha and Dhingra, 1993), *Sapium sebiferum* (Siril and Dhar, 1997), and *Bombax ceiba* (Chand and Singh, 1999). Though single buds were developed in PGR supplemented media, the explants failed to initiate bud break in hormone-free medium. The newly sprouted axillary buds developed into shoots with distinct nodes and internodes. The developed shoots were subcultured on to the same medium for multiplication and the elongated shoots (2-3 cm) were used for all further experiments. Decapitated and intact shoots explants in the medium showed varied responses. The intact shoot explants though elongated, scarcely produced axillary branches, while the decapitated explants showed frequent sprouting of axillary buds and therefore were selected as potential explants. In *Musa,* decapitation of apical dome was found necessary for the development of new shoots (Ma and Shii, 1972; Doreswamy *et al.,* 1983), while longitudinally split shoot halves showed better multiplication than whole explant in *Curcuma haritha* (Bejoy *et al.,* 2006), *Allium wallichi* (Wawrosch *et al.,* 2001) and *Opuntia polyacantha* (Mauseth and Halperin, 1975), whereas rhizome bud explants longitudinally divided into four equal parts showed better response in *C. longa* (Parthanturarug *et al.,* 2003). The present study showed that the removal of apical meristem facilitated sprouting of axillary buds overcoming the apical dominance reminiscent of the *in vivo* systems.

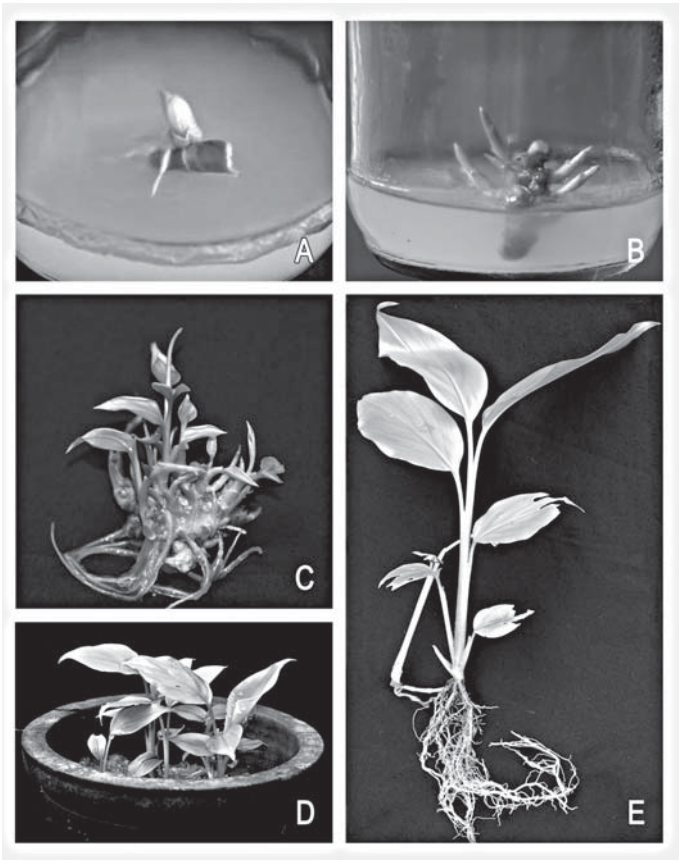

Fig. 1. *In vitro* propagation of *A. hypoleucum* from shoots of rhizome origin: 1A. Proliferating axillary bud from rhizome; 1B. Shoot development from decapitated shoot; 1C. Induction of multiple shoots with roots in BA (3mg L^{-1}) + TDZ (1mg L^{-1}) supplemented medium; 1D. Initial establishment of *in vitro* plantlets after one month; 1E. Well established plantlet showing newly formed rhizome developing into shoot.

Shoot multiplication: The growth response of shoots in terms of shoot elongation was better in BA alone supplemented media.

Table 1. Effect of plant growth regulators on morphogenetic response of shoot explants of *A. hypoleucum**

BA (mg L^{-1})	TDZ (mg L^{-1})	IAA (mg L^{-1})	Number of of shoots**	Shoot length (mm)**	Number of roots**
0.5			1.8 ±0.4a	50.8±7.3n	20.0±2.5q
1.0			2.8±0.6ab	41.1±5.5lm	18.0±3.5p
2.0			4.3±0.6bcd	44.4±4.6m	23.8±3.4r
3.0			3.7±0.4bcd	31.5±2.7ikj	14.4±1.4no
	0.01		2.7±0.4ab	40.2±6.2l	13.0±3.9mn
	0.05		3.1±0.6ab	32.7±4.2jk	4.6±1.0ghi
	0.10		3.8±0.8bcd	31.3±4.2ijk	5.3±1.3hi
	0.50		3.7±0.8bcd	22.7±4.4fg	4.0±0.6fgh
	1.0		5.0±0.7bcd	15.8±2.3abc	0.8±0.4a
	2.0		5.8±0.9cd	13.9±1.5a	3.2±0.8efg
	3.0		6.3±0.8d	17.6±1.7cde	1.6±0.6abc
	4.0		4.6±0.9bcd	15.3±1.9ab	2.0±1.3bcd
2.0	0.5		4.0±0.8bcd	21.2±2.0def	4.2±1.1ghi
2.0	1.0		5.2±0.7bcd	19.8±1.7def	3.6±0.6fgh
2.0	2.0		6.3±0.8d	17.2±1.3bcd	3.0±0.7efg
3.0	0.5		4.3±1.0bcd	18.7±2.8cde	2.0±0.9efg
3.0	1.0		9.2±0.8e	18.0±1.6cde	2.8±1.0efg
3.0	2.0		4.1±0.5bcd	18.3±2.6cde	1.0±0.5ab
1.0		0.1	3.2±0.8abc	16.9±4.6abc	8.8±2.1j
1.0		0.5	3.8±1.2bcd	32.0±3.8jk	4.3±0.8ghi
1.0		1.0	5.2±0.9bcd	34.8±4.4k	15.2±2.4o
2.0		0.1	3.0±0.7ab	49.0±3.9n	12.0±2.3lm
2.0		0.5	3.5±0.6abc	30.2±3.0ij	9.0±3.4jk
2.0		1.0	4.8±0.4bcd	21.7±2.5ef	10.6±2.1kl
	1.0	0.1	3.8±0.8bcd	28.1±4.1hi	2.1±0.4cde
	1.0	0.5	5.1±0.5bcd	25.6±2.8gh	2.5±1.0efg
	1.0	1.0	6.3±0.8d	18.6±1.3cde	2.3±0.7def
	2.0	0.1	4.3±0.6bcd	23.0±1.9fg	4.0±0.6fgh
	2.0	0.5	5.2±0.7bcd	15.0±1.4ab	6.0±0.9i
	2.0	1.0	4.6±0.7bcd	21.3±5.0ef	1.0±0.4ab

*Basal medium: MS + 3% sucrose + 0.7% agar & pH 5.7; all data after 60 days of incubation; **Mean ± Standard error followed by different letters within a column denotes significant difference at P =0.05 by Duncan's multiple range test.

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Maximum elongation of *in vitro* shoots was achieved in 0.5 mg L^{-1} BA (50.8 mm). The shoots produced in medium supplemented with TDZ above 3 mg L^{-1} were short, stout and occasionally produced malformed leaves. Though BAP initiated multiple shoots, TDZ was found to be more effective. Thidiazuron, a substituted phenylurea (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea) has been identified for its cytokinin-like activity in various explants cultured *in vitro* (Mok *et al.,* 1982; Huetteman and Preece, 1993). As per available literature, TDZ has been very rarely tested in members of Zingiberaceae, except in *C. longa* (Parthanturarug *et al.,* 2003). However, cytokinins were individually effective, their combined use improved the rate of multiplication. Addition of BA along with TDZ was the most potential hormonal regime for evoking almost 50% more multiplication (Table 1). The explants developed an average 9.2 shoots when treated with 3 mg L^{-1} BA and 1 mg L^{-1} TDZ in 9 weeks. This is in line with the reports of Vincent *et al.* (1992) and Anish *et al.* (2008), where the synergistic action of two cytokinins resulted in maximum shoot production. In contrast, cytokinin supplemented with an auxin was found optimal in other species, *C. amada* (Prakash *et al.,* 2004) and *C. longa* (Salvi *et al.,* 2002). Application of an auxin (IAA) along with BA did not improve the rate of multiplication in *A. hypoleucum*.

Effect of nutrient formulations: Mineral nutrition has also a controlling influence over morphogenic response of explants cultured *in vitro* (Preece 1995; Ramage and Richard, 2002). Hence, effect of four basal media (MS, B5, Nitsch and WPM) with constant PGR regime (3 mg L^{-1} BA and 1 mg L^{-1} TDZ) was investigated (Fig. 2). There were significant differences in the culture responses depending on the nutrient media used. The rate of multiplication was 9.2, 5.2, 4.6 and 2.8 in MS, Nitsch, WPM and B5 formulations, respectively revealing the superiority of MS medium in shoot multiplication for *A. hypoleucum*. Improved regeneration using MS has been reported in many members of Zingeberaceae such as *A. microstephanum* (Thoyajaksha and Rai, 2006), *C. haritha* (Bejoy *et al.,* 2006), *Kaempferia rotunda* (Anand *et al.,* 1997) and *Z. officinale* (Balachandran *et al.,* 1990). Shoot elongation (25.4 mm) was better in B5 compared to 17.8 mm in MS medium although the shoots developed in B5 medium showed pale yellow and narrow leaves when compared to other media. In contrast, B5 was found to be the best nutrient formulation for shoot multiplication in *C. amada* (Barthakur and Bordoloi, 1992). Rhizogenesis also varied in accordance with the nutrient composition. In the present study, Nitsch medium induced maximum number of roots (17.0) when compared to WPM (7.8), MS (2.8) and B5 (2.5).

Rooting and *ex vitro* establishment: In *A. hypoleucum,* about 70 percent of the shoots rooted in multiplication media. This is in agreement with reports in other Zingiberaceae members such as *C. haritha* (Bejoy *et al.,* 2006), *Paracautleya bhatii* (Rai and Thoyajaksha, 2001), *B. pulcherrima* (Anish *et al.,* 2008) etc, where rooting was achieved during shoot multiplication. Such concurrent root formation along with shoot development, reported in many Zingiberaceae members, may be due to an inherent root inducing capability of rhizomatous tissues. Simultaneous root development from microshoots also made the multiplication system easier and cost effective as reported in *Anthurium andraeanum* (Bejoy *et al.,* 2008). Since 30 percent shoots did not show simultaneous root development, a rooting medium was also adopted. Shoots without roots were rooted with the help of rooting hormones such as NAA and IBA (Table 2). The time required for root initiation was lesser (12-14 days) in NAA supplemented medium, when compared to that with IBA. Maximum number of roots per shoot (8.3) was also observed in 0.5 mg L^{-1} NAA. The necessity of a separate rooting phase in Zingiberaceae has been very rarely reported (Thoyajaksha and Rai, 2006).

Table 2. Effect of auxins on *in vitro* rooting response in *A. hypoleucum**

NAA (mg L^{-1})	IBA (mg L^{-1})	Days to root initiation	Percent response	Number of roots/shoot**
0.1	-	12-14	98	6.2 ± 1.2^{ab}
0.5	-	12-14	99	8.3 ± 1.1^{b}
-	0.1	15-18	95	5.4 ± 0.6^{a}
-	0.5	14-16	97	7.8 ± 0.6^{ab}

* Basal medium: MS+3% sucrose+0.7% agar & pH 5.7; all data after 4 weeks of incubation; **Mean ± Standard error followed by different letters within a column denotes significant difference at ∞ =0.05 by Duncan's multiple range test.

After 4-5 weeks of incubation in the rooting media, plantlets with good root system were deflasked and transplanted to the *ex vitro* condition. The young plants showed 85% survival and started producing new leaves in 6-8 weeks in community pots (Fig. 1D). They were then transferred to 8 inch clay pots containing river sand and soil mixture. The plantlets grew to 30-45 cm size in 6-7 months and developed fresh rhizome with vigorous shoot system (Fig. 1E). The regenerated plants were morphologically similar to their mother plants *in vivo*.

This is the first report on *in vitro* multiplication of *A. hypoleucum*. The multiplication programme established for the species has considerable practical importance to establish new population for the restoration of this rare species with minimum samples, suitable for its conservation.

Acknowledgement

We thank the Director, TBGRI for facilities and Dr. P.J. Mathew for encouragement. We also gratefully acknowledge Western Ghats Cell, Planning and Economic Affairs Department, Govt. of Kerala for financial support and Kerala Forest Department for permission for collection.

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Journal of Applied Horticulture, 12(1): 50-53, January-June, 2010

Effect of nitrogen concentration and growth regulators on growth and nitrate content of lettuce

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Abstract

Lettuce plants (*Lactuca sativa* L. cv. 'Paris Island') were grown in an unheated plastic greenhouse to determine the effects of solution nitrogen concentration and growth regulators (gibberellin and kinetin) on growth (fresh and dry head weight) and tissue nitrate content (TNC). The plants were grown in plastic containers with perlite and supplied with a basic nutrient solution supplemented with nitrogen (N) corresponding to 50, 100, 150, and 200 ppm NO_3-N. Growth regulators; gibberellin (GA_3) and kinetin were applied at different doses independently and in combination. Fresh, dry weight and TNC were responsive to N application level. However, fresh and dry weights were similar at 150 and 200 ppm and TNC at 100 and 150 ppm nitrogen supply. Gibberellin (GA_3) and gibberellin and kinetin in combination (GA_3+kinetin) enhanced fresh and dry weight and TNC compared to the control. There were few differences in response to application rates. Therefore, where lettuce plants are grown in similar conditions and low NO_3 accumulation is desirable, together with high yield and good size, the best N application level is 150 ppm NO_3-N and growth regulators application may enhance yield. However their use in reducing the nitrate content is not recommended.

Key words: *Lactuca sativa*, nitrogen, gibberellin, kinetin, growth, TNC

Introduction

The presence of nitrates in vegetables (Peck *et al.*, 1971), as in water and generally in other food products is a serious threat to man health (WHO, 1985). Not so much due to its toxicity, which is low, but for giving rise to the dangerous compounds in the organism (*e.g.* methaemoglobin, nitrosamines) (Van Der Boon *et al.*, 1986, 1990; Boink and Speijers, 2001).Vegetables constitute the major source of nitrate providing 72-74% of the average daily human dietary intakes (Santamaria, 1997). Generally leafy vegetables tend to accumulate nitrates and the European Commission has issued the Commission Regulation (EC) No. 563/2002 setting maximum acceptable nitrate concentration in lettuce and spinach. For winter grown lettuce the value is 4500 mg kg^{-1} fresh weight and for summer-grown lettuce 2500 mg kg^{-1} fresh weight.

To find ways to reduce nitrate accumulation in lettuce, many experiments were conducted by controlling some factors like the nitrogen form and concentration, light intensity, CO_2 concentration, genetic variation and cultivation techniques. (Blom-Zandstra and Lampe, 1985; Reinink and Groenwold, 1987; Reinink *et al.*, 1987; Kaiser and Brendle-Behnisch, 1991; Economakis, 1992; Jaervau, 1994, Pasda *et al.*, 2001; Premuzic *et al.*, 2002, 2003).

There are reports focusing on nitrate content in lettuce and other leafy vegetables known as nitrate accumulators by growth regulators (Rozek *et al.*, 1989; Rozek and Wojciechowska, 1990; Myczkowski *et al.*, 1990, 1991; Sady *et al.*, 1995). Encouraged by these observations, EU recommendations to lower nitrate content of vegetables and the growing interest in soilless culture, our research was focused on the influence of solution nitrogen concentration and two plant growth regulators (gibberellin and kinetin) on growth and tissue nitrate content (TNC) in lettuce grown in soilless culture under eastern Mediterranean climatic conditions (Cyprus).

Materials and methods

Plant material and culture: The experiment was conducted at the Agricultural Research Institute of Cyprus (32°24′ E, 35°09′ N) in a plastic greenhouse under natural daylight conditions and temperature. Lettuce seedlings at four true leaves stage (cv. 'Paris Island', a romaine type) were transplanted into plastic containers filled with 2 kg of horticultural perlite. The N treatments corresponded to nutrient solutions nitrogen concentration of 50, 100, 150, and 200 ppm NO_3-N and electrical conductivity values 1.5, 2.0, 2.6 and 2.8 mS cm^{-1}. It does appear that although lettuce plants can be grown over a wide range of solution conductivities, EC levels within 2.0-3.0 mS cm^{-1} give more satisfactory results (Economakis, 1991). The pH of the nutrient solutions was adjusted around 6.5 (Bres, 1992). Treatments were arranged in a randomized complete block design. Each nitrogen treatment corresponded to a block with 32 plants and replicated three times. Each block consisted of four rows with four plastic containers in each row and two plants per container. Within every block the growth regulators (GA_3, kinetin, GA_3+kinetin) were applied.

In-line drip irrigation, providing 2 L h^{-1}, was placed at the top of the containers. Small holes were cut on the bottom of the plastic containers to allow excess nutrient solution to flow out and the plastic containers were laid on a catchment plate to gather the excess nutrient solution. The nutrient solution applied and the number of trickling turns per day was adjusted depending on the amount of over drained solution. The plants were fertigated with a basic nutrient solution (Table 1) supplemented with the nitrogen concentration corresponding to the treatment.

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Table 1. Nutrient solution basic composition

Nutrient	Concentration (mg L^{-1})
P	30
K	150
Mg	25
Ca	50
Hoagland solution*	50 mL m^{-3}

*Micro-nutrients were supplied from the micro-nutrient Hoagland stock solution ($MnSO_4.H_2O$=34 g L^{-1}, $CuSO_4.5H_2O$=1 g L^{-1}, $ZnSO_4.7H_2O$=2.2 g L^{-1}, $(NH_4)_6Mo_7O_{24}.4H_2O$=1 g L^{-1}).

The air temperature and relative humidity (R.H.) inside the greenhouse are given in Table 2. The greenhouse was ventilated when the temperature exceeded 25°C. Global solar radiation fluctuated from 19.3 to 20.4 MJ m^{-2} and sunshine duration from 7.9 to 9.8 h day^{-1} over the whole experiment. There were no disease problems during the growth cycle. The plants were sprayed once, at an early stage of growth, with the systematic insecticide Mospilan (Acetamiprid) to prevent any pest problems. On the 29th day the plants were sprayed with 40 mL per plant solution of gibberellic acid (GA_3) or kinetin (Kinetin®, Sigma) independently (20, 100 and 200 ppm) or in combination (100 + 100, 20 + 200 and 200 + 20 ppm). Plants were harvested after a growing period of 40 days with a good market size and a relatively well-formed head (romaine type). At harvest, the lettuce heads were weighed, damaged leaves were removed, and the marketable weight was determined. The dry weight and tissue nitrate content were recorded.

Table 2. Mean minimum and maximum air temperature (°C) and relative humidity (%) inside the greenhouse during the growing period

Growing period (days)	Air temperature (°C)		RH(%)	
	Maximum	Minimum	Maximum	Minimum
1-8	37.8	5.0	95.1	32.1
9-16	40.6	5.7	85.0	20.0
17-24	36.1	4.9	95.0	22.1
25-32	38.9	6.1	89.5	18.9
33-40	40.7	7.7	94.2	27.1

Sample preparation: Lettuce heads were cut in four pieces put in paper bags and placed in an oven at 65°C temperature and air circulation to dry until reaching a constant weight. The dried material was recorded, finely mixed, ground and closed hermitically in glass vessels.

Determination of nitrates in plant tissue: For the determination of TNC a method reported by Paul and Carlson (1968), modified by Baker and Smith (1969) and Hadjidemetriou (1982) was used. An amount of 0.25 g of dried ground head was added to 25 mL extracting solution (0.02 N copper sulphates containing the amount of silver sulphate needed for the precipitation of chlorides), homogenized by shaking for 15 min and filtered through Watman (No.2) filter paper. The samples potential (me) was measured with a nitrate combination ion selective electrode (924-300, Jenway, UK) connected with the pH and conductivity meter apparatus measuring in millivolt (mv) with the help of a standard solution of known concentrations of nitrate. To avoid interference by chloride ion, silver sulphate was added in the extractant for precipitation of chlorides (Hadjidemitriou, 1982) because chlorides are possibly the most serious interfering ion (Baker and Smith, 1969).

Statistical analysis: The yield data and nitrate content in plant tissue were subjected to ANOVA in SAS (SAS Inc., Cary, NC) for analysis of variance. Means were compared using Duncan's multiple range test at the 5% level of significance.

Results and discussion

Effect of solution nitrogen concentrations: The effect of nitrogen concentrations on fresh and dry weight per head and TNC of lettuce plants are shown in Table 3. Average head weight (fresh weight) was very responsive to N application level (Pearson correlation coefficient r = 0.917*). Kunsch *et al.* (1993) reported that lettuce heads can be determined as commercial when the fresh weight of the lettuce head is above 250 g. According to this, the lettuce heads of the treatment 50 ppm NO_3-N were not as per commercial standards whereas the lettuce heads of the treatments 150 and 200 ppm NO_3-N had more marketable heads. The dry matter weight per head increased significantly related to the amount of N applied (Pearson correlation coefficient r = 0.882*) but differences were not significant between application levels of 150 and 200 ppm NO_3-N (Table 3). This could be expected based on fresh weight results. Increasing the N application level increased the TNC (Table 3) with a significant correlation coefficient (r = 0.798*). The maximum value was observed at 200 ppm NO_3-N whereas; at 100 and 150 ppm NO_3-N TNC remained at significant lower values (Table 3). The lowest significant value was at 50 ppm NO_3-N but growth was consistently depressed in contrary with results of Koleilat (1993) suggesting that lettuce can be grown at low nitrogen level (50 ppm) without reduction in yield. Finally, it could be stressed that TNC in lettuce heads was below the recommended limit by the European Commission at all nitrogen concentrations levels.

Effect of GA_3, kinetin and GA_3+kinetin: The effect of growth regulators, GA_3, kinetin and GA_3+kinetin on fresh and dry weight per head and TNC of lettuce plants are shown in Table 3. GA_3 and GA_3+kinetin treatments had the highest averages of fresh, dry weight and TNC. Control gave significant lower values and kinetin had non significant differences (Table 3). The interaction between N application level and growth regulators was not significant (Table 3).

There were few differences in response to application rates. GA_3 at different application rates had a non significant influence on fresh and dry weight. However, increased supply of GA_3

Table 3. Effect of nutrient solution nitrogen concentration (NC) and growth regulators (GR): gibberellin (GA_3), kinetin and GA_3+kinetin, on fresh head weight (F.H.W.), dry head weight (D.H.W.) and tissue nitrate content (TNC) of lettuce

Nitrogen concentration	F.H.W. (g)	D.H.W. (g)	TNC [mgNO_3 (kg F.W.)$^{-1}$]
N-50 ppm NO_3-N	212c	10.9c	381c
N-100 ppm NO_3-N	325b	12.2b	1173b
N-150 ppm NO_3-N	553a	19.0a	1204b
N-200 ppm NO_3-N	590a	19.7a	1371a
GR			
Control	380b	14.3b	893c
Kinetin	416ab	15.2ab	1002bc
GA_3 + Kinetin	430a	16.1a	1073ab
GA_3	454a	16.3a	1161a
NS × GR	NS	NS	NS

NS: non-significant at $P \leq 0.05$.

Mean values followed by the same letter in the same column are not significantly different at $P \leq 0.05$.

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Table 4. Effect of growth regulators (GR): gibberellin (GA_3), kinetin and GA_3 + kinetin combination at different application rates on fresh head weight (F.H.W.), dry head weight (D.H.W.) and tissue nitrate content (TNC) of lettuce

GR (ppm)	F.H.W. (g)	D.H.W. (g)	TNC [mgNO_3 (kg F.W.)$^{-1}$]
GA_3			
20	417	15.3	1095b
100	478	17.3	1141ab
200	466	16.3	1248a
Significance	NS	NS	*
Kinetin			
20	418	15.3	978
100	428	15.3	983
200	403	15.0	1044
Significance	NS	NS	NS
GA_3 + kinetin			
100 + 100	409	15.5	1317a
20 + 200	415	15.5	906b
200 + 20	466	16.2	996b
Significance	NS	NS	*

NS, non-significant, * significant at $P \leq 0.05$.
Means within columns for a given treatment followed by the same letter are not significantly different at $P \leq 0.05$.

enhanced the accumulation of nitrates (Table 4). The TNC was highest at 200 ppm and lowest at 20 ppm GA_3 application. The GA_3 at 100 ppm gave non significant differences. The effects of kinetin application rates on fresh and dry weight and TNC of lettuce plants were not significant as shown in Table 4. Similarly, the effects of GA_3+kinetin at different application rates on fresh and dry weight were not significant. However, the exposure of plants at application rate of 100+100 ppm resulted in significantly higher TNC (Table 4).

Gibberellin and GA_3+kinetin enhanced fresh and dry weight per head and similar results were reported by Rozek *et al.* (1989) and Neocleous *et al.* (2007), who found that growth regulators GA_3, kinetin and GA_3+kinetin combination stimulated fresh weight growth in lettuce heads, being the most intensive in plants sprayed with the combination of the two regulators. In addition, GA_3 and GA_3+kinetin enhanced TNC. These results are in agreement with those found by Neocleous *et al.* (2007) but in conflict with those found by Rozek and Wojciechowska (1990), Myczkowski *et al.* (1990) and Sady *et al.* (1995) who reported that lettuce plants sprayed with GA_3 and kinetin had the greatest reduction in nitrate content. Attributed to different weather conditions (light intensity, sunshine duration and temperature) and reports from the literature (Roorda-Van-Eysinger, 1984; Blom-Zandstra and Lampe, 1985; Kaiser and Forster, 1989; Van der Boon *et al.*, 1990; Talon *et al.*, 1991; Lacertosa *et al.*, 1997; Neocleous *et al.*, 2007) showing that there is an interaction between the amount of light or time of the year in which crop is grown and the nitrate content, it can be argued that under east Mediterranean (Cyprus) climatic conditions, where light intensity and temperature are high even in winters, the role of growth regulators in lowering the tissue nitrate content might be less important. Similarly, Behr and Wiebe (1992) reported a close negative correlation between photosynthesis and nitrate content in lettuce cultivars and this explaination may be suitable in present study also. Tissue nitrate content was between 381 and 1371 mg NO_3 (kg fresh weight)$^{-1}$ all over the experiment which is lower than European standards and comparable with biologically produced lettuce (Leuzzi *et al.*, 1997).

In conclusion, in lettuce plants low NO_3 accumulation is desirable together with high fresh and dry head weight for which the best N-application level is 150 ppm NO_3-N. Also the use of growth regulators, GA_3 and GA_3+kinetin, may enhance yield. However, their use for reducing the nitrate content is not recommended.

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Journal of Applied Horticulture, 12(1): 54-58, January-June, 2010

Fruit ripening of Solo Sunrise, Tainung #2 and Red Lady papaya at two temperatures

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Abstract

The process of ripening was evaluated in three papaya cultivars, Solo Sunrise (SS), Tainung #2 (T2) and Red Lady (RL) with different mean fruit weights of 387, 1364 and 2266 g and fruit cavity void volumes of 56, 334 and 502 mL and fruit weight/cavity void volume ratios of 6.9, 3.8 and 4.3 g mL^{-1}, respectively. The evaluation was done by comparing physiological determinants of the ripening process; ethylene (C_2H_4) generation and respiratory CO_2 production, measured at two temperature ranges, 20-22°C and 28-30°C, by sampling cavity void volumes, with physico-chemical quality characteristics of ripening: skin colour, flesh firmness and pH. Fruit ripening of the three cultivars was delayed at the lower temperature range as measured both by physiological determinants including pre-and peak climacteric rates and physico-chemical quality characteristics. However, cv RL showed slower ripening than cvs SS and T2 at both temperature ranges, probably partly related to its low fruit weight/cavity void volume of 4.3 g mL^{-1}. Moreover, there were negative temporal displacements for skin degreening compared with those for flesh softening, respiration and ethylene generation in fruit of the three cultivars. Fruits of cvs SS and T2 were fully ripened in 8 days after harvest (DAH) and RL fruits in 10 DAH at the lower temperature range. Values for C_2H_4 generation and CO_2 production measured in the fruit cavity are judged to be sensitive indicators of the progress in the process of ripening.

Key words: Papaya, ripening, climacteric, ethylene, colour, firmness

Introduction

Papaya (*Carica papaya* L.; *Caricaceae*) is a native of Tropical America but is cultivated throughout the tropics, where there are several cultivars retained by inbreeding in their countries of selection. These include *Solo* in Hawaii, *Betty* in Florida, *Hortus Gold* in South Africa and *Improved Petersen* in Australia (Simpson, 1980). Moreover, although *Solo* originated in Barbados in the Caribbean, several improved lines were developed in Hawaii *e.g.* cvs Solo 5, Solo 8, Bush and Sunrise, where papaya is cultivated on a commercial scale for export to the United States and Canada.

The papaya fruit is a fleshy, hollow berry of 0.5 to 3.0 kg in weight. At the lower end of the weight range, the Solo cultivars are hermaphrodites with yellow flesh fruit except for the preferred cvs Bush and Sunrise, with red flesh fruits. Alternatively, cv *Hortus Gold* is dioecious with firm golden flesh fruits and weights of 1.5 -2.0 kg (Salunkhe and Desai, 1984). Most of the postharvest research on papaya including changes in physiology, composition and sensory characteristics with ripening has been done on fruits of cv Solo in Hawaii (Wills and Widjanarko, 1995). However, the wide range of respiratory rates in papaya fruit 3-32 mL CO_2 kg^{-1} hr^{-1} was noted by Biale and Barcus (1970) and recommended storage conditions of temperature and relative humidity reviewed by Thompson (1996).

Solo fruit are exported on a large scale from Hawaii, Malaysia and to a lesser extent from Jamaica in the Caribbean to the U.K., U.S. and Canada. Internationally, they are most popular because of their small size, red flesh colour and other quality attributes, suitable for use as a fresh fruit. However, because of their susceptibility to Bunchy Top, a microplasmic disease transmitted by a leaf hopper, several hybrids have been produced worldwide. Two of these hermaphroditic hybrids, cvs T2 and RL are cultivated in the Caribbean. They vary considerably in size and eating quality from cv SS but are sold locally and exported to regional and international markets (Zhang and Paull, 1990). Unfortunately, little work has been published on the ripening of cvs.T2 and RL. Accordingly, the objective of this study was to conduct a comparative evaluation of the effect of storage temperature on ripening as indicated by parameters of ethylene generation and carbon dioxide production as well as by quality changes of skin colour, flesh firmness and pH in order to detect characteristics of the process of ripening of fruits of the three cultivars, which may be useful in further studies. Gases were measured in the fruit cavity rather than in the void volume of containers in which the fruits were stored to ripen, because preliminary investigations revealed earlier detection of C_2H_4 and CO_2 in the former system by the gas chromatograph used in the investigations.

Materials and methods

Mature papaya fruit of *cvs*. Solo Sunrise, Tainung #2 and Red Lady at the stage of colour break with yellow streaks comprising 6 - 8% of the fruit surface were harvested at the University Field Station and packed in cardboard boxes lined with shredded paper. Fruits were transported to the Food Biology Laboratory, Department of Food Production, at the University of the West Indies, St. Augustine within one hour of harvest. At the laboratory, the fruits were hand washed to remove surface debris. Forty fruits from each cultivar were treated for 20 minutes with hot water at 40°C in separate 150 L metal drums each equipped with

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two VWT Scientific thermo-regulators (VWR Scientific, model #1222, Niles Illinois) on opposite ends. To keep fruit submerged, a padded stainless steel grill was placed at the surface level of the water. Fruits were cooled for 30 minutes in tap water, air-dried and allowed to ripen at 28-30°C, 85-90% RH (ambient room temperature) and 20-22°C, 50-60% RH on trolleys lined with foam of 2.22 cm thickness in an air conditioned room. The postharvest quality of the fruits was assessed over an eight day storage period. Carbon dioxide and ethylene evolution rates were measured at two-day intervals while physical and biochemical parameters were measured at 4-day intervals.

CO_2 production and C_2H_4 generation: External CO_2 and C_2H_4 concentrations from fruits were first attempted by enclosing fruit in sealed plastic buckets and extracting gas samples through a rubber septum for analysis. Since detection of C_2H_4 by this method was inconsistent, CO_2 production and C_2H_4 generation from the internal fruit cavity was adopted and production rates were determined by the use of a Finnegan gas chromatograph (Model #9001, Finnegan Corp. Austin, TX). Ethylene was measured using a Flame Ionization Detector (FID) set at 250°C, while carbon dioxide was measured using a Thermal Conductivity Detector (TCD) set at 200°C and analyzed simultaneously. A hollow 4-cm long hypodermic needle was inserted into the cavity and the area around the needle was sealed with petroleum jelly. Approximately 0.3 mL gas sample was withdrawn with a 1.0 mL syringe as described by Salveit (1982). Samples of gas were injected through a rubber septum in the gas chromatograph with oven temperature set at 28°C using helium as the carrier gas with a flow rate of 25 mL/min. The flow rates of hydrogen and air were 15 mL/min and 175 mL/min, respectively. Megabore columns of 0.53 mm diameter and 30 m in length were used. Quantification of carbon dioxide and ethylene production was done by calculation against instrument responses to standard gas mixtures.

Skin colour: Skin colour was measured with a portable tristimulus Minolta Chroma meter (Model CR-200, Minolta Corp., Ramsey, NJ), the meter was calibrated with "L" and "a" coordinates. The "L" values represented the lightness of colour and were greater for lighter colour whilst the "a" values were negative for green and positive for red. Colour was measured at four evenly spaced points along the equatorial region of each fruit.

Flesh firmness: Flesh firmness was determined using a Koehler digital penetrometer (Model #K 19550, Koehler Instrument Company, Bohemia, N.Y) using a K 20500 brass probe with a hardened stainless steel tip which was used to measure penetration depth. The probe had a mass of 2.5 g and the standard plunger used was 47.5 g in mass. A 2.2 cm slice was taken from the midpoint between the stem and the calyx end of the fruit and the plunger vertically pressed into the flesh at four equatorially spaced points along the surface; penetration was expressed in mm sec^{-1}. Increasing depths of penetration represented continuous loss of firmness in fruit.

Fresh fruit weight and cavity volume: For measurement of C_2H_4 and CO_2, harvested fruits of each variety were weighed individually on a Mettler Toledo calibrated digital scale (Model #SB 8001 Toledo SD 8001, Schwerzenbach, Switzerland) to determine fresh weight. Each fruit was subsequently cut in half, seeds removed and weighed. Each half was then filled with distilled water which was poured into a graduated cylinder for cavity volume measurement. In a separate sample of papaya fruit of each cultivar, total fruit weight was measured and fruit void volumes determined as before. Seeds were then removed and weighed and fruit flesh weight calculated by subtracting seed weight from total fruit weight.

pH: The pulp pH was determined using an Orion Research digital pH meter (EA 920 Orion Research Inc. Boston, MA) standardized with two buffer solutions of pH 7.01 and 4.01. The extract was obtained by macerating 25 g of pulp with 100 mL demonized water (Products Corp. Hartford, CT).

Experimental design and statistical analysis: The experiment consisted of two replicates with each replicate containing four fruits. Data were analyzed as a completely randomized design with a factorial arrangement of variables and subjected to Analysis of Variance using Minitab. Comparisons of the means were done using the least significant difference (LSD) method at the 5% level.

Results

Fruit fresh weight and cavity volume: The fresh weights (fwt), cavity volume (cvo) and the fwt/cvo ratios of the fruit (Table 1) showed that there were considerable differences between values for fruit of the three cultivars. Thus, although mean fruit weights and cavity volumes of cv SS were considerably smaller than those of cvs T2 and RL, the fwt/cvo ratio of cv SS (6.9 g mL^{-1}) was 1.8 and 1.6 times those of cvs T2 and RL, respectively. The possible effect of these dimensional differences on C_2H_4 and CO_2 accumulation in the cavity and ripening will be described below. Since CO_2 production and C_2H_4 generation were measured in the fruit cavity, these differences could affect both the accumulation of gases and the interpretation of their effects on ripening.

Respiratory CO_2 and C_2H_4 generation changes with ripening: The time courses of the changes in climacteric patterns of (CO_2) respiration rate and ethylene (C_2H_4) generation varied with cultivars and storage temperatures (Fig. 1 and 2). Examination of initial rates over 0-2 DAH indicated that at both ambient temperature (28-30°C) (Fig. 1C) and at (20-22°C) (Fig. 2C) cv RL showed slower rates of CO_2 and C_2H_4 generation than cvs SS and T2 (Figs. 1A, 1B). Whereas rates of CO_2 and C_2H_4 generation in fruit of each cultivar were similar at ambient temperature (Figs. 1 A, B and C). C_2H_4 generation proceeded at a more rapid rate than CO_2 evolution at 20-22°C particularly in fruit of cvs SS and T2 (Figs. 2 A, B). Thus, the preclimacteric period of C_2H_4 generation occurred over 0-2 and 0-4 DAH at 28-30°C and 20-22°C, respectively in cv RL but was not recorded in cvs SS and

Table 1. Dimensional characteristics of fruit of three papaya cultivars

Cultivar	Fresh weight (g)			Fruit cavity void vol (mL)	Frut pulp weight/ cavity void volume (g mL^{-1})
	Whole fruit	Fruit seeds	Fruit pulp		
Solo Sunrise (SS)	387b	ND[Z]	387b	56a	6.9b
Training #2 (T2)	1364e	87a	1277d	334b	3.8a
Red Lady (RL)	2266g	124a	2142f	502c	4.3a
LSD (P=0.05)		109.3		81.5	1.3

[Z]ND: No data due to seedless nature of cultivar

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Table 2. Effect of temperature, storage duration and cultivar upon colour development ("L" and "a" values) of papaya fruit

Colour/Day	28-30°C			20-22°C		
	Solo Sunrise	Tainung #2	Red Lady	Solo Sunrise	Tainung #2	Red Lady
"L" value						
0 DAH	33.65 cd	34.65 d	30.35 a	32.32 b	33.94 cd	31.23 ab
4 DAH	56.43 h	61.15 i	48.75 e	51.77 f	52.33 f	47.70 e
8 DAH	70.08 k	70.46 k	61.21 i	64.35 j	63.71 j	54.03 g
LSD (P=0.05)			1.13			
"a" values						
0 DAH	-11.67 a	-12.34 a	-1.59 d	-10.52 ab	-10.89 a	-12.93 a
4 DAH	-1.66 d	-0.72 d	-3.67 c	-5.19 c	-5.32 c	-8.21 b
8 DAH	5.25 e	7.85 f	0.64 d	4.02 e	4.66 e	-5.12 c
LSD (P=0.05)			2.54			

T2 at either temperatures (Figs. 1 and 2). Preclimacteric CO_2 evolution occurred over 0-2 DAH for cv RL at 28-30°C and over the same period for cvs SS and T2 at 20-22°C.

The climacteric peak of autocatalytic C_2H_4 generation occurred at 2 DAH in cvs SS and T2 at 28-30°C but at 4 DAH at 20-22°C. Alternatively, for cv RL fruits, this peak occurred on 4 and 6 DAH at 28-30°C and 20-22°C, respectively (Figs. 1 and 2). At 28-30°C, the respiration and ethylene peak heights were similar for cvs T2 and RL fruits but higher than those for cv SS fruits (Figs. 1B and C). At the lower storage temperature (20-22°C), the CO_2 and C_2H_4 peak heights were highest for cv T2 fruits despite their occurrence at the same time (4 DAH) as cv SS fruits and 2 days earlier than cv RL fruits (Figs. 2A and 2B). Temperature seemed to have greater inhibitory effect on initial respiration over 0-2 DAH than on C_2H_4 generation (Fig. 2) particularly in cvs SS and T2 fruits.

The lower peak heights for C_2H_4 and CO_2 accumulation in cv SS fruits at both temperatures were surprising in view of the lowest

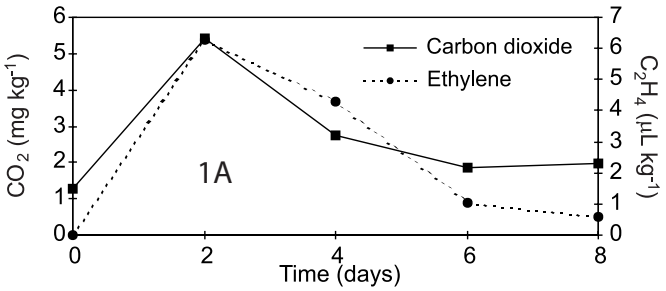

Fig.1A. Respiration and ethylene production rates of papaya cv. Solo Sunrise stored at 28-30°C. LSD (P=0.05) CO_2 = 1.11; C_2H_4 = 0.69.

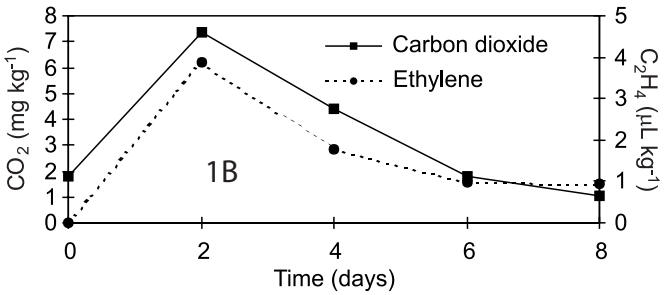

Fig.1B. Respiration and ethylene production rates of papaya cv. Tainung # 2 stored at 28-30°C. LSD (P=0.05) CO_2 = 1.11; C_2H_4 = 0.69.

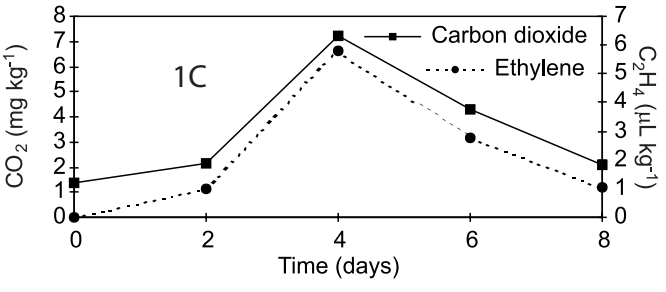

Fig.1C. Respiration and ethylene rates of papaya cv. Red Lady stored at 28-30°C. LSD (P=0.05) CO_2 = 1.11; C_2H_4 = 0.69.

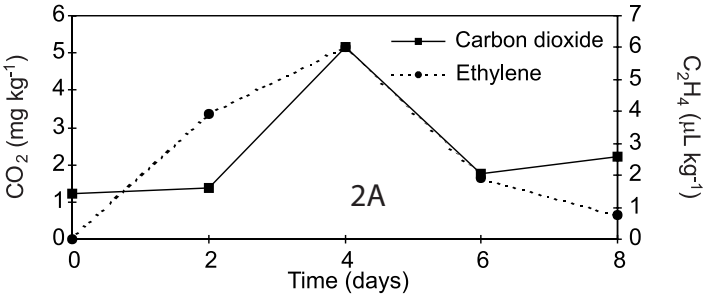

Fig. 2A. Respiration and ethylene rates of papaya cv. Solo Sunrise stored at 20-22°C. LSD (P=0.05) CO_2 = 1.11; LSD (P=0.05) C_2H_4 = 0.69.

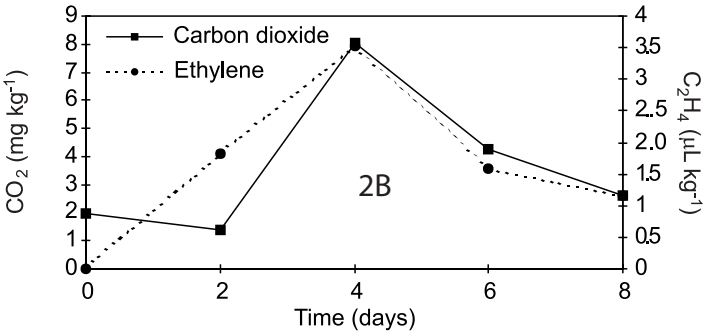

Fig. 2B. Respiration and ethylene production rates of cv. Tainung #2 stored at 20-22°C. LSD (P=0.05) CO_2 = 1.11; LSD (P=0.05) C_2H_4 =

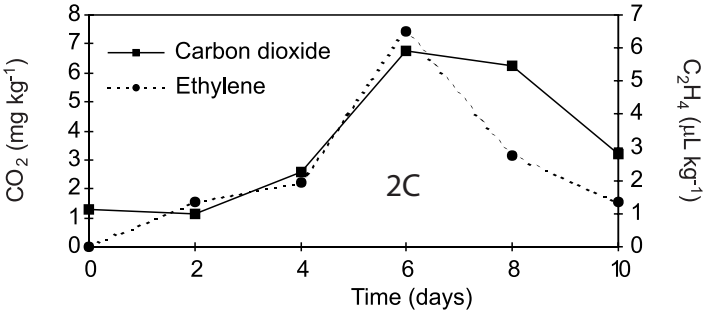

Fig. 2C. Respiration and ethylene production rates of cv. Red Lady stored at 20-22°C. LSD (P=0.05) CO_2 = 1.11; LSD (P=0.05) C_2H_4 = 0.69.

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Table 3. Effect of storage duration and cultivar on pH of papaya

Cultivar	pH		
	0 DAH	4 DAH	8 DAH
Solo Sunrise (SS)	5.14bc	4.64a	4.49a
Training #2	5.60cd	4.86ab	4.53a
Red Lady (RL)	5.63e	4.92ab	4.52a
LSD (P=0.05)		0.47	

Table 4. Interaction effects of cultivar x storage duration upon firmness of papaya

Cultivar	Firmness		
	0 DAH	4 DAH	8 DAH
Solo Sunrise (SS)	16.19b	87.76d	99.91e
Training #2	13.89ab	86.46d	104.37f
Red Lady (RL)	10.19a	66.47c	88.05d
LSD (P=0.05)		3.92	

Table 5. Interaction effects of temperature x storage duration upon firmness of papaya

Temperature (^{0}C)	Firmness		
	0 DAH	4 DAH	8 DAH
28-30^{o}C	13.38a	84.89c	101.19e
20-22^{o}C	13.48a	75.68b	93.70d
LSD (P=0.05)		3.20	

void cavity volume (56 mL) and highest flesh fwt/cvo ratio (6.9 g mL^{-1}) recorded in fruit of this cultivar. Alternatively, the extension of the preclimacteric phase in cv RL to 4 DAH at 20-22^{0}C could possibly be related to the low flesh fwt/cvo ratio (4.3 g mL^{-1}) of this cultivar. In cvs SS and T2, there were preclimacteric phases of respiratory CO_2 production over 0-2 DAH at 20-22 ^{0}C but autocatalytic C_2H_4 generation had apparently already began at 2 DAH. This early separation of CO_2 and C_2H_4 production rates did not occur in cv RL.

Quality changes in colour, texture and pH with ripening: Degreening measured by "L" and "a" values progressed significantly (P<0.05) as storage time increased for each cultivar, more so at 28-30^{o}C than at 20-22^{o}C, as expected (Table 2). Changes in flesh colour, although not measured paralleled skin degreening. Colour development in both skin and flesh was also cultivar specific. Thus at 28-30^{o}C cvs SS and T2 changed from colour-break to a bright uniform yellow colour (skin and flesh) at 8 DAH but cv RL developed a bright orange colour 2 days later. At 20-22^{o}C, these changes were realized at 10 DAH. There was also evidence of decay in some fruit particularly at the 28-30^{o}C temperature range at 10 DAH.

At the climacteric phase over 0-2 DAH at 28-30^{o}C cvs SS and T2 showed an estimated 30% (approx.) yellow skin colour, which increased to 60% at the post climacteric stage over 4-6 DAH. These colour changes accounted for almost a doubling of "L" values and much greater increases for "a" values (Table 2). The orange colour changes in cv RL proceeded similarly but at a slower rate as indicated by the lower "L" and "a" values at both temperatures compared to cvs SS and T2. The rate of fruit softening as measured by penetration depths (Tables 4 and 5) increased with increasing storage durations and was affected by cultivar and temperature and indicated by significant interactions (Tables 4 and 5). As observed previously in the development of skin colour and rise in CO_2 and C_2H_4 evolution, cvs SS and T2 showed similar rates of softening while in cv RL softening was significantly less pronounced. The pH values decreased in fruit of all three cultivars during ripening (Table 3).

Discussion

Ripening in climacteric fruit is the collective process in which many physiological and physico-chemical changes occur, more or less in parallel to each other and in a time relation to the climacteric. However these changes are themselves not necessarily causally related (Pratt and Goeschl, 1969). The physico-chemical changes *e.g.* skin degreening and flesh softening are those by which fresh fruit quality is judged and the physiological changes (*e.g.* respiratory CO_2 production and C_2H_4 generation) are those which determine the time relation of the physico-chemical changes to the completion of the ripening process and hence to the storage life of the fruit.

In this study, ripening of fruit of the commercial cv SS was compared with that in two disease resistant hybrids of greater size, cvs T2 and RL recently introduced to the Caribbean from Taiwan. This was done by measuring C_2H_4 generation and CO_2 in the fruit cavity of the hollow berry as well as skin colour and flesh texture of the ripening fruit. It had previously been demonstrated that C_2H_4 generation in papaya occurred in the pericarp tissue of the fruit (Allong *et al.,* 2001). It is generally agreed that C_2H_4 plays a stimulatory role, albeit imprecisely defined, in fruit ripening, either by its increasing concentration or by increased sensitivity of mature-green fruit tissues to a threshold level of C_2H_4. In either case, the low fresh fruit weight/cavity volume ratio in cv RL (4.3 g mL^{-1}) could have delayed realization of the threshold level of C_2H_4 needed to stimulate ripening. However, whilst the high value of this ratio in cv SS (6.9 g mL^{-1}) could explain its more rapid ripening than cv RL, the lowest ratio (3.8 g mL^{-1}) and rapid ripening in cv T2 did not support the explanation of the influence of cavity dimensions on the ripening process. Moreover, autocatalytic production of C_2H_4 has also been considered to be a product of the ripening process. Notwithstanding the possible role of cavity volume in realized CO_2 and C_2H_4 accumulation and concentration, the effect of temperature on such accumulation differed for the two gases. Thus, at 28-30^{o}C no preclimacteric period was recorded for either gas in cvs SS and T2 but such periods were recorded for both gases in cv RL. Alternatively, at 20-22^{o}C (Fig. 2) preclimacteric periods occurred for CO_2 production in all three cultivars but only for C_2H_4 generation in cv RL. This could mean either that respiratory inhibition was affected to a greater extent by lowered temperature than C_2H_4 generation, leading to 2.33 to 2.66 times greater levels of C_2H_4 in cavities of cvs SS and T2 respectively than that in cv RL by 2 DAH at 20-22^{o}C (Fig. 2). Thus at this temperature, increase in C_2H_4 generation preceded that in respiration in cvs SS and T2 and hence could have stimulated the climacteric respiratory response in fruit of these cultivars.

Differences in CO_2 and C_2H_4 production rates among commercial lines of papaya were also reported in previous studies on "Kapoho" (Akamine and Goo, 1976, 1979), "Solo Sunrise"(Paull and Chen, 1983) and in "Taiping" and "Bentong" (Nazeeb and Broughton, 1978).

Even after optimal ripeness, cv RL had firm flesh which was judged to be ideal for fresh-cut purposes (Allong *et al.,* 2001).The

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slower softening rate of cv RL fruit would also allow for greater flexibility in harvesting and handling of the fruit throughout the marketing chain. The intensity of the orange skin colour and corresponding firmer flesh have the added advantage of allowing harvesters to pick fruits at a later stage when more sugar has accumulated. According to Chan *et al*. (1979) papaya fruits on the whole have no starch reserves for production of soluble sugars after harvest. Thus, a fruit that remains on the tree accumulates more sugars and develop a higher quality (Zhang and Paull, 1990). Although the mode of action of slower ripening in cv RL fruits is unknown it could provide material for generating commercial lines with longer shelf life as well as to conduct research on the genetic regulation of flesh softening in papaya ripening either through changes in pectin or pectolytic enzyme activity.

The negative temporal displacement of skin degreening, compared to softening, respiration and ethylene production rates in this study is in agreement with Zhang and Paull (1990) and appears to be unique when compared both with results of Grumet *et al.* (1981) and Tigchelaar *et al.* (1978) in tomatoes as well as those with Brecht *et al.* (1984) with nectarines. However, in view of the rapid degreening response of most fruits to low C_2H_4 concentrations, the apparent inhibition of degreening in our investigation could be due to the lower C_2H_4 concentration in the laboratory atmosphere compared with that in the fruit cavity.

The pH values decreased in all three cultivars during storage. This may be explained by the observation that papayas are one of the few fruits in which ascorbic acid content increased with ripening (Selvaraj *et al.*, 1982). However, storage temperature had no significant effect on these changes.

Fruit of three papaya cultivars, *cvs* SS, T2 and RL stored at the 20-22^{o}C temperature range showed distinct pre-climacteric phases, delayed climacteric peaks (2 days later) and ripened more slowly, than fruit stored at 28-30^{o}C as assessed by C_2H_4 generation and CO_2 production as indicated by the physico-chemical quality characteristics measured. Such fruit were also more attractive as judged by even colour, ripened firmness and reduced incidence of disease lesions. They were more likely to be acceptable to consumers. As a result, the 20-22^{o}C temperature range was used in the following experiments on the inhibition of ripening by 1-MCP. However, fruit of cv RL ripened more slowly than those of cvs SS and T2. This could have been induced by higher concentrations of C_2H_4 in the fruit cavity of cv RL than in that of cv SS; associated with a lower fresh fruit/cavity volume ratio in cv RL. However, the rapid ripening/low ratio characteristics of fruit of cv T2 did not support this interpretation.

The negative temporal displacement of skin degreening compared with C_2H_4 generation and CO_2 production and flesh softening was explained by the difference in C_2H_4 concentration between the fruit cavity and the laboratory environment. It was concluded that occurrence of preclimacteric C_2H_4 and CO_2 concentrations and climacteric peak levels of the fruit cavity were sensitive indicators of the progress of ripening in papaya fruit and may be used in studies of the effect of 1-MCP on ripening.

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Journal of Applied Horticulture, 12(1): 59-61, January-June, 2010

Effect of winter foliar application of urea on some quantitative and qualitative characters of flower and fruit set of orange cv 'Valencia'

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Abstract

Yield of fruit tree is determined primarily by flowering intensity and subsequent fruit set. Flower number and fruit set are also influenced by endogenous nitrogen level. This research was concerned with the effect of winter foliar application of urea on flowering and fruit-set of 30-year-old 'Valencia' orange on sour orange rootstock at Safiabad Agricultural Research Center of Dezful. Treatments included urea foliar application at 3 levels (0, 0.5, 1%) and 2 times of application (6 and 9 weeks before full bloom). The experimental design was a factorial randomize complete block with 4 replications. Nitrogen percentage in leaf, flower number, ovary diameter and fruit set were studied. Results showed that winter application of urea increased the level of N for 2 weeks. Different levels of urea increased the number of flowers, ovary diameter and fruit set. The higher concentration of urea (1%) had more effect. Considering the time of application, urea spray 9 weeks before full bloom had the highest effect on flowering but urea spray 6 weeks before full bloom resulted in higher ovary diameter and fruit set.

Key words: Nitrogen, flowering, fruit set, 'Valencia' Orange

Introduction

Flowering is the most important physiological process in fruit trees. Citrus yield can be closely related to total number of flowers in spring bloom even though most of the flowers don't set fruit that remain until harvest (Moss, 1971). Nitrogen could influence the number of flowers and fruit set (Albrigo, 1999). During flowering and fruit set, high nutrient demand occur and also the soil temperature is low. Low soil temperature reduces root metabolic activity, solubility of nutrients in the soil solution and nutrient transport stream (Lovatt, 1999). During and before flowering, the trees may not be able to translocate sufficient major nutrients (nitrogen, phosphorus, and potassium) for the need of up to 50,000 to 100,000 flowers per tree (Erickson and Brannaman, 1960). Therefore, at low N situation, the pre-blossom urea spray augments the reserve N pool since it is readily accessible and metabolizable (Rabe, 1994).

Researches with citrus have provided evidence of a relationship between ammonium, its metabolites and flowering and fruit set. Leaf concentrations of several C and N compounds before, during and after low temperature or water deficit stress treatments have been quantified in 5-year-old rooted cuttings of 'Washington' navel orange and commercially producing 16-year-old 'Frost Lisbon' lemon trees that were designed to induce flowering (Lovatt *et al.,*1988a; 1988b). Ali and Lovatt (1994) showed mid-January and mid-February application of low biuret urea to 30-year-old 'Washington' navel orange trees, significantly increased yield per tree above the control trees receiving soil applied urea. Mid-January foliar application of low biuret urea significantly increased the number of fruit per tree over the control trees.

It was demonstrated by El-Otmani *et al.*(2004) that urea application to the foliage of 'Cadox' clemantine mandarin trees during the periods of flower initiation-differentiation, fruit set and 'June drop', lead to increased leaf area, leaf specific dry weight, leaf N level and total yield. The yield increase was due to an increase in both, fruit number per tree and fruit size.

The purpose of this study was to evaluate the effect of urea spray (rate and time of application) as a source of nitrogen on flowering and fruit set of 'Valencia' orange.

Materials and methods

Location: The experimental orchard was located in the Safiabad Agricultural Center of Iran, characterized by a subtropical climate with rainfall of 300-400 mm per year with mild winter (10-12°C) and warm summer (35-45°C).

Plant material: Thirty-year-old 'Valencia' orange trees on sour orange rootstock were used. The factorial experiment was arranged in randomize complete block design with 4 replications. Treatments were foliar application of urea in 3 rates (0, 0.5, 1%) and 2 times of application (23 January, 13 February 2006, 9 and 6 week before full bloom, respectively). Urea (46% N) was applied to the foliage to the point of runoff.

Physiological parameters: To determine leaf total N concentration, 30 old spring-cycle leaves from nonfruiting terminals shoots were collected per tree weekly, from 9 weeks before full bloom until 1 week after full bloom. Leaves were washed with 1% HCl and rinsed thoroughly with distilled water and oven dried at 60°C. Total N was determined using micro-kjehldahl method (Ali and Lovatt, 1994).

Flowers and fruits were counted on 10 randomly selected shoots (all sides of the tree), at full bloom and after June drop (10 July), respectively. Percentage of fruit set was calculated by:

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Fruit set (%) = (Number of fruits/ Number of flowers) ×100

To determine ovary diameter, before full bloom, 100 flowers were collected from each tree and ovary diameter was measured by digital slide caliper.

Statistical analysis: Data were subjected to an analysis of variance and mean separation was carried out using Duncan's multiple range test at $P<0.01$.

Results and discussion

Leaf nitrogen concentration: From the beginning of experiment on January 23. (first week from the beginning of experiment) until full bloom on March 28 (tenth week from the beginning of experiment) there was a loss of N from the old leaves (Figs.1 and 2).

As most commercial citrus cultivars are evergreen, leaves are the major source of N that support spring and summer growth flushes, since root uptake may not be able to fulfill immediate growth, flowering and fruit set requirement at this time. Citrus leaves, therefore, constitute a major reserve of mobile N (Lea-Cox *et al.*, 2001).

Winter urea sprays markedly increased leaf N level for a short time, second and third weeks from the beginning of experiment (Fig. 1) and fifth and sixth weeks from the beginning of experiment (Fig. 2). This transient increase in the leaf N status has been reported by Rabe (1994). Following application, urea rapidly accumulates in the leaf and transform into amino acids and proteins in order to function as a N source in the citrus trees (Sirvastava and Singh, 2003).

Flower opening (tenth week from the beginning of experiment), period of high nutrient demand (Lovatt, 1999), coincides with minimum N values in the leaves (Figs.1and 2). There is a competition for nutrient between flowers, fruitlets and newly developing spring leaves. This agrees with earlier results of Sanz *et al.* (1987) who reported that mineral elements (N, P, K) in old leaves of 'Washington Navel' orange decreased during the spring flush of growth and reached minimum values at flower opening, coinciding with a peak in flower abscission.

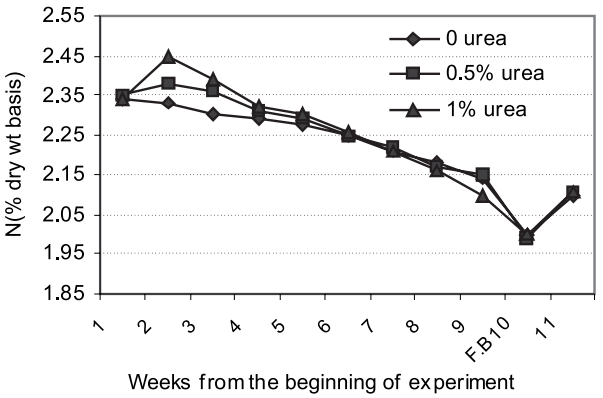

Fig.1. Changes in leaf nitrogen (urea spray: 9 weeks B.F.B)

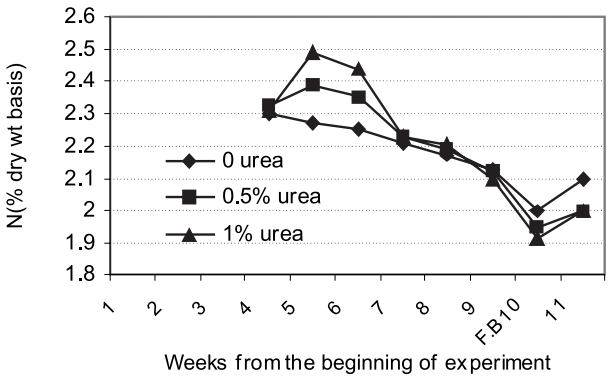

Fig.2. Changes in leaf nitrogen (urea spray: 6 weeks B.F.B)

Urea spray effect on flowering, fruit set and ovary diameter: Foliar urea spray at 1 and 0.5% concentration significantly ($P<0.01$) affected growth parameters. The greatest number of flower, fruit set and ovary diameter were by 49.2 flower m^{-1} shoot length, 2.32 mm and 2.99%, respectively at 1% urea treatment (Table 1).

Application of urea, 9 weeks before full bloom significantly ($P<0.01$) increased flower number by 24.28% compared to 6 weeks before full bloom (Table 2). But urea spray, 6 weeks before full bloom significantly ($P<0.01$) increased fruit set and ovary diameter by 16.83 and 50% compared to 9 weeks before full bloom (Table 2). The combined effects of urea concentration and time of application treatments are shown in Table 3.

The larger ovary diameter (2.66mm), flower number (57.8 flower m^{-1} shoot length) and fruit set (3.7%) were obtained by urea spray at 1% concentration, 6 and 9 weeks before full bloom, respectively (Table 3).

Moallemi and Dadpour (2004) showed that flower bud differentiation in 'Valencia' orange at Safiabad starts in February. Therefore, urea spray 9 and 6 weeks before full bloom (23 January, 13 February) in this experiment coincide with flower bud induction and differentiation, respectively. In addition to low temperature in the region, which is the most important climatic stress factor inducing citrus flowering, urea application resulted into increased number of flower. Urea spray, 6 weeks before full bloom, at differentiation stage, promotes ovary growth.

Winter foliar fertilizer application increases flowering, fruit set and yield, because essential nutrients for flowering and fruit set are limiting due to reduced transpiration and nutrient acquisition by roots when air and soil temperatures are low (Lovatt,1999). Earlier researches provided evidence that foliar urea applied during or after low temperature or water deficit period increased citrus flowering by elevating the ammonia metabolites status of the tree (Lovatt *et al.,* 1988a, 1988b) and increased the polyamine content, growth rate and size of developing citrus fruit as well as their potential to set (Lovatt *et al.*, 1992).

Following application, urea is transformed into amino acids specially arginine and polyamines (Srivastava and Singh, 2003). Polyamines are involved in many plant developmental processes, including cell division, embryogenesis, reproductive organs development, floral initiation and development and fruit development. They stimulate DNA replication, transcription and translation (Kaur-Sawhney *et al.*, 2003). Evidences indicate a close connection between polyamines and reproductive development in olive (Diose *et al.*, 2006), damson plum (Prista *et al.*, 2004) and *Arabidopsis thaliana* (Applewhite *et al.*, 2000).

Results of our experiment demonstrated that winter application of urea in January or February significantly increased leaf nitrogen status, compared to control trees. Urea spray increased

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Table 1. Effect of urea concentration on flowering, ovary diameter and fruit set

Urea concentration (%)	Number of flower (flower m^{-1} shoot length)	Ovary diameter (mm)	Fruit set (%)
0	32.73[b]	1.92[b]	1.25[b]
0.5	43.10[a]	2.16[a]	1.99[b]
1.0	49.20[a]	2.32[a]	2.99[a]

Means within a column with the same letter are not significantly different by Duncan's multiple range test (P<0.01)

Table 2. Effect of time of urea spray on flowering, ovary diameter and fruit set

Time of urea application	Number of flower (flower m^{-1} shoot length)	Ovary diameter (mm)	Fruit set (%)
9 weeks B.F.B.[1]	46.25[a]	1.96[b]	1.66[b]
6 weeks B.F.B.	37.10[b]	2.29[a]	2.49[a]

Means within a column with the same letter are not significantly different by Duncan's multiple range test (P<0.01). Before Full Bloom

Table 3. The combined effects of urea concentration and time of application treatments on flowering, ovary diameter and fruit set.

Parameter	Time of application (weeks B.F.B.)	Urea concentration		
		Control	0.5%	1%
Flower number (flower m^{-1} shoot length)	9 weeks	33.85[cd]	47.10[b]	57.80[a]
	6 weeks	31.36[d]	39.10[c]	40.60[bc]
Ovary diameter (mm)	9 weeks	1.93[c]	2.00[c]	1.98[c]
	6 weeks	1.91[c]	2.32[b]	2.66[a]
Fruit set (%)	9 weeks	1. 32[c]	1.46[c]	2.29[b]
	6 weeks	1.27[c]	2.52[b]	3.70[a]

Means within a column with the same letter are not significantly different by Duncan's multiple range test (P<0.01)

the number of flowers, ovary diameter and fruit set. The highest concentration of urea (1%) had the highest effect. Considering the time of application, urea spray, 9 weeks before full bloom, had the highest effect on flowering but urea spray 6 weeks before full bloom resulted in higher ovary diameter and fruit set. It seems increase in leaf N as a result of urea spray in January or February promotes flower induction (at induction stage) and ovary growth (at differentiation stage), respectively, that lead to increased flowering and fruit set. Therefore, it is important to determine the optimal level and time of application of nutrients to stimulate a specific physiological process in ctrus.

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Journal of Applied Horticulture, 12(1): 62-64, January-June, 2010

Management of root-knot nematode (*Meloidogyne incognita* (Kofoid and White) Chitwood) in ashwagandha (*Withania somnifera* Dunal.) and senna (*Cassia angustifolia* Vahl.) using non-chemicals

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Abstract

Experiments were conducted for the management of root knot nematode, *Meloidogyne incognita* using non-chemicals under controlled and field conditions in medicinal crops *viz.,* ashwagandha (*Withania somnifera*) and senna (*Cassia angustifolia*). All the treatments comprising of bioagents, organic amendments and humic acid were effective to suppress *M. incognita* population and to increase the plant biomass and yield of economic parts of these crops. Among the treatments, the use of plant growth promoting rhizobcaterium, *Pseudomonas fluorescens* available commercially in talc formulation (2.6 X 10^6 cfu g^{-1}) at 2.5 kg ha^{-1} as soil application recorded the lowest nematode population accompanied with highest economic yield.

Key words: *Cassia angustifolia, Meloidogyne incognita, Pseudomonas fluorescens, Withania somnifera,* non-chemicals

Introduction

In recent years, there has been an increased interest in the cultivation of medicinal plants to meet the requirements of pharmaceutical and cosmetic industries and to earn foreign exchange through export (Husain, 1983). Attention has also been given on plant parasitic nematodes which have been recognized as serious constraints in the productivity of medicinal plants (Alam *et al.,* 1978; Butool and Hasseb, 1993). Pandey (1998) proved that the root knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood is widespread in medicinal crops and causes both qualitative and quantitative yield loss. Hence, the present study was undertaken for the management of *M. incognita* using non-chemicals considering the export value of the medicinal crops *viz.,* ashwagandha (*Withania somnifera* Dunal.) and senna (*Cassia angustifolia* Vahl.).

Materials and methods

Glasshouse experiment: The seeds, surface sterilized with mercuric chloride (0.5%), were sown in pots (10 kg capacity) filled with steam sterilized pot mixture. The established plants were thinned to two per pot at 21 DAS (days after sowing) followed by the inoculation of second stage juveniles (J2) of *M.*

Table 1. Management of *M. incognita* in Ashwagandha (*W. somnifera*) under glasshouse conditions

Treatments	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Economic yield of root weight per plant (g)	No. of galls g^{-1} of root	No. of females g^{-1} root	No. of eggs per egg mass	Gall index	Soil nematode population (200 cc)
T_1 - Soil application of *P. fluorescens* @ 2.5 kg ha^{-1}	52.1a	55.7a	22.5a	10.7a	2.0a	1.0a	186.7a	1.0a	45.7a
T_2 - Soil application of *T. viride* @ 2.5 kg ha^{-1}	45.0b	49.0b	16.6b	7.0cde	2.7a	1.7a	220.0b	1.7a	65.7a
T_3 - Combined application of *P. fluorescens* + *T. viride* (each @ 1.25 kg ha^{-1})	49.7a	53.7a	20.8a	9.0b	2.3a	1.3a	190.0a	1.3a	54.0ab
T_4 - Soil application of neem cake @ 1 ton ha^{-1}	43.3bc	40.7c	12.4c	8.3bc	8.0b	8.7b	266.7c	3.3b	136.7b
T_5 - FYM with recommended dosage of fertilizers	43.4bc	34.7de	11.6cd	7.7bcd	9.3bc	9.0bc	290.0cd	3.7bc	313.3c
T_6 – Soil drenching with 4% Panchkavya	41.5cd	35. 3d	13.6bc	6.7de	10.7cd	9.7bc	300.0d	4.3cde	336.7c
T_7 – Foliar application of Panchkavya @ 4%	40.0d	30.3f	11.9cd	6.0e	11.3cd	10.0c	296.7d	4.7de	366.7c
T_8 – Application of humic acid at 1%	36.9e	30.7ef	12.3c	5.7e	12.7d	10.0c	283.3cd	4.0bcd	293.3c
T_9 - control	30.6f	20.3g	8.4d	3.3f	18.3e	16.0d	336.7e	5.0e	536.7d

Means in columns followed by the same letter are statistically not significant (Duncan's multiple range test, P=0.05)

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incognita (one g^{-1} soil). The treatments were given as furnished in Table 1. All the treatments were replicated thrice in a completely randomized design and watered regularly.

Observations were made on yield attributes at the time of termination of the experiment at 180 DAS. Nematode population was assessed in soil and roots in terms of number of galls and females g^{-1} of root and number of eggs per eggmass (Cobb, 1918; Schindler, 1961; Taylor and Sasser, 1978).

Field experiments: The above experiments were conducted in nematode prone area under field conditions with initial population of *M. incognita* at more than one J2 g^{-1} soil, to confirm the results of earlier experiments. The plot size was 9 m^2 and the design adopted was RBD. The data collected were subjected to statistical analysis (Gomez and Gomez, 1984).

Results and discussion

All the treatments recorded significantly higher growth parameters *viz.,* shoot length and weight, root length and weight as economic yield by lowering the nematode population under glasshouse as well as field conditions in the present study. Among the treatments, *P. fluorescens* at 2.5 kg ha^{-1} as soil application at 21 DAS registered the lowest nematode population in soil and roots and the highest growth parameters and economic yield of the crop. It was followed by the combined application of *P. fluorescens* + *T. viride* (each at 1.25 kg ha^{-1}). The other non-chemicals *viz.,* neem cake, FYM, panchakavya and humic acid evaluated in the present study were also effective but not as effective as bioagents *viz., P. fluorescens* and *T. viride* in the suppression of *M. incognita* population and to improve the plant growth (Table 1 to 4).

Table 2. Management of *M. incognita* in Ashwagandha (*W. somnifera*) under field conditions

Treatments	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Economic yield of root weight per plant (g)	No. of galls g^{-1} of root	No. of females g^{-1} root	No. of eggs per egg mass	Gall index	Soil nematode population (200 cc)
T_1 - Soil application of *P. fluorescens* @ 2.5 kg ha^{-1}	66.7a	43.3a	27.7a	15.0a	2.0a	1.0a	186.7a	1.0a	45.0a
T_2 - Soil application of *T. viride* @ 2.5 kg ha^{-1}	61.7a	40.7a	24.0b	14.0ab	2.7a	1.3a	203.3a	1.3a	63.3ab
T_3 - Combined application of *P. fluorescens* + *T. viride* (each @ 1.25 kg ha^{-1})	65.0a	43.3a	26.3a	14.3a	2.3a	1.3a	193.3a	1.0a	51.7ab
T_4 - Soil application of neem cake @ 1 ton ha^{-1}	60.0a	38.3ab	22.3b	12.7b	9.0bc	8.0bc	263.3b	3.3b	126.7b
T_5 - FYM with recommended dosage of fertilizers	43.3b	33.3bc	19.0c	10.7c	10.7cd	10.0cd	290.0bcd	4.3c	306.7c
T_6 – Soil drenching with 4% Panchkavya	43.3bc	31.7c	18.0cd	10.0cd	10.0bcd	8.7bc	290.0bcd	5.0d	326.7c
T_7 – Foliar application of Panchkavya @ 4%	41.7c	31.7c	18.7cd	10.0cd	10.3bcd	9.0bc	293.3cd	5.0d	360.0c
T_8 – Application of humic acid at 1%	45.0bc	39.3bc	19.7c	10.7c	8.0b	7.3b	273.3bc	4.7cd	300.0c
T_9 - control	41.7c	30.0c	17.0d	9.00d	12.3d	12.0d	306.7d	5.0d	533.3d

Means in columns followed by the same letter are statistically not significant (Duncan's multiple range test, P=0.05)

Table 3. Management of *M. incognita* in Senna (*C. angustifolia*) under glasshouse conditions

Treatments	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Economic yield per plant		No. of galls g^{-1} of root	No. of females g^{-1} root	No. of eggs per egg mass	Gall index	Soil nematode population (200 cc)
					Leaf (g)	Pod (g)					
T_1 - Soil application of *P. fluorescens* @ 2.5 kg ha^{-1}	50.2a	14.4a	26.5a	7.2a	122.6a	65.8a	6.7a	4.3a	233.3a	1.3a	23.3a
T_2 - Soil application of *T. viride* @ 2.5 kg ha^{-1}	41.0b	13.0bcd	22.1c	6.7bc	118.4bc	61.1c	8.3a	5.0a	256.7b	2.3ab	66.7bc
T_3 - Combined application of *P. fluorescens* + *T. viride* (each @ 1.25 kg ha^{-1})	46.8a	13.7ab	24.7b	7.0ab	120.2ab	63.4b	7.3a	4.3a	245.0b	1.7a	36.7ab
T_4 - Soil application of neem cake @ 1 ton ha^{-1}	41.5b	12.7cd	20.6cd	6.4c	116.3cd	60.6c	13.0b	11.7b	300.0bc	3.3bc	100.0c
T_5 - FYM with recommended dosage of fertilizers	38.4bc	12.5cd	12.3ef	5.8d	114.1de	57.9d	13.3cd	15.7c	286.7bc	4.0cd	243.3d
T_6 – Soil drenching with 4% Panchkavya	36.6c	13.2bc	18.2f	5.3e	112.9e	55.5e	18.3d	16.3c	310.0bc	4.7d	306.7e
T_7 – Foliar application of Panchkavya @ 4%	39.1bc	13.1bc	19.0def	5.9d	116.3cd	59.4cd	16.0c	17.3c	280.0bc	4.3cd	280.0de
T_8 – Application of humic acid at 1%	35.4c	12.3d	19.9de	5.5de	115.6cde	58.4d	17.0cd	16.0c	276.7bc	4.3cd	300.0e
T_9 - control	30.7d	10.2e	16.1g	3.7f	104.4f	50.7f	23.0e	20.7d	323.3c	5.0d	430.0f

Means in columns, followed by the same letter are statistically not significant (Duncan's multiple range test, P=0.05)

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Table 4. Management of *M. incognita* in Senna (*C. angustifolia*) under field conditions

Treatments	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Economic yield per plant		No. of galls g^{-1} of root	No. of females g^{-1} root	No. of eggs per egg mass	Gall index	Soil nematode population (200 cc)
					Leaf (g)	Pod (g)					
T_1 - Soil application of *P. fluorescens* @ 2.5 kg ha^{-1}	58.7a	18.5a	31.7a	9.6a	223.3a	76.7a	6.3a	5.0a	230.0a	1.7a	15.0a
T_2 - Soil application of *T.viride* @ 2.5 kg ha^{-1}	53.2b	15.5c	28.7b	9.2ab	171.7b	73.3a	6.7a	5.0a	246.0ab	2.3b	60.0a
T_3 - Combined application of *P. fluorescens* + *T.viride* (each @ 1.25 kg ha^{-1})	52.2bc	16.9b	29.3b	8.7bc	176.7b	73.3a	6.0a	4.3a	170.7a	2.0ab	46.7a
T_4 - Soil application of neem cake @ 1 ton ha^{-1}	50.2c	16.7b	25.3c	8.3c	150.0c	65.0b	13.3b	12.0b	266.7bc	4.0c	168.3b
T_5 - FYM with recommended dosage of fertilizers	45.2de	13.5d	24.7cd	7.2d	120.0d	65.0b	17.0bc	15.0bc	211.0de	5.0e	340.0c
T_6 – Soil drenching with 4% Panchakavya	47.12d	13.5d	24.0cd	7.0d	125.0d	46.7c	18.0c	16.3cd	266.7cd	4.7de	366.7c
T_7 – Foliar application of Panchakavya @ 4%	44.2e	13.0de	23.3d	6.7 e	120.0d	45.0c	18.7c	17.7cd	280.0cd	5.0e	356.6c
T_8 – Application of humic acid at 1%	47.2d	13.3d	24.7cd	6.9de	121.7d	45.0c	17.0bc	16.7cd	286.7cd	4.7cd	326.6c
T_9 - control	41.3f	12.3e	19.7e	6.2e	118.3d	43.3c	20.7c	19.3d	313.3e	5.0e	426.6d

Means in columns followed by the same letter are statistically not significant (Duncan's multiple range test, P=0.05)

Earlier, several studies have proved the efficacy of *P. fluorescens* in suppressing *M. incognita* in many field crops *viz.,* tomato (Jonathan *et al.,* 2000), chickpea (Khan *et al.,* 2001) and turmeric (Srinivasan *et al.,* 2001). Several mechanisms were attributed to the suppression of phytonematodes by the application of *P. fluorescens* such as induced systemic resistance, production of antibiotics and siderophores, competition for nutrients and alteration of specific root exudates such as polysaccharides and amino acids which modify nematode behaviour (Oostendorp and Sikora, 1990; Aalten *et al.,* 1998).

Thus, the present study indicated that the use of *P. fluorescens* treatment is effective to reduce *M. incognita* population and to enhance the yield. Hence *P. fluorescens* can be used as an efficient and eco-friendly bionematicide in ashwagandha and senna.

Acknowledgement

The authors are grateful to the National Medicinal Plants Board, New Delhi for providing the funds to carry out the study.

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Journal of Applied Horticulture, 12(1): 65-68, January-June, 2010

Studies on the suitability of cling-stone and free-stone low chilling peach cultivars for canning and other processed products

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Abstract

Two cling stone peach cultivars "Shan-i-Punjab" and "Tropic Beauty" and one free stone cultivar "Florda Grande" were processed for canning (whole and halves) and beverages (squash and nectar). Fruits of Shan-i-Punjab were found to have the best characteristics for canning (as whole) and for making pulp based beverages (nectar and squash) with maximum ascorbic acid content (17 mg/100 g) and pulp yield (47.62%). Organoleptically Shan-i-Punjab fruits were found to have the highest acceptability scores *i.e.* 8.50 for canned peaches, 8.58 for squash and 8.38 for nectar among all the cultivars studied on a 9-point Hedonic scale judged by eight semi-trained panelists and general consumers. Florda Grande also received the higher acceptability scores (8.50) for its canned peach halves because of its tough texture. The fruits of Florda Grande were not much suitable for pulp based beverages, whereas those of Tropic Beauty were found acceptable for canning as well as for making beverages at zero time and also after six months storage.

Key words: Cling stone, peach, Shan-i-Punjab, cultivar, canning

Introduction

Peach (*Prunus persica* (L) Batsch.) is a popular temperate zone fruit, but with the introduction of high quality and heavy bearing low chilling peach cultivars, it has become possible to grow it in subtropical areas of Punjab, Haryana, Delhi and Western U.P. In Punjab, peach is grown over an area of 1367 hectares with the production of 20505 metric tones of fruit annually (Anonymous, 2007). In India, stone fruits are cultivated on nearly 0.31 million ha area with annual production of 2.52 million tonnes and average productivity of 8.13 tonnes/ha (2002-03). As per FAO production data 2006, peach production in India was 1.5 lakhs tonnes.

The demand for stone fruits and their processed products has increased because of rise in health concerns and nutritional awareness. The demand of these fruits is expected to improve further in near future. Peach is an excellent source of minerals like potassium, phosphorus and iron and a good source of vitamins and low calorific diet (Gopalan *et al.*, 1996). Peach comes in the market for a short period of time from end of April to May and moreover the fruit is highly perishable at ambient temperature. Its palatability and utilization can be increased by processing fruit either into canned form or into different processed products. Some work has been done on canning of peaches (Aggarwal *et al.,* 1992, Srivastava and Arora, 1994) but the studies are not available on the processing suitability of these cultivars into canned and pulp based products.

Therefore, the present investigation was carried out with the objective to find out the suitability of cling stone and free stone peach varieties for canning and other processed products. Selected varieties may be propagated on a wider scale for distribution to the farmers for sufficient production to supply processing industry and to develop some new products from peaches for domestic and export markets.

Materials and methods

Peach varieties, two cling stone "Shan-i-Punjab" and "Tropical Beauty" and one free stone "Florda Grande", were procured from Department of Horticulture, PAU, Ludhiana and were processed into canned peaches (whole and halves) and beverages (squash and nectar).

Canning: Selected hard matured and well riped fruit of all the peach varieties weighing 20 and 25 kg each were washed under running water with gentle rubbing of the skin with hands. The fruit was lye peeled in 1% solution for 1 minute (which was pre standardized by using various concentrations of lye solution *i.e.* 0.5, 1.0, 1.5 and 2% for 1 and 2 minutes of boiling. After 1 minute treatment of fruit in boiling lye solution, it was immediately cooled under running water followed by a dilute acid treatment. Peeled fruits were filled in pre sterilized A 2-1/2 tall cans. The fruits were covered with hot syrup (40 and 50°B). Cans were steam exhausted until the temperature in the center of the cans reached 85°C and sealed immediately. Cans were boiled in water for 30 minutes and were quickly cooled after processing under running water.

Pulp: Pulp from all the peach varieties was extracted by superfine pulper (Raylons, India). Two extractions were made from each variety to have better pulp yield, which was stored for further use with 2000 ppm of potassium meta bisulphite. The pulp was further used for making squash and nectar.

Nectar: Sugar syrup was prepared in a stainless steel jacketed kettle. Pulp (20%) was added into the sugar syrup which was then strained through a muslin cloth. Total soluble solids were adjusted to 15° B. Acidity of nectar was adjusted to 0.32 % with the addition of citric acid. 200 mL capacity bottles were filled with peach nectar at the temperature of 80-82°C. Bottles were corked

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and processed in boiling water for 20 minutes. These were then air cooled and stored at room temperature for use.

Squash: Syrup was prepared by mixing and heating sugar, acid and water. Total soluble solids were adjusted to 45°B. The mixture was filtered through muslin cloth. Pulp was added to the sugar syrup after cooling. Potassium meta bisulphite was added in the squash after dissolving in small amount of squash and then mixing in bigger lot. Squash was filled into 650 mL capacity bottles and crown corked. The following recipes were used for the preparation of squash and nectar from the pulp of all the peach varieties.

Standardized recipes for peach squash and peach nectar

Sr. No.	Ingredients	Quantity (Approx)	
		Squash	Nectar
1	Juice	1.00 kg	1.00 kg
2	Sugar	1.70 kg	0.65 kg
3	Water	1.25 kg	3.30 kg
4	Citric acid	41.0 g	9.00 g
5	Potassium meta bisulphite	700 ppm	-

Analytical Methods: Peach pulp of all the varieties was analysed for total solids, total soluble solids, acidity, ascorbic acid, total sugars and reducing sugars (Ranganna,1994). Cut out analysis of canned peaches was done. Canned peaches, squash and nectar were analysed for their sensory parameters by a panel of eight semi-trained judges periodically on the basis of 9 point hedonic scale (Amerine *et al.*, 1965). Sensory evaluation was also done by general consumers on a simple format. Results were analysed statistically for their interpretation using completely randomized design experiment as discussed by Cochran and Cox (1957).

Results and discussion

All the varieties *i.e.* Shan-i-Punjab, Florda Grande and Tropical Beauty were processed into canned and bottled products. Data regarding the various chemical and sensory parameters of the peach products are presented in Tables 1 to 5.

Canned peaches: In cut out analysis of canned peaches, external and internal conditions of the cans were found satisfactory (Table 1). No swelling and rusting was observed in any can. Vacuum in cans ranged between 10 inch to 11 inch Hg. Head space was also within permissible limits. Drained weight of the fruit ranged from 64.4 to 50 per cent, thus meeting the FPO specifications.

Canned whole peaches from variety Shan-i-Punjab were scored the highest for appearance (8.63) whereas peach halves from variety Florda Grande (free stone variety) were scored the highest for its texture (8.63) and taste (8.50) by a panel of eight semi-trained judges at zero time. Fruits of Shan-i-Punjab were quite big and of bright yellow colour which was liked by most of the consumers (semi-trained panel and general consumers). After six months storage the texture of the two varieties Shan-i-Punjab and Florda Grande was found the same, and both the varieties were very much liked by the panel. A simple proforma was prepared to take the views of general consumers. Excellent remarks were received for Florda Grande and Shan-i-Punjab and good for Tropical Beauty.

Table 2. Effect of variety and storage on the organoleptic evaluation* of canned peaches

Variety	Storage (months)	Appearance	Texture	Taste	Overall mean
Shan-i- Punjab	0	8.63	8.50	8.38	8.50
	3	8.50	8.38	8.25	8.38
	6	8.38	8.38	8.25	8.34
	Mean	8.50	8.42	8.29	8.41
Tropical Beauty	0	7.38	7.50	7.50	7.46
	3	7.25	7.38	7.38	7.34
	6	7.25	7.13	7.25	7.21
	Mean	7.29	7.33	7.38	7.34
Florda Grande	0	8.38	8.63	8.50	8.50
	3	8.50	8.50	8.38	8.46
	6	8.25	8.38	8.25	8.29
	Mean	8.38	8.50	8.38	8.42
LSD (*P*=0.05)					
Varieties		0.33	0.34	0.35	0.09
Storage		NS	NS	NS	0.09

*Mean values of eight panelists.

Table 3. Effect of varieties on the yield and *bio-chemical parameters of peach pulp

Variety	Yield (%)	TS (%)	TSS (°B)	Acidity (%)	Ascorbic acid (mg/100g)	Total sugars (%)	Reducing sugars (%)
Shan-i-Punjab	47.62	10.04	8.2	0.75	17.00	5.61	5.08
Tropical Beauty	46.06	13.41	11.5	0.76	5.00	7.56	6.69
Florda Grande	30.0	14.25	11.5	0.73	3.10	6.78	6.14

* Mean of three values.

Pulp: Shan-i-Punjab gave the highest percentage of pulp yield *i.e.* 47.62% among all the varieties (Table 3). Ascorbic acid was found to be maximum in Shan-i-Punjab *i.e.* 17.00 mg/100g. Florda-Grande gave the lowest pulp yield *i.e.* 30% because it was quite hard textured variety, which was not found much suitable for making pulp.

Squash: Squash prepared from Shan-i-Punjab had bright yellow colour whereas Florda Grande was pale yellow which caused lowering of scores for appearance (Table 4). Squash from Shan-

Table 1. Cut out analysis* of canned peaches from different varieties

Variety	Can wt (g)	Drained wt (g)	Syrup wt (g)	Drained wt (%)	Syrup wt (%)	Vacuum (Hg")	Head space (cm)	TSS (°B)	Acidity (%)
Shan-i-Punjab	986.50	561.5	309.00	64.40	35.50	11	1.20	27.2	0.50
Tropical Beauty	955.70	421.1	420.60	50.05	49.95	11	1.45	27.2	0.44
Florda Grande	961.30	421.9	467.70	50.25	49.74	10	1.40	27.2	0.37

*Average of three values.

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Table 4. Effect of variety and storage on the organoleptic quality* of squash

Variety	Storage (months)	Appearance	Flavour	Texture	Overall mean
Shan-i- Punjab	0	8.50	8.75	8.50	8.58
	3	8.25	8.50	8.25	8.33
	6	8.38	8.25	8.38	8.25
	Mean	8.38	8.50	8.46	8.42
Tropical Beauty	0	8.13	8.25	8.25	8.21
	3	8.00	8.13	8.13	8.09
	6	7.75	7.88	8.25	7.96
	Mean	7.96	8.08	8.21	8.09
Florda Grande	0	7.00	8.25	8.25	7.83
	3	6.88	8.00	8.38	7.75
	6	6.13	7.63	8.25	7.34
	Mean	6.67	7.96	8.29	7.64
LSD(P=0.05)					
Varieties		0.27	0.29	NS	0.52
Storage		0.27	0.29	NS	NS

*Mean values of eight panelists.

Table 5. Effect of variety and storage on the organoleptic quality* of peach nectar

Variety	Storage (months)	Appearance	Texture	Flavour	Overall mean
Shan-i- Punjab	0	8.38	8.25	8.38	8.50
	3	8.21	8.00	8.25	8.38
	6	8.17	7.75	8.25	8.50
	Mean	8.25	8.00	8.29	8.45
Tropical Beauty	0	8.00	8.00	7.88	8.38
	3	8.04	8.25	7.63	8.25
	6	7.83	7.50	7.75	8.25
	Mean	7.99	7.92	7.75	8.29
Florda Grande	0	7.96	7.38	8.00	8.50
	3	7.63	6.25	8.25	8.38
	6	7.21	5.50	7.75	8.38
	Mean	7.60	6.38	8.00	8.42
LSD(P=0.05)					
Varieties		0.32	0.27	NS	NS
Storage		0.32	NS	NS	NS

*Mean values of eight panelists.

i-Punjab was scored the highest for its appearance (8.50) and flavour (8.75). Other two varieties were also found acceptable by a panel of eight judges with 8.21 overall mean scores for Tropical Beauty and 7.83 scores for Florda Grande. During storage of squash, appearance scores were found to decrease significantly ($P \leq 0.05$) in case of Florda Grande and Tropical beauty. Appearance of squash prepared from Florda Grande was not appreciable therefore scored the lowest with 6.13 scores after six months storage whereas Shan-i-Punjab was very much acceptable with 8.38 scores for appearance and 8.25 for flavour. Squash from Tropical Beauty was also liked by the panel with 7.75 score for appearance and 7.88 for flavour even after six months storage. There was no significant ($P \leq 0.05$) change in the body of the squash prepared from three cultivars neither as fresh nor during storage.

Table 6. Cost of products (cost per can/ bottle in Rs)

Ingredients	Can	Nectar	Squash
Fruit	6.0	0.80	3.25
Sugar	4.0	0.60	4.80
Citric-acid and other chemicals	0.10	0.10	0.10
Empty bottles / can	10.00	1.70	5.00
Processing charges including depreciation cost	1.00	0.30	0.30
Total	21.10	3.50	13.45

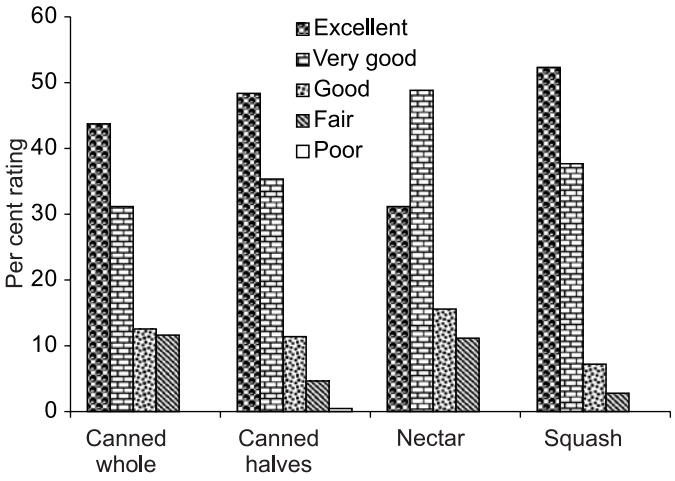

Fig.1. Percent rating of general consumer acceptance for peach products

Nectar: Significant ($P \leq 0.05$) difference in appearance and flavour scores of the nectar prepared from three cultivars of peach was found. Nectar prepared from Shan-i-Punjab had highest scores *i.e.* 8.25 for appearance and 8.38 for flavour. Nectar from Florda Grande was scored the lowest in its appearance (7.38) because of its dull yellow colour. Tropical Beauty was also liked very much with 8.0 scores for appearance and 7.88 for flavour by the panel of eight judges. Storage showed significant ($P \leq 0.05$) decrease in the appearance scores of nectar prepared from three cultivars, however it remained acceptable during six months storage. Nectar from Shan-i-Punjab was liked very much even after six months storage with 8.25 scores for flavour and 7.75 for appearance. After six months storage nectar from Florda Grande was scored quite low for its appearance *i.e.* 5.5 but it was acceptable due to its flavour with 7.75 scores. Nectar from Tropical Beauty did not show much decrease in sensory scores during storage. However. the maximum liking was for nectar prepared from Shan-i-Punjab. No significant effect of storage was found on the flavour, body and overall acceptability of nectar prepared from three cultivars.

General consumer acceptance: Samples of canned peach halves of Florda grande, canned whole peaches, nectar and squash of cultivar Shan-i-Punjab were fed to around 200 general consumers to judge their preferences for the products. Canned peach halves and wholes were rated excellent by 48.3, 43.75%, very good by 35.28, 31.25% and good by 11.37, 12.5 % and fair by 4.55, 11.60% consumers. 52.3 and 31.1 % consumers rated squash and nectar as excellent drinks, respectively. None of the consumers rated squash and nectar as a poor drink (Fig 1.). Cost of the products was

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calculated as 21.10, 3.50, 13.45 rupees/ can or bottle respectively for can, nectar and squash (Table 6).

Therefore, it is concluded that commercially grown variety Shan-i-Punjab has a very good potential for making squash, nectar and canning as whole peaches. Other free stone variety Florda Grande which was found excellent for making canned peach halves was found very attractive and convenient to eat by all the taste panel and general consumer. All the products showed very good acceptability even after six months of storage.

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Journal of Applied Horticulture, 12(1): 69-70, January-June, 2010

Spectrophotometric determination of total alkaloids in some Iranian medicinal plants

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Abstract

A simple spectrophotometric method based on the reaction with Bromocresol Green (BCG) was developed for determination of total alkaloids in medicinal plants. A yellow complex forms and is easily extractable by chloroform at pH 4.7. The absorbance of the complex obeys Bear 's law over the concentration range of 4-13 µg atropine per mL of chloroform. This procedure can be carried out in the presence of other compounds without interference.

Key words: BCG, total alkaloids, medicinal plants, determination

Introduction

The alkaloids represent a group of natural products that had a major impact on the economic, medical and social affairs of humans. Many of these agents have potent physiological effects on mammalian systems as well as other organisms, and as a consequence, some constitute important therapeutic agents. Atropine, morphine, quinine and vincristine are representative of a host of agents used to treat a range of disease conditions that range from malaria to cancer. Therefore determination of total alkaloids is very important related to the quality of medicinal plants (Robbers *et al.,*1996).

The methods reported for the determination of alkaloids include official methods (Kartal, 2001; Levent, 2002;), high-performance liquid chromatography (HPLC)(Tomonari *et al.,* 1994; Salvadori *et al.,* 1994; Qi *et al.,* 2002; Kanazawa *et al.,* 2000), fluorimetry (Masatoki *et al.,* 1989; Andio *et al.,* 1987), ion-chromatography (Qing-Chun *et al.,* 2001), coulometry (Qing-qin *et al.,* 2002), gas-chromatography (Pagliariussi *et al.,* 2002; Chernyseva *et al.,* 2001), and electrochromatography. Most of the reported spectrophotometric methods have disadvantages such as narrow range of determination, require heating or extraction, a long time is needed for the reaction to be completed, and the coloured product formed is unstable. The purpose of the current work was to provide a simple, sensitive, and rapid spectrophotometric method for the determination of total alkaloids in medicinal plants. The method is based on the reaction of alkaloid with Bromocresol Green, forming a yellow-colored product. The method offers the advantages of sensitivity and stability.

Materials and methods

Plant material: Plant material including *Acroptilon repens* L. (aerial parts), *Berberis vulgaris* L. (aerial parts, fruits), *Biebersteinia multifidia* DC. (aerial parts, root), *Calendula officinalis* (flower), *Chelidonium majus* L. (aerial parts), *Echium amoenum* Fish & Mey (flower), *Equisetum arvense* L. (aerial parts), *Hyoscyamus niger* L. (aerial parts), *Hypecoum pendulum* L. (aerial parts), *Malva sylvestris* L. (aerial parts), *Scrophularia striata* Bioss. (root) and *Stachys lavandulifolia* Vahl. (aerial parts), was collected from local market of Tehran province, in May 2003. All plants were identified in the Herbaruim of Faculty of Pharmacy, Tehran University of Medical Sciences.

Bromocresol green solution (1×10^{-4}): Bromocresol green (69.8 mg) was warmed with 3 mL of 2N NaOH and 5 mL distilled water until compeletely dissolved and diluted to 1000 mL with distilled water.

Phosphate buffer (pH 4.7): pH of 2 M sodium phosphate (71.6 g Na_2HPO_4 in 1 L distilled water) was adjusted to pH 4.7 with 0.2 M citric acid (42.02 g citric acid in 1 L distilled water).

Atropine standard solution: One mg pure atropine (Sigma Chemical Co.) was dissolved in 10 mL distilled water.

Preparation of standard curve: Accurately measured aliquots (0.4, 0.6, 0.8, 1 and 1.2 mL) of atropine standard solution were transfered to different separatory funnels. Five mL phosphate buffer (pH 4.7) and 5 mL BCG solution were added. Mixture was shaken with 1, 2, 3 and 4 mL of chloroform. The extracts were collected in a 10 mL volumetric flask and then diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above but without atropine.

Extraction: The plant material (100g) was grinded and then extracted with methanol for 24 h in a continuous extraction (soxhlet) apparatus. The extract was filtered and methanol was evaporated on a rotary evaporator under vacuum at a temperature of 45°C to dryness. A part of this residue was dissolved in 2 N HCl and then filtered. One mL of this solution was transferred to a separatory funnel and washed with 10 mL chloroform (3 times). The pH of this solution was adjusted to neutral with 0.1 N NaOH. Then 5 mL BCG solution and 5mL phosphate buffer were added to this solution. The mixture was shaken and the complex formed

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was extracted with 1, 2, 3, and 4 mL chloroform by vigorous shaking. The extracts were collected in a 10 mL volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 417 nm vs. similarly prepared blank.

Results and discussion

A yellow-coloured complex with a maximum absorption was developed. This complex was completely extractable by chloroform at pH 4.7. A calibration curve was plotted for various concentration of atropine (Fig.1). Bear 's law was followed over the concentration range of 4-13 µg atropine per mL of chloroform. The effect of temperature and pH were studied. A pH of 4.7 gave optimum results and different temperatures had no effect on complex formation and extraction. The complex was very stable in chloroform and began to fade slowly only after 10 days. Before the extraction, the mixture was put in a boiling water bath for 3 min. The absorbance did not change after extraction with chloroform. Table 1 shows the amount of total alkaloid in tested plant materials determined by BCG-complex formation method.

Table 1. Determination of total alkaloids in tested plant materials (100g) by BCG-complex formation

No.	Plant	Part used	Amount (mg)	Amount (M mol)
1	*Acroptilon repens* L.	Aerial parts	13.35	0.023
2	*Berberis vulgaris* L.	Aerial parts	40.58	0.070
3	*Berberis vulgaris* L.	Fruit	19.70	0.034
4	*Biebersteinia multifidia* DC.	Aerial parts	204.56	0.353
5	*Biebersteinia multifidia* DC.	Root	1688.47	2.920
6	*Calendula officinalis* L.	Flower	16.14	0.028
7	Chelidonium majus L.	Aerial parts	248.09	0.430
8	*Echium amoenum* Fish & Mey	Flower	18.44	0.320
9	*Equisetum arvense* L.	Aerial parts	255.02	0.440
10	*Hyoscyamus niger* L	Aerial parts	324.09	0.560
11	*Hypecoum pendulum* L.	Aerial parts	39.20	0.068
12	*Malva sylvestris* L.	Aerial parts	35.06	0.060
13	*Scrophularia striata* Bioss.	Root	7.90	0.014
14	*Stachys lavandulifolia* Vahl.	Aerial parts	9.73	0.017

A few methods with different sensitivities have been developed for determination of alkaloids in plant materials for example gravimetric and titrimetric methods. These methods lack the adequate sensitivity and have some problems. As with most gravimetric methods, the residue obtained is found to be impure since more than one spot is revealed by TLC. In titrimetric assay, the end-point is masked by the color of the extract. On the other hand, there is no constant method applicable for all alkaloids. Methods with high sensitivity such as HPLC are not routine methods for determination of total alkaloids and these methods are very costly and need special equipment. Spectrophotometric determination of total alkaloids with bromocresol green is a simple and sensitive method and do not need very special equipment. The proposed method has the advange of being less time consuming, with the assay requiring an average of 1 h.

Acknowledgement

We are grateful to the Faculty of Pharmacy, Tehran University of Medical Sciences, for the financial support of this investigation.

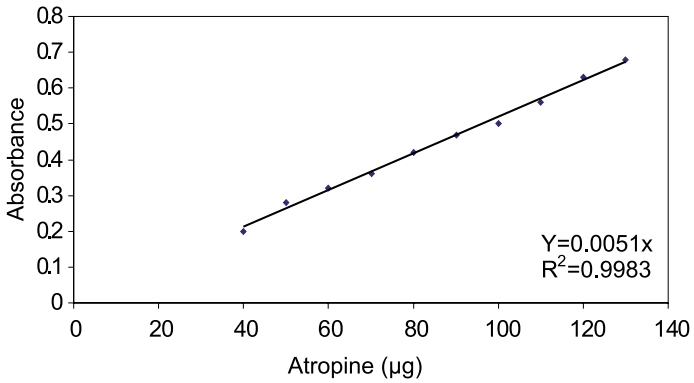

Fig 1. Variation of the absorbance vs. atropine concentration at 470 nm

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Journal of Applied Horticulture, 12(1): 71-74, January-June, 2010

Characterisation of promising okra genotypes on the basis of Principal Component Analysis

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Abstract

Twenty selected genetically diverse okra strains were evaluated using Principal Component and cluster analysis for the extent of variability and relationship between various economically important traits for the purpose of genetic improvement. The trial was laid out in a randomized block design (RBD). Positive significant correlation for days to 50% flowering (DF) with days to first harvest (DFH), number of pod per plant (NP) with pod yield per plant (PY) and pod yield per plot (PYP) ($P \leq 0.001$) and PY with PYP ($P \leq 0.001$) and negative correlation was observed for pod weight (PW) with NP ($P \leq 0.01$). The analysis of extracted components, component pattern and Eigen values revealed that the first two principal components alone accounted for 53.25% of variance. First component was found heavily loaded with days to 50% flowering (DF), days to first harvest (DFH), pod length (PL), pod diameter (PD) and pod weight (PW), which comprised of fourteen genotypes in three clusters. The matrix obtained from principal component analysis revealed that the genotype Pb- 57 and HRB-9-2 found their positions in same cluster in principal space. Dominating similar prominent phenotypic characters formed separate place in principal space as coherent cluster. Cluster based inter breeding of genotypes would exhibit high hetrosis and is also likely to produce new recombinants with desired characters in okra.

Key words: Okra, *Abelmoschus esculentus,* correlation, variance, principal component.

Introduction

Okra [*Abelmoschus esculentus* (L.) Moench] is one of the oldest cultivated vegetable crops. *Abelmoschus* genus contains about 150 known species with *esculentus* as the species of okra (Thompson and Kelly, 1957), also known as lady's finger or gumbo in England (Tindall, 1986) and bhindi in India, is believed to be native to tropical Africa and belongs to family Malvaceae. It is grown in all parts of the tropics during spring, summer and rainy season as kharif crop (Baloch, 1994). It is available all year-round, with a peak season during the summer months (Anon., 2003).

The crop is highly nutritious and also considered as power house of valuable nutrients. This vegetable provides protein, carbohydrate and an important input of vitamins and mineral salts, including calcium; which are often lacking in diet of developing countries. The young tender pods of okra are used in different ways; as boiled vegetables (Magness *et al.,* 1971), fresh, canned, frozen or dried and used as soup thickeners (Tindall, 1986) or cooked in curries, stewed and used in soups. Considerably high quantity of both soluble and insoluble fibre is present in okra. Nearly half of which is soluble fibre in the form of gums and pectin, which helps in lowering the serum cholesterol and thus reducing the risk of coronary heart diseases. The other half is insoluble fibre, which helps to keep the intestinal tract healthy (Jeff, 2002) and prevent the symptom of irritable bowel syndrome (IBS). The mucilaginous extract of okra is often used for clearing the cane juice from which jaggary or brown sugar is manufactured (Prasad and Nath, 2002). The stem of plant provides non digestible strong linear fiber, which finds uses in the paper, packaging and textile industries (Baloch, 1994).

Stability with consistent performance under predictable and unpredictable environment is the main concern in screening of phenotypically stable genotypes. Large variations among important traits are desirable. To improve particular attribute including yield characteristic information on genetic variability and inter-relationship among different traits is necessary as improvement is proportional to its magnitude of genetic variability present in the germplasm. Genetic diversity is one of the important tools to qualify genetic variability in both cross- and self-pollinated crops (Murty and Arunachalam, 1966; Gaur *et al.,*1978). Therefore, the present study was undertaken to analyze the genetic divergence in 20 okra genotypes, based on multivariate approach, which is not possible through univariate analysis. The aim was also to find out the characteristic pattern prevailed so as to get divergence information that may be useful for the varietal improvement.

Materials and methods

In pursuance of the envisaged objectives, the present study was carried out with twenty okra genotypes collected from different agro climatic regions of India. Seeds of selected genotypes were planted in randomized block design at three different sowing time scattered uniformly of twenty days interval during the kharif season starting from mid June with three replications. Standard techniques were adopted in data collection for 5 competitive plants in each genotypes on 10 characters *viz.*, plant height (PH), number of branches per plant (NB), days to 50% flowering (DF), days to first harvest (DFH), number of pods per plant (NP), pod length (PL), pod diameter (PD), pod weight (PW), pod yield per plant (PY) and pod yield per plot (PYP).

The data on various genotypes of okra under kharif season was processed using MINITAB v 13.2 and SPSS v 11.0 for multivariate analysis and Microsoft Excel v 2000 for descriptive statistics. The

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Principal component and Hierarchical cluster analysis was applied to analyze characteristic pattern to classify the selected genotypes.

Results and discussion

The comparative performance of twenty okra genotypes for variability in different traits was compared as the overall mean response of genotypes in respect of overall performance of genotypes as mean line (Fig. 1). The analysis of variance revealed that the differences in genotypes were significant for characters indicating the presence of variability among them. The results indicated that genotypes, HOE-202 (XIV), BO-2 (X) and Sel.-10 (XII) had the highest plant height (PH) with mean of 133.68, 134.14 and 138.67 cm, respectively. Highest values of number of branches (NB) for Sel.-7, days to 50% flowering (DF) and days to first harvest (DFH) for HOE-301, number of pods per plant (NP) for HRB-9-2, pod length (PL) for Sel. 10, pod diameter (PD) for Pb-57, pod weight (PW) for 71-14 and pod yield per plant (PY) & pod yield per plot (PYP) for HRB 9-2 (Fig. 1). These results may support the varietal improvement program in selecting parents.

Positive significant correlation could be observed (Table 1) for days to 50% flowering (DF) with days to first harvest (DFH), number of pod per plant (NP) with PY and PYP ($P \leq 0.001$) and PY with PYP ($P \leq 0.001$) and negative correlation was observed for pod weight (PW) with NP ($P \leq 0.01$). The strong correlation is indicative for the genotypic character association and the feeble correlation is influenced by environmental factors and limiting the yield (Subramanyan *et al.,* 1995). The results are in agreement with earlier studies on *Hibiscus sabdariffa* L (Banarjee *et al.,* 1988).

The data of observed measurements was subjected to Principal Component Analysis (PCA). The Eigen values given in Table 2 indicate that four principal components (PCs) account for 80.45% of the total variance in the

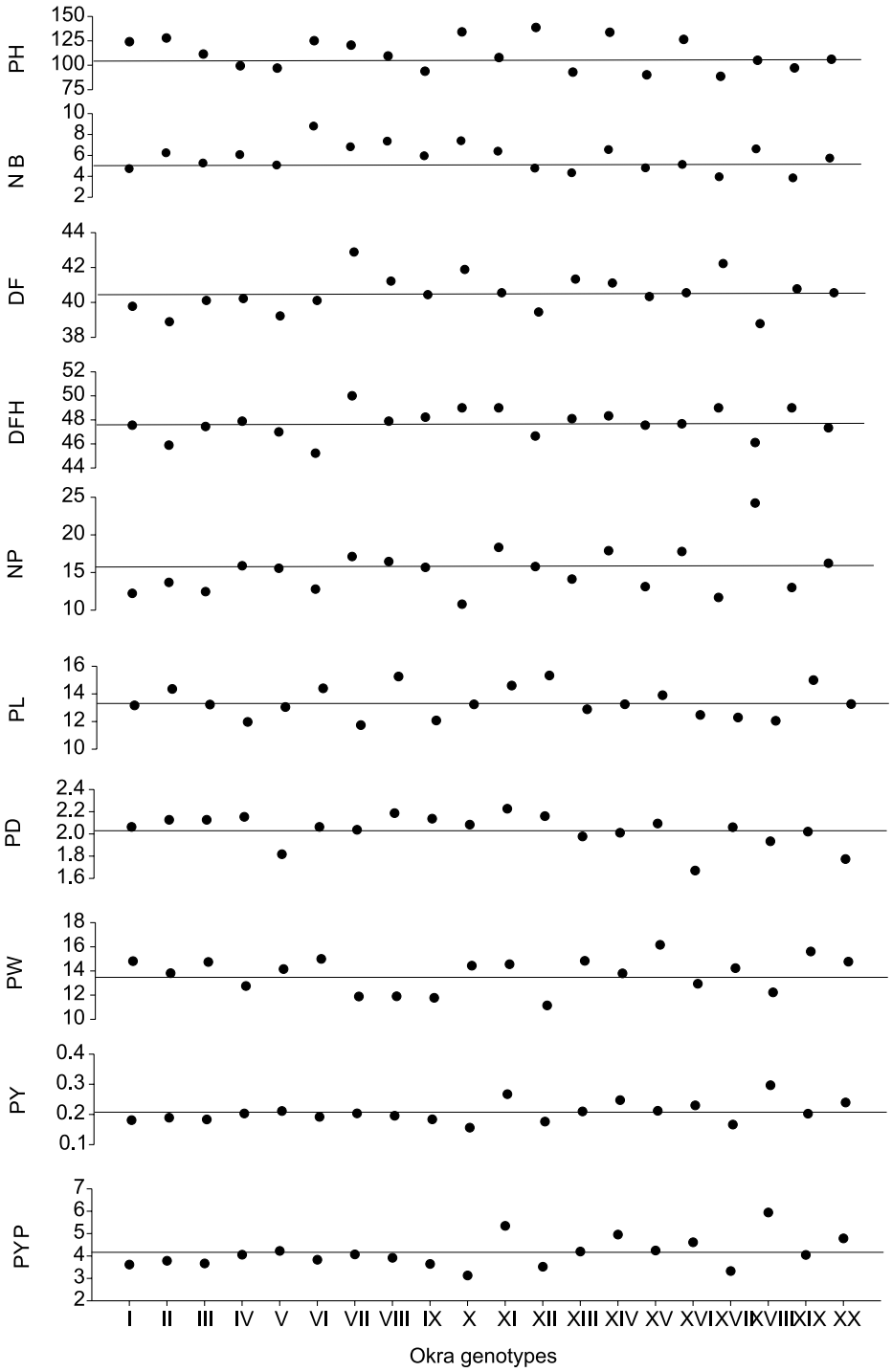

Fig. 1. Characteristic performance of twenty okra genotypes

Table 1. Correlation Matrix of performance variables of okra genotypes

Variables	PH	NB	DF	DFH	NP	PL	PD	PW	PY	PYP
PH	1.000									
NB	0.441	1.000								
DF	-0.077	0.041	1.000							
DFH	-0.211	-0.241	0.816***	1.000						
NP	0.014	0.228	-0.259	-0.120	1.000					
PL	0.264	0.062	-0.236	-0.254	-0.226	1.000				
PD	0.028	0.210	0.066	0.128	-0.258	0.368	1.000			
PW	-0.265	-0.250	0.020	0.004	-0.544**	0.198	-0.109	1.000		
PY	-0.126	0.126	-0.271	-0.116	0.853***	-0.130	-0.354	-0.034	1.000	
PYP	-0.121	0.128	-0.268	-0.116	0.852***	-0.127	-0.356	-0.031	0.999***	1.000

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Table 2. Eigen values and their contribution in variation

No.	Eigen value	Individual Percent
1	3.249	32.50
2	2.075	20.75
3	1.666	16.66
4	1.054	10.54
5	0.912	9.12
6	0.656	6.56
7	0.301	3.01
8	0.081	0.81
9	0.003	0.04
10	0.001	0.01

Table 3. Distribution of 20 okra genotypes among four clusters

Group/ cluster No.	No. of genotypes	Genotypes in different cluster
1	3	Vaishali Vadhu (IV), HOE-3019 (VII), HRB-55 (IX)
2	4	BO-1 (II), Sel.-7 (VI), Sel.-4 (VIII), Sel.-10 (XII)
3	7	AROH-1 (I), Sel.-2 (III), BO-2 (X), KS-3 (XIII), 71-14 (XV), NDO-25 (XVII), Sel.-8 (XIX)
4	6	H-1-87-16 (V), Pb-57 (XI), HOE-202 (XIV), D-1-87-5 (XVI), HRB-9-2 (XVIII), Pusa Sawani (XX)

data. The loading of component on principal axes indicated a high degree of genetic diversity among the selected genotypes (Fig. 2). To study the pattern of the attributes and to identify factors that are substantively meaningful, a multivariate approach was used. The loading plots are shown in Fig. 2, for the three principal components. It can be seen that the first component explaining 32.50 % of variance is highly correlated both positively (PD, DF and DFH) and negatively (NP, PY and PYP). Thus it classifies and distinguishes the score of okra genotypes on the basis of these components. The second principal component explaining 20.75% of total variance is highly correlated with DF and DFH in positive direction and with PH, NB, and PL in negative direction. While, the third component has explained only 16.66% of total variance is positively correlated with PW and negatively with PH, NB and DF. Fig. 2 illustrates the biplot of scores and loadings of the three principal components of observed parameters of okra genotypes, which further affects their positions according to their characteristics with respect to the extracted principal components. The Biplot of PC1 and PC2 shows the separation of the okra genotypes according to their respective scores. First and fourth quadrant of the plot contains samples having positive PC1 scores. The genotypes of this quadrant are heavily loaded with days to 50% flowering (DF), days to first harvest (DFH), pod length (PL), pod diameter (PD) and pod weight (PW), which comprised of fourteen genotypes of cluster 1, 2 and 3 (Table 3). Second and third quadrant is expressing samples with negative PC1 scores and heavily loaded mainly with the phenotypic and yield related characters and contains the high yielding promising genotype HRB 9-2. The loading pattern thus obtained with respect to the position of the genotype in principal space may be considered as one of the imperative indicator for finding out the characteristic divergence or similarity, which is essentially required for genetic improvement through breeding.

Hierarchical cluster analysis (HCA) using Ward's minimum variance using Euclidean distance method (Ward, 1963) as dendogram is shown in Fig. 3. As a result of applying HCA to

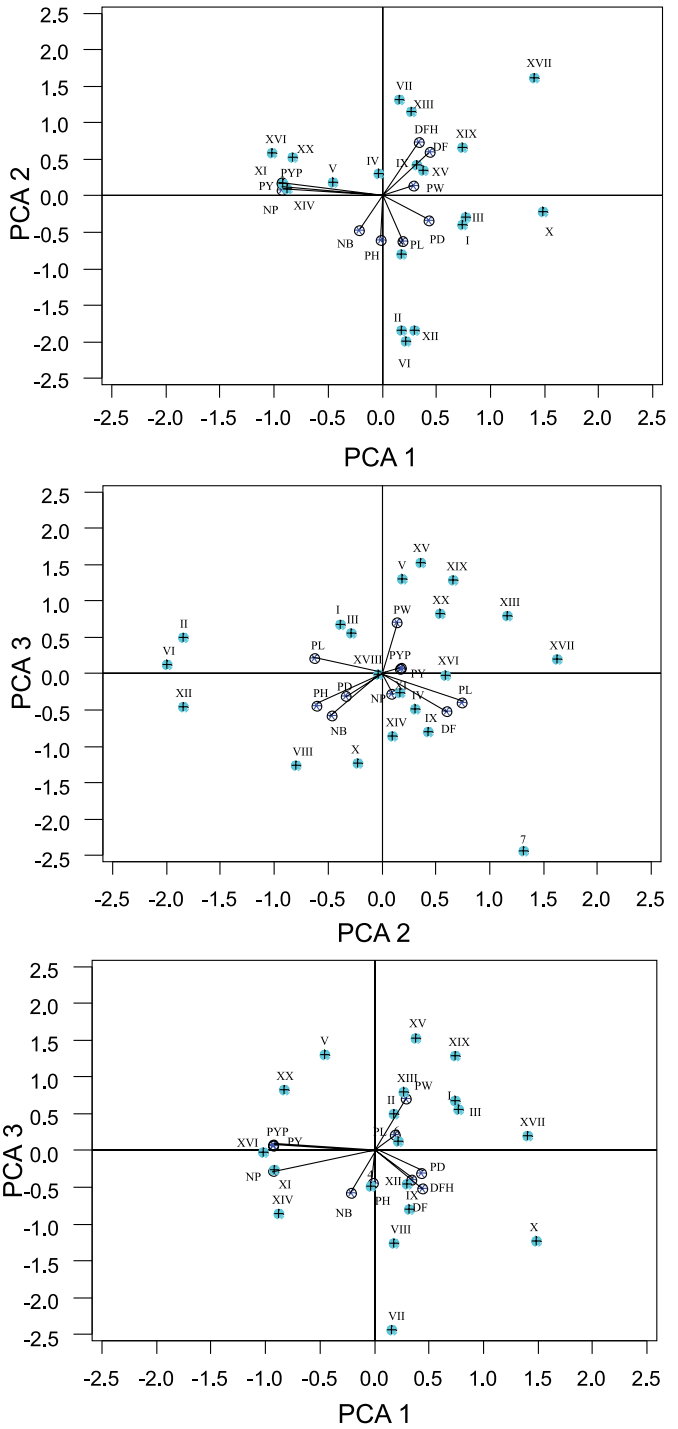

Fig. 2. Biplot of loadings and scores for okra genotypes on principal axes 1, 2 and 3

the principal component score matrix, the okra genotypes were grouped into four different clusters (Table 3). It was revealed that clusters 1, 2, 3 and 4 have 3, 4, 7 and 6 genotypes in clusters, respectively. The genotype Pb -7, a high yielding and promising genotype, showed complete resistance to yellow vein mosaic virus and fell in the cluster 4 where another high yielding variety HRB 9-2 occupied the position in the space. Clustering pattern of okra genotypes showed considerable genetic diversity among themselves by occupying four clusters. The data pertaining to the phenotypic characters showed diverse nature towards various parameters studied. The combination of all quality attributes in single genotype as presented in Fig. 1 does not give a firm output on univariate analysis. Thus the above characterizations

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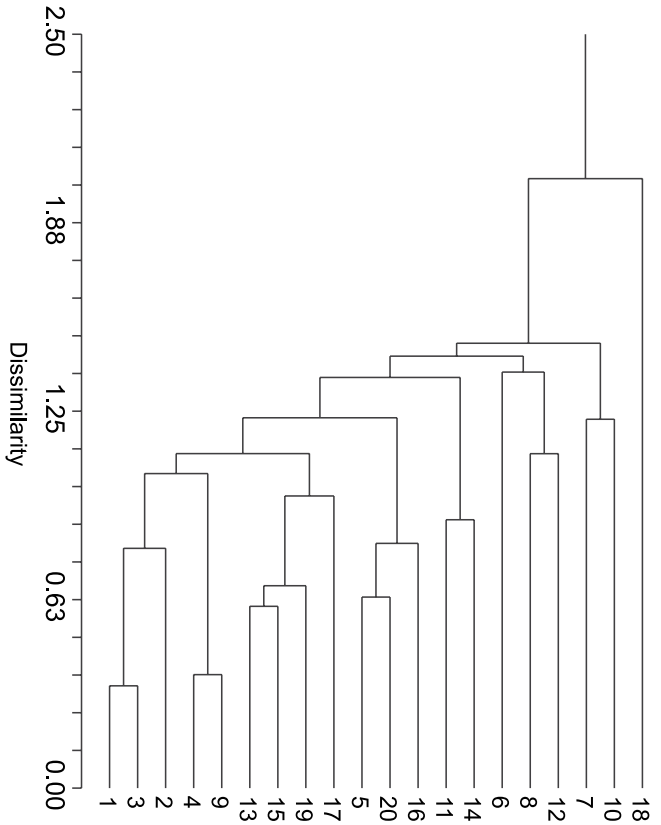

Fig. 3. Dendogram of cluster analysis for selected okra genotypes

of okra genotypes on the basis of dissimilarity in scores with respect to extracted principal components indicates the existence of variability and confirm the same among the clusters for the selected attributes.

From the results obtained it was concluded that genetic variation existed among the genotypes in all characters. Genotypes Pb-57 and HRB 9-2 showed a greater potentials in terms of yielding attributes as it out performed the other cultivars, indicating the usefulness as promising genotypes in kharif crop and crosses among the other clusters would exhibit high hetrosis and is also likely to produce new recombinants with desirable characters in okra.

Acknowledgement

Authors would like to acknowledge the support of Coordinators of "All India Coordinated Research Projects on Vegetables" for their timely support in completion of present work.

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Journal of Applied Horticulture, 12(1): 75-80, January-June, 2010

Efficacy of some essential oils on controlling powdery mildew on zinnia (*Zinnia elegans* L.)

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Abstract

A field experiment was carried out during two successive seasons at the Experimental Farm of the Faculty of Agriculture, Kafr El- Sheikh University to evaluate some essential oils as biocontrol agents for powdery mildew on *Zinnia elegans*, L. Marjoram, clove, cinnamon, garlic, ginger and fennel oil were used as a foliar spray at 2 levels (250 and 500 ppm) beside Kema zein 75% and distilled water as a control. The plants were sprayed four times beginning from June 15th with one week interval by a hand atomizer as soon as the first sign of powdery mildew detected on plants. Disease incidence and severity as well as vegetative parameters such as plant height, number of branches per plant, leaf area, fresh and dry weights of shoots, root length and fresh and dry weights of roots were determined in the two seasons. Peroxidase and polyphenol oxidase activities were determined after 24 hour from the last spray in leaves samples. The highest significant decrease in disease incidence and severity and the best results for most of the studied growth and flowering parameters and total green colour were recorded when plants were sprayed with ginger, cinnamon and clove oils, respectively each at 500 ppm compared to the other treatments in both seasons. In addition, the activities of peroxidase (POX) and polyphenol oxidase (PPO) enzymes increased as a result of oil spray on plants. In conclusion, these findings provide a rational basis for possible utilization of these essential oils as a safe and alternative method to fungicides for controlling powdery mildew in zinnia plants.

Key words: *Zinnia elegans,* essential oils, powdery mildew, biocontrol agents.

Introduction

Zinnias are one of the easy to grow herbaceous summer annual flower, blooming from mid-summer all the way until frost. About 10 species of zinnia are garden flowers but only the *Zinnia elegans* is the most popular. *Z. elegans* belongs to family Asteraceae and native to the Southwest United States, Mexico and Central America and therefore, likes an warm-hot climate. Zinnia plant's leaves are lance-shaped, sandpaper like in texture, stalkless, and have erect stems that bear opposite leaves and terminal flower heads. Zinnias come as yellow, orange, white, red, rose, pink, purple, lilac and multi-colored blooms. Zinnia varieties include both miniatures and giants that range from about a foot to over three feet tall. However**,** zinnia plants are subjected to many pathogens, which cause various diseases, *Erysiphe cichoracearum* is one of the important pathogens since it causes one of the most serious disease of zinnia plants namely powdery mildew. This pathogen attacks all plant parts destroying most leaves and flowers, finally the remaining flowers become unmarketable.

The present work aimed to study the efficacy of certain essential oils in controlling powdery mildew on zinnia caused by *E. cichoracearum*.

Material and methods

A field experiment was carried out during the two successive seasons of 2006 and 2007 at the Experimental Farm of the Faculty of Agriculture, Kafr El- Sheikh University to evaluate some essential oils as biocontrol agents for powdery mildew on *Z. elegans* L. Seeds were sown in nursery beds on March 15th in both years and seedlings were transplanted in May 1st to a clay soil in plots 1 x 1.5m^2 at 50 cm apart as a twins in the hill, and each bed was divided into two parts (1 x 0.75 m^2). Each part contained 12 plants (6 hills) and was considered as a replicate. Therefore, every treatment consisted of 36 plants (18 hills) in the three replicates. The experiment was arranged in a completely randomized block design. The plants including control were fertilized with the recommended dose of N, P and K (100, 200 and 100 kg/ application, respectively), beginning from May 15th and repeated three times with two weeks interval. The used fertilizers were ammonium sulphate "20% N", calcium super phosphate "15.5% P_2O_5" and potassium sulphate "48% K_2O". The common agricultural practices *i.e.,* watering, weed control, etc. were done whenever plants needed.

Marjoram, clove, cinnamon, garlic, ginger and fennel oils were added as a foliar spray at 2 levels of 250 and 500 ppm. Tween-20 was used as a surfactant at the rate of 0.1% v/v. Kemah zein 75% was used at recommended dose (2 g/L). The plants were sprayed four times beginning from June 15th with one week interval by a hand atomizer as soon as the first sign of the symptoms were observed. For control treatments, plants were sprayed with distilled water only. Percentage of disease incidence and severity were determined after 7 days from the last spray according to the scale reported by Horsfall and Barrett (1945) and Biswas *et al.* (1992).

Enzymes extraction and assay: Leaf samples of each treatment, healthy and infected, were collected after 24h of the treatment for peroxidase and polyphenol oxidase enzymes activity assay. In addition, untreated healthy and infected leaves were used as control. Enzyme extract was obtained by grinding leaf tissue in 0.1 M sodium phosphate buffer at pH 7.1 (2g leaf tissues) in a

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porcelain mortar. The extracted tissues were strained through four layers of cheesecloth. Filtrates were centrifuged at 3000 rpm for 20 min at 6°C. The clear supernatants were collected and considered as crude enzyme extract. Peroxidase (POX) activity was determined according to the method of Allam and Hollis (1972) by measuring the oxidation of pyrogallol to pyrogalline in the presence of hydrogen peroxide. Peroxidase activity was measured following the changes in absorbance at 425 nm every 1 min up to 4 min. Polyphenol oxidase (PPO) was determined according to Maxwell and Batman (1976). The changes in absorbance were measured spectrophotometrically at 495 nm, and recorded every 1 min up to 4 min. All measurements were assayed using Beckman Spectrophotometer Du®7400. The details of essential oils used as treatments are given in Table 1.

At the end of the experiment, plant height (cm), branch number/ plant, leaf area (cm^2), shoots fresh and dry weights /plant (g), root length (cm), roots fresh and dry weights /plant (g), flower number /plant, flower diameter (cm), flower fresh and dry weights (g), total green colour (SAPD) were recorded. Means between treatments were compared with Duncan's Multiple Range Test according to Snedecor and Cochran (1982).

Results

Effect on disease incidence and severity: In this investigation, the essential oil of marjoram, clove, cinnamon, garlic, ginger and fennel plants were used to control powdery mildew disease of zinnia plants under field conditions**.** Data presented in Table 1 show that all essential oils and Kemah zein 75% treatments significantly decreased disease incidence (average number of powdery mildew spots/leaf) and disease severity (percent surface infected area) on zinnia plants more than distilled water treatment in both seasons. Results indicated that, the best control of the studied disease was obtained when the essential oils were used at 500 ppm compared to 250 ppm in most cases. The oil of ginger at 500 ppm was the most efficient treatment than others on disease incidence and severity in both seasons. It significantly decreased disease incidence and severity from 30.78 to 1.2% and 87.2 to 6.0%, respectively in the first season and from 35.69 to 1.6% and 91.5 to 6.5%, respectively in the second one. This was followed by cinnamon oil treatments.

The treatment of oil of Marjoram was the least effective treatment used at either 25 or 50% in both seasons. In general, all used treatments gave better or similar results with those obtained when the fungicide Kema zein 75% was used.

Effect on peroxidase and polyphenol oxidase activity: Data presented in Table 2 show that peroxidase activity significantly increased as a results of these treatments. The higher activity of peroxidase was observed with ginger oil at 500 ppm followed by fennel oil at 500 ppm.

Polyphenol oxidase activity presented in Table 3 reveal that all used oils and Kemah zein 75% increased polyphenol oxidase activity over control. It also indicates that high concentration of all used essential oils caused higher activity of peroxidase and polyphenol oxidase than lower one in both seasons. The highest values for polyphenol oxidase activity were recorded with cinnamon oil at 500 ppm followed by ginger oil at 500 ppm. The lowest values in both seasons resulted from the treatment of marjoram oil at both concentrations.

Table 1. Effect of some essential oils and Kemah zein 75% treatments on incidence and severity of powdery mildew disease of *Zinnia elegans* L. during 2006 and 2007 seasons

Treatment	Concentration	Average number of spots/leaf (Disease incidence)		Percent of surface infected area (Disease severity)	
		2006	2007	2006	2007
Distilled water	-	30.78a	35.69a	87.2a	91.5a
Kemah zein 75%	2g /L	2.7e	2.3g	8.9e	10.2f
Marjoram oil	250 ppm	9.6c	11.9b	23.5b	24.5b
	500 ppm	9.7b	10.8c	19.2c	20.4c
Clove oil	250 ppm	1.9j	2.1h	7.4i	7.8k
	500 ppm	1.6l	1.8j	7.0l	7.5n
Cinnamon oil	250 ppm	1.8k	1.9i	7.3j	7.6m
	500 ppm	1.4m	1.7k	6.7m	6.8o
Garlic oil	250 ppm	2.3h	2.4f	8.5f	10.4e
	500 ppm	2.6f	2.5e	8.2g	8.7j
Ginger oil	250 ppm	2.0i	1.9i	7.2k	7.7l
	500 ppm	1.2n	1.6l	6.0n	6.5p
Fennel oil	250 ppm	3.2de	3.4d	9.1d	11.5d
	500 ppm	2.4g	2.1h	7.5h	9.5i

Means within a column having the same letters are not significantly different in Duncan's Multiple Range Test.

Table 2. Activity of peroxidase in leaves of *Zinnia elegans* L. after 24 hours from the last treatment with essential oils 75%

Treatments	Concentration	Peroxidase activity/minute after 24 h			
		1	2	3	4
Distilled water	-	0.403g	0.405m	0.409k	0.413e
Kemah zein 75%	2g /L	1.112cde	1.115j	1.121h	1.125cd
Marjoram oil	250 ppm	0.677fg	0.701l	0.713j	0.723e
	500 ppm	0.723efg	0.748k	0.759i	0.766de
Clove oil	250 ppm	1.461bcd	1.575f	1.606e	1.629bc
	500 ppm	1.556def	1.672e	1.687d	1.692cd
Cinnamon oil	250 ppm	1.411bcd	1.506h	1.533f	1.572bc
	500 ppm	1.767ab	1.892d	1.907c	1.914ab
Garlic oil	250 ppm	1.235cd	1.250i	1.357g	1.369c
	500 ppm	1.464bcd	1.561f	1.600e	1.626bc
Ginger oil	250 ppm	1.531bc	1.544g	1.549f	1.555bc
	500 ppm	2.115a	2.146a	2.149a	2.155a
Fennel oil	250 ppm	1.771ab	1.943c	1.981b	1.991ab
	500 ppm	1.961a	1.980b	1.981b	1.988ab

Means within a column having the same letters are not significantly different in Duncan's Multiple Range Test.

Plant height: Data presented in Table 4 revealed that all oils and Kemah zein 75% treatments significantly increased plant height over control in both the seasons. The tallest plants in both seasons resulted from the treatment of ginger oil (500 ppm), Kemah zein 75% recorded 196.00, 177.58, and 194.50, and 177.51 cm followed by clove oil (500 ppm) than cinnamon oil (500 ppm) in the first season. The second rank was of clove oil (250 ppm) and (500 ppm) in the second season which produced188.00 and 175.36 cm plant height, respectively. The shortest plants were obtained from the treatment of marjoram oil (250 ppm) and (500 ppm) and recorded 160.33 and 158.44 cm, respectively against 116.00 and 125.71 cm for control in both seasons.

Branch number: All essential oils and Kemah zein75% treatments significantly increased number of branches over control in the two seasons (Table 4). Both cinnamon oil concentrations (250 and 500 ppm) and Kemah zein75% gave the highest number of

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Table 3. Activity of polyphenol oxidase in leaves of *Z. elegans* L. after 24 hours from the last treatment with essential oils and Kemah zein 75%

Treatments	Concen-tration	Polyphenol oxidase activity/minute			
		1	2	3	4
Distilled water	-	0.043d	0.071h	0.073f	0.077h
Kemah zein 75%	2g /L	0.113a	0.117b	0.119b	0.082gh
Marjoram oil	250 ppm	0.072bc	0.073gh	0.076ef	0.078h
	500 ppm	0.050d	0.097cde	0.100bc	0.102cdef
Clove oil	250 ppm	0.058cd	0.090efg	0.092cde	0.095efgh
	500 ppm	0.087b	0.102bcde	0.104bc	0.105bcde
Cinnamon oil	250 ppm	0.111a	0.112bcd	0.114b	0.116bc
	500 ppm	0.115a	0.145a	0.154a	0.157a
Garlic oil	250 ppm	0.074bc	0.111bcd	0.114b	0.115bcd
	500 ppm	0.088b	0.115bc	0.119b	0.123b
Ginger oil	250 ppm	0.082b	0.094def	0.095cd	0.097defg
	500 ppm	0.114a	0.143a	0.152a	0.155a
Fennel oil	250 ppm	0.050d	0.077fgh	0.081def	0.085fgh
	500 ppm	0.111a	0.112bcd	0.114b	0.115bcd

Means within a column having the same letters are not significantly different in Duncan's Multiple Range Test.

branches as 10.67, 10.33 and 10.30, respectively in the first season while in the second one, the treatments of cinnamon oil at 500 ppm and Kemah zein 75% gave 8.55 and 8.49, respectively. In the second rank were the oils of cinnamon (250 ppm) and clove (500 ppm) in the first and second seasons which gave 10.33 and 8.12 branches, respectively. The lowest branch number resulted from the treatments of marjoram oil (250 and 500 ppm) and fennel oil (250 ppm) in the first season and marjoram oil (250 ppm) in the second one as recorded 6.00, 6.33 and 6.00 and 5.85, respectively.

Leaf area: It is obvious from Table 4 that all the oils at both concentrations and Kemah zein 75% produced the wide leaves than control in both the seasons. The widest leaves resulted from plants sprayed with cinnamon oil at 500 ppm in both seasons and Kemah zein75% in the second one. This was followed by ginger oil at 500 ppm and Kemah zein75% in the first season and ginger oil at 250 ppm and Kemah zein75% in the second one. The smallest leaves resulted from the treatments of garlic oil 250 ppm in the first season and marjoram oil at 250 ppm in the second one which gave 29.33 and 31.79 cm^2 leaf area, respectively against 21.58 and 27.25 cm^2 for control in both seasons.

Shoot's fresh and dry weights: It is clear from Table 5 that all oils and Kemah zein75% treated plants had significantly high values for both fresh and dry weight of shoots than control in both seasons. The heaviest fresh and dry shoots /plant in the first season resulted from the treatments of ginger oil at 500 ppm and Kemah zein 75%. Whereas in the second one were the treatments of clove oil at 500 ppm as recorded 286.08 and 41.17 g, respectively. This was followed by the treatments of cinnamon oil at 500 ppm for fresh weight and ginger oil at 250 ppm for dry weight in the first season. The lightest fresh and dry shoots /plant in both seasons were obtained in the treatment of marjoram oil at 250 ppm.

Root length: Data in Table 5 reveal that all used treatments gave significantly longer roots than control in both the seasons. The long roots in the first season were recoreded with clove oil at 500 ppm, ginger oil at 500 ppm and cinnamon oil at 500 ppm and recorded 17.67, 17.62 and 17.00 cm, respectively against 11.67 cm for control without significant differences among themselves in most cases. Whereas in the second season root length was more in clove oil at 500 ppm.

Root's fresh and dry weights: Data presented in Table 6 show that essential oils and Kemah zein75% treatments significantly increased both root fresh and dry weights over control in both seasons. The heaviest fresh roots resulted from ginger oil at 500 ppm and Kemah zein75% in the first season and clove oil at 500 ppm in the second season. The lightest fresh roots resulted from the treatment of cinnamon oil at 250 ppm in the first season and fennel oil at 250 ppm in the second season (15.16 and 9.10 g, respectively) against 11.40 and 8.58 g for control in both the seasons.

Heavy roots were produced under the treatments of clove oil at 250 ppm, clove oil at 500 ppm and ginger oil at 500 ppm in the first season. There were no significant differences among most treatments in the second season.

Flower number: Data in Table 6 indicated that all used treatments significantly increased the flower number over control in both seasons. In the first season, there were non-significant differences among most treatments. The highest flower number resulted from the treatments of clove oil at 500 ppm, Kemah zein75%, cinnamon oil at 500 ppm, ginger oil at 500 ppm, cinnamon oil at 250 ppm, ginger oil at 250 ppm and clove oil at 250 ppm against control. In contrast to this, the significantly higher flower number in the second season resulted from the treatment of ginger oil at 500 ppm. The lowest flower number resulted from the treatments of marjoram oil at 250 ppm and fennel oil at 250 ppm in the first

Table 4. Effect of essential oils and Kemah zein 75% on plant height, branches number and leaf area of *Zinnia elegans* L.

Treatments	Concentration	Plant height (cm)		Branch number / plant		Leaf area (cm^2)	
		2006	2007	2006	2007	2006	2007
Distilled water	-	116.00j	125.71l	3.67f	4.05l	21.58m	27.25l
Kemah zein 75%	2g /L	194.50a	177.51a	10.30ab	8.49a	48.95b	48.55ab
Marjoram oil	250 ppm	160.33i	164.31i	6.00e	5.85k	30.60j	31.79k
	500 ppm	164.00h	158.44k	6.33e	6.10i	31.80i	34.27i
Clove oil	250 ppm	188.00c	171.48e	7.33d	7.54e	35.50f	37.22g
	500 ppm	190.00b	175.36c	8.00c	8.12b	37.63e	46.55d
Cinnamon oil	250 ppm	165.00gh	170.64g	10.33ab	7.90c	44.23c	47.84c
	500 ppm	175.00e	176.98b	10.67a	8.55a	52.20a	48.66a
Garlic oil	250 ppm	165.30g	165.48h	7.00d	6.56g	29.33l	39.12f
	500 ppm	170.00f	170.71f	8.00c	7.13f	34.67g	41.59e
Ginger oil	250 ppm	183.00d	175.23d	8.00c	7.66d	42.37d	48.50b
	500 ppm	196.00a	177.58a	10.00b	8.17b	49.57b	47.88c
Fennel oil	250 ppm	166.00g	162.70j	6.00e	5.94j	30.23k	32.45j
	500 ppm	170.00f	164.31i	7.00d	6.27h	33.52h	35.20h

Means within a column having the same letters are not significantly different in Duncan's Multiple Range Test.

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Table 5. Effect of some essential oils and Kemah zein 75% on shoots fresh and dry weights (g) /plant and root length (cm) of *Zinnia elegans*, L. during 2006 and 2007 seasons

Treatments	Concentration	Shoots fresh weight / plant (g)		Shoots dry weight plant (g)		Root length (cm)	
		2006	2007	2006	2007	2006	2007
Distilled water	-	99.25m	112.52l	9.04m	11.42l	11.67d	7.52m
Kemah zein 75%	2gm/L	326.09a	280.55b	40.12a	36.88b	16.58b	12.95bc
Marjoram oil	250 ppm	178.04l	244.48k	20.27l	25.15k	16.00c	10.45k
	500 ppm	187.63j	259.80h	21.50k	30.28f	16.33bc	11.60f
Clove oil	250 ppm	289.05f	277.62c	30.77h	36.92b	16.60bc	12.88c
	500 ppm	305.77d	286.08a	33.74e	41.17a	17.67a	13.15a
Cinnamon oil	250 ppm	295.64e	268.53g	32.75f	26.85i	16.33bc	12.47e
	500 ppm	315.62b	277.32d	37.22c	35.49c	17.00ab	13.02b
Garlic oil	250 ppm	242.51h	245.83j	31.53g	28.61g	16.03c	11.26h
	500 ppm	268.06g	268.97f	34.33d	26.52j	16.08c	10.67j
Ginger oil	250 ppm	312.78c	275.24e	38.27b	32.62e	16.67bc	11.45g
	500 ppm	336.08a	283.78b	42.23a	35.22d	17.62a	12.74d
Fennel oil	250 ppm	182.06k	258.81i	22.63i	26.83i	16.00c	10.26l
	500 ppm	188.45i	269.13f	21.77j	27.75h	16.01c	11.08i

Means within a column having the same letters are not significantly different in Duncan's Multiple Range Test.

season and marjoram oil at 250 ppm in the second season.

Flower diameter: Table 7 showed that all used treatments increased flower diameter over control in both seasons. The biggest flower in the first season resulted from plants treated with ginger oil at 250 ppm, ginger oil at 500 ppm, fennel oil at 500 ppm and cinnamon oil at 500 ppm without significant differences among themselves in most cases against control. Whereas, in the second year plants treated with ginger oil at 250 ppm, followed by cinnamon oil at 250 ppm recoreded increased diameter.

Fresh and dry weights of flower: Table 7 indicate that all the used essential oils at both concentrations and Kemah zein75% gave the heavier fresh and dry weights of flower than control in both seasons. The heaviest fresh and dry weights in the first season resulted from the plants treated with ginger oil at 500 ppm. Whereas in the second year, the plants treated with both cinnamon oil at 250 ppm and ginger oil at 250 ppm recorded higher fresh weight. The lightest fresh and dry weights in the first season resulted from plants treated with garlic oil at 250 ppm. However, in the second year this resulted from the plants treated with fennel oil at 500 ppm.

Effect on total green colour: Table 8 revealed that all treated plants were greener than untreated ones in both seasons. The greenest plants were those treated with ginger oil at 500 ppm and Kemah zein75% in the first season (33.85 and 3380 SAPD value, respectively). Cinnamon oil at 500 ppm gave 38.72 SAPD value in second season. The palest plants were those treated with marjoram oil at 250 ppm in the first season and marjoram oil at 500 ppm in the second season.

Discussion

In this study, results indicated that disease incidence and severity of powdery mildew on *Z. elegans* L. was significantly decreased by spraying some essential oils as ginger, cinnamon and clove each at 500 ppm, four times beginning from June 15th with 7 days interval. These three treatments significantly surpassed others in most cases and the essential oils treatments gave better or similar results with those obtained when the fungicide Kemazein 75% was sprayed.

These findings could be explained as per previous workers who revealed that essential oils have important ecological functions. One of these functions is to protect the plant against infection by pathogens (Taiz and Zeiger, 1991; El-Kazzaz *et al.*, 2003). The mycelia growth of *Aspergillus flavus* Link was completely inhibited and the hyphal diameter decreased and hyphal wall appeared as precipitates and disappeared in some regions when oil of *Cymbopogon citratus* L. was used. In addition, oil treatment

Table 6. Effect of some essential oils and Kemah zein 75% on roots fresh and dry weights (g)/ plant and flower number of *Zinnia elegans*, L. during 2006 and 2007 seasons

Treatments	Concentration	Roots fresh weight / plant (g)		Roots dry weight / plant (g)		Flower number / plant	
		2006	2007	2006	2007	2006	2007
Distilled water	-	11.40i	8.58l	1.55g	1.18f	10.01f	11.21k
Kemah zein 75%	2gm/L	16.20a	10.88b	2.26b	1.50a	26.95a	17.85b
Marjoram oil	250 ppm	15.38g	9.66j	2.27b	1.35cd	18.00de	15.19j
	500 ppm	15.46f	10.24f	2.20d	1.45b	19.00cd	15.61i
Clove oil	250 ppm	15.83d	10.83c	2.30a	1.48ab	25.67ab	16.21h
	500 ppm	16.01b	11.13a	2.31a	1.53a	27.00a	17.81b
Cinnamon oil	250 ppm	15.16h	10.34e	2.10f	1.48ab	26.33a	16.81g
	500 ppm	15.58e	10.91b	2.24c	1.51a	27.00a	17.75c
Garlic oil	250 ppm	15.38g	9.75i	2.17e	1.32d	20.67c	16.88f
	500 ppm	15.46f	10.14g	2.10f	1.38c	24.33b	17.20e
Ginger oil	250 ppm	15.90c	10.00h	2.20d	1.36cd	26.00ab	17.52d
	500 ppm	16.24a	10.55d	2.30a	1.45b	26.67a	18.11a
Fennel oil	250 ppm	15.33g	9.10k	2.10f	1.27e	17.00e	15.58i
	500 ppm	15.35g	9.77i	2.20d	1.34cd	17.37de	16.92f

Means within a column having the same letters are not significantly different in Duncan's Multiple Range Test.

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Table 7. Effect of some essential oils and Kemah zein 75% on flower diameter (cm) and flower fresh and dry weights (g) of *Zinnia elegans*, L. during 2006 and 2007 seasons

Treatments	Concentration	Flower diameter (cm)		Flower fresh weight (g)		Flower dry weight (g)	
		2006	2007	2006	2007	2006	2007
Distilled water	-	4.00e	3.21l	1.95j	2.52i	0.32i	0.51h
Kemah zein 75%	2gm/ L	6.62b	4.85c	2.90b	4.86bc	0.65b	0.83c
Marjoram oil	250 ppm	5.50d	4.66f	2.65h	4.71de	0.55gh	0.80c
	500 ppm	6.40b	4.82cd	2.71ef	4.80c	0.57e	0.78cd
Clove oil	250 ppm	5.67d	4.31h	2.65h	4.68e	0.55fgh	0.71ef
	500 ppm	5.83cd	4.08i	2.70g	4.37g	0.56efg	0.68ef
Cinnamon oil	250 ppm	6.33bc	5.87b	2.89c	5.13a	0.63c	0.96b
	500 ppm	6.73ab	4.50g	2.93b	4.80c	0.65b	0.73de
Garlic oil	250 ppm	5.50d	4.01j	2.50i	4.40g	0.54h	0.66fg
	500 ppm	6.33bc	4.87c	2.74e	4.75cd	0.57ef	0.83c
Ginger oil	250 ppm	7.17a	6.01a	2.77d	5.18a	0.66b	1.03a
	500 ppm	6.97a	4.81d	3.03a	4.92b	0.71a	0.81c
Fennel oil	250 ppm	6.33bc	4.72e	2.72f	4.52f	0.59d	0.51h
	500 ppm	6.83ab	3.95k	2.76d	4.28h	0.61c	0.83c

Means within a column having the same letters are not significantly different in Duncan's Multiple Range Test.

caused plasma membrane disruption and mitochondrial structure disorganization (Helal *et al.*, 2007). Scarito *et al.* (2007) studied the effect of essential oils of oregano and clove at 0.125 and 0.5 mL L^{-1} concentration on roses. Other investigators reported that the essential oils contained antifungal compounds and fungitoxic agents that can inhibit the growth of certain microorganisms (Farag *et al.*, 1989; Zambonelli, 1996; El-Shoraky, 1998; Chao *et al.*, 2000; El-Shazly, 2000; Abd El-Kader *et al.*, 2003; Voda *et al.*, 2003; Moleyar and Narasimham, 2004; Sheng *et al.*, 2005; Krishna Kishore and Pande, 2007).

Results also showed that spraying zinnia plants with these essential oils induced higher activity of peroxidase and polyphenol oxidase enzymes. The higher activity of enzymes was associated with decreased infection of powdery mildew disease. This means that spraying plants with essential oils gave a defense to plants from invasion with pathogen. Many investigators explained these results since they reported that peroxidase is known to be involved in the oxidation and polymerization of hydroxycinnamyl alcohols to yield lignin and cross-linking isodityrosine bridges in cell wall. It also produces free radicals and hydrogen peroxide which are toxic to many microorganisms (Vance *et al.*, 1980; Fry, 1982; Pena and Kuc,1992). Also, Ride (1983) and Tarred (1983) stated that the increase in peroxidase activity enhances lignifications in response to infection with pathogens which may restrict fungal penetration.

Table 8. Effect of essential oils and Kemah zein 75% on total green colour (SAPD) of *Zinnia elegans* L.

Treatments	Concentration	Total green colour (SAPD)	
		2006	2007
Distilled water	-	23.85k	28.84m
Kemah zein 75%	2g /L	33.80a	37.49b
Marjoram oil	250 ppm	25.44j	34.71j
	500 ppm	25.94i	33.65l
Clove oil	250 ppm	28.98g	35.88g
	500 ppm	31.61c	37.55b
Cinnamon oil	250 ppm	30.46e	36.81d
	500 ppm	31.45d	38.72a
Garlic oil	250 ppm	30.06f	35.34i
	500 ppm	31.69c	37.21c
Ginger oil	250 ppm	33.39b	35.44h
	500 ppm	33.85a	36.75e
Fennel oil	250 ppm	25.95i	34.66k
	500 ppm	26.48h	36.40f

Means within a column having the same letters are not significantly different in Duncan's Multiple Range Test.

The study revealed that spray of ginger, cinnamon and clove oil at 500 ppm reduced disease incidence. In addition, the activities of peroxidase and polyphenol oxidase increased as a result of oil spray on plants. These findings provide a basis for possible utilization of these essential oils as a safe and alternative method to fungicides for controlling powdery mildew in zinnia plants.

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Journal of Applied Horticulture, 12(1): 81-84, January-June, 2010

The valuation of olive orchards: A case study for Turkey

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Abstract

Valuation of orchards is an important issue in condemnation, taxation, loan, insurance, inheritance, and purchase-sale cases. The approach to be used for orchards may vary according to the purpose of appraisal, age of the establishment, obtainable data, and according to the current regulations. In this study, land and tree values of olive orchards in a selected region from Turkey were determined by the periodic income capitalization approach. For this aim, four villages were selected and data was collected from 55 farmers selected randomly. While determining the value of the olive orchards with trees, past values approach was used. The capitalization rate for the income capitalization approach was determined as 5.32%. The value of bare land of olive orchards over periodic net income was calculated to be $ 19,684.87/ha. Tree values per hectare varied between $ 9,189.86 and $ 16,768.13 according to tree ages.

Key words: Capitalization rate, orchards, income capitalization approach, olive, valuation.

Introduction

The olive which is the symbol of peace has been the most important plant in Mediterranean and Aegean Regions since ancient times (Sesli and Tokmakoglu, 2006). According to 2007 FAO statistics, olive production in the world was realized in 7.7 million ha area and production quantity was 17.4 million tonnes. The most important share in olive production of the world belongs to Spain (33%) followed by Italy (20%), Greece (15%), and Turkey (9%) (FAO, 2007). In Turkey, 65-70% of olives are produced for oil and the rest are reserved for table olives. Further, 85% of table olive production in Turkey is black olive (Tunalioglu and Karahocagil, 2005).

Olives grow best in warm temperatures, and cannot tolerate extreme climatic conditions. The Mediterranean region, owing to its mild climate, contains 98% of the olive harvest and 95% of the olive oil production in the world. The annual production of olives depends on climatic conditions and the alternate bearing nature of the olive tree, which yields one-year high/one-year low amounts (periodicity). Nevertheless, Turkey enjoys the advantages of being one of the major producer of olives in the Mediterranean basin. Turkey stands in second place in table olives production (first in black olives) and fourth in olive oil production in the world (Göksu, 2008).

According to 2007 data of Turkish Statistical Institute, there are 144.3 million olive trees in Turkey (TURKSTAT, 2007). 65% of olive trees in Turkey are in the Aegean region. Manisa which is located in the Aegean region is an important place in olive production of Turkey. Manisa is situated in the western part of Turkey between 38° 04′ N and 27° and 27° 08′ E. According to 2007 data of Turkish Statistical Institute, there are 4.4 million olive trees in Manisa and its olive production is 34,249 tonnes (TUKSTAT, 2007).

In recent years, many studies have been made on economics of olive and olive oil production in Turkey (Olgun ve Akgungor, 1998; Artukoglu, 2002; Tunalioglu and Gokce, 2002; Dizdaroglu *et al.*, 2003; Tunalioglu and Karahocagil, 2005; Ozden, 2006; Karsli, 2006; Erdoğan, 2008; Artukoglu and Olgun, 2008). However, there has not been much research examining valuation of olive and other orchards in Turkey (Angin, 1989; Ozudogru, 1998; Engindeniz, 2001; Engindeniz, 2003; Keskin, 2003; Engindeniz, 2007). Therefore, there is still need for study, especially in local level.

The purpose of valuation should be known to carry out valuation accurately, to explain the results, and to interpret them correctly. The approach to be used for valuation of real estates varies according to the purpose and different approaches give different results (Murray *et al.*, 1983). Valuation of orchards is mainly carried out for condemnation, taxation, loan, insurance, inheritance, and purchase-sale purposes (Casler and White, 1982; Anonymous, 1983; Buchwald, 1986; Verger *et al.*, 1989; Kennedy *et al.*, 1995; Henning *et al.,* 1996; Correra, 1997; Kennedy *et al.*, 1997).

In this study, the periodic income capitalization approach that can be applied in determining the land and tree values of orchards was theoretically examined, and then application of this approach was performed for olive orchards in Manisa, Turkey.

Material and methods

Data was collected from 55 non-irrigated olive orchard owners (farmers) by randomly sampling method in four villages (Caglayan, Karayakup, Malkoca, and Tepekoy) selected from Gordes district of Manisa province by survey method. According to 2007 data of Turkish Statistical Institute, there are 93,000 olive trees in Gordes district and its annual olive production is 180 tonnes (TURKSTAT, 2007). All the data surveyed from the farmers were the data of 2008. Size of olive orchards varied between 0.3-3.6 hectares and average olive orchard size was 1.7 hectares.

Various data of this study were collected by survey method. To serve the purpose, two different survey forms were used. The

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first one was filled with owners of olive orchards and the second one with people dealing with purchase-and-sales of land in every location.

To determine the land and tree values of olive orchards, the periodic income capitalization approach was used. According to the periodic income capitalization approach, in order to find the value of a real estate, all incomes of that real-estate in future were estimated and added to the value on valuation time. The standpoint in this approach is the income of the appraised goods. Therefore, to apply this approach the appraised goods should have a continuous income (Boykin and Ring, 1993; Ventolo and Williams, 2001; Anonymous, 2001). Basically, it is possible to appraise land and tree values of orchards both over average annual net income and over periodic net income.

In calculation of average annual net income of orchards; total establishment and maintenance (production) costs were subtracted from total production value (gross production value) to be obtained. Compound interest factor $(1+f)^n$ was used in calculating the added establishment and production costs at the end of rotation period.

In determination of land value over average annual net income, the following equation was used (Murray *et al.*, 1983);

$$D_0 = \frac{R}{f} \qquad (1)$$

where: D_0 – is land value of orchard; R – was obtained average annual net income from orchard; f – is capitalization rate.

For appraisal of tree value according to age; gross income value was used and all paid costs at the end of economic life should be updated to the valuation time. At this stage, a discount factor

$$\left[\frac{1}{(1+f)^n}\right]$$

was used. As a second step, total cost was subtracted from gross income. In determining the value of land with trees according to their age, values determined according to age were also added to the land value. Because, orchards are perennial, in valuation of orchards by the income capitalization approach, it is better to take the periodic net income capitalization as the basis (Casler and White, 1982; Murray *et al.*, 1983; Correra, 1997). This is not the only reason; other important factors are that tree ages and thus yield (income from trees) differ. Therefore, periodic net income should be calculated instead of annual net income for valuation of orchards.

The following equation was used in determination of value of bare land of orchard using total periodic net income (Mulayim, 2001):

$$D_0 = \frac{\sum_0^n (R)}{q^n - 1} \qquad (2)$$

where: D_0 – is value of bare land of orchard; $\sum_0^n (R)$ – is total periodic net income obtained from orchard at the end of economic life; n – is economic life of orchard; q – is compound interest factor $(1+f)^n$.

In determining value of land with trees in orchards over periodic net income, generally two approaches *viz.*, Past value or Future value approach are used (Rehber, 2008).

In calculating the value of land covered with trees by using past values approach, the value of bare land was updated to "t" year and then the updated total income is subtracted from this value. This equation can be written as follows:

$$D_t = \left(D_0 \cdot q^t\right) - \sum_0^t (R) \qquad (3)$$

In future values approach; the value of bare land covered with trees at "t" age is calculated by adding the updated land value (n–t) and net income updated to "t". This equation can be written as follows:

$$D_t = \left(D_0 \cdot \frac{1}{q^{n-t}}\right) + \sum_n^t (R) \qquad (4)$$

In principle, same or similar results are obtained by past and future approaches. Therefore, past values approach was only used in this study. However, it is recommended to use past values approach if the orchard to be appraised is close to establishment years and future values approach if it is close to end of economic life.

In determination of tree values according to age, value of bare land was subtracted from the calculated value of land with trees according to age and divided by number of trees per hectare.

Results

According to 2007 data of Turkish Statistical Institute, yield of olive per tree in Gordes, Manisa and Turkey were 8, 14, and 10 kg, respectively (TURKSTAT, 2007). The average yield of olive in normal production period was found 2,959.60 kg per hectare and 10.57 kg per tree in this study. Productive years of olive had been accepted to be 100 years.

Olive is marketed by sales co-operatives, wholesalers, commissioners, olive oil companies, and retailers in Turkey. In the selected area, most olives of producers were marketed to olive oil companies. The producer price of olives varied between $ 0.73/kg and $ 1.17/kg according to results of this study. Average price was calculated to be $ 1.05/kg (Table 1).

The establishment cost cover all the expenses that is relating with the period of the trees having productive capacity and were included for six years in olive orchards. These are generally related with the costs of labour and machines (maintenance, energy, etc). On the other hand, production cost consists of both operating (variable) and fix cost. Labor is used for harvesting, transporting and classification in this production branch.

In this study, average gross production value of olive orchards for productive years was found to be $ 3,107.58 per year and $ 11.10 per tree in normal production period. Gross production value is expressed by the total yield multiplied with the average price of olive. Net income was calculated by deduction from the total gross production value all such costs within the production period. This value was the income of fix assets. Net income of olive orchards in normal production period was found to be $ 1,960.18/year and $ 7.00/tree (Table 1).

Generally, land values of olive orchards can be appraised according to sales comparison and income capitalization approaches. In Turkey, farmers rarely sell their land. Therefore, in using of sales comparison approach, only data of six non-irrigated orchards that have undergone purchase-sales procedures lately were used. Sales price per hectare of six non-irrigated orchards varied between

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Table 1. Average net income of olive orchards by age of trees

Periods	Olive production			Number of tree in a ha	Average price of oil ($/kg)	Gross production value ($/ha)	Total costs ($/ha)	Net income ($/ha)
	Age of trees	kg/tree	kg/ha					
Establishment period	1	-	-	280	-	-	1,247.06	-1,247.06
	2	-	-	280	-	-	469.93	-469.93
	3	-	-	280	-	-	571.07	-571.07
	4	-	-	280	-	-	571.07	-571.07
	5	-	-	280	-	-	571.07	-571.07
	6	-	-	280	-	-	571.07	-571.07
Increasing period	7	2.21	618.80	280	1.05	649.74	1,147.40	-497.66
	8	4.07	1,139.60	280	1.05	1,196.58	1,147.40	49.18
	9	6.92	1,937.60	280	1.05	2,034.48	1,147.40	887.08
Normal production period	10-96	10.57	2,959.60	280	1.05	3,107.58	1,147.40	1,960.18
	97	8.39	2,349.20	280	1.05	2,466.66	1,147.40	1,319.26
Decreasing period	98	7.86	2,200.80	280	1.05	2,310.84	1,147.40	1,163.44
	99	7.45	2,086.00	280	1.05	2,190.30	1,147.40	1,042.90
	100	6.96	1,948.80	280	1.05	2,046.24	1,147.40	898.84

Table 2. Land and tree values of olive orchards by the periodic income capitalization approach

Age of tree	Value of bare land (D_0) ($/ha)	Value of land with tree (D_t) ($/ha)	Total value of trees (D_t- D_0) ($/ha)	Value per tree ($/tree)
10	19,684.87	36,453.00	16,768.13	59.89
20	19,684.87	36,309.47	16,624.60	59.37
30	19,684.87	36,077.40	16,392.53	58.54
40	19,684.87	35,681.33	15,996.46	57.13
50	19,684.87	35,016.53	15,331.66	54.76
60	19,684.87	33,898.80	14,213.93	50.76

$14,666.67 and 19,166.67. Average sales price per hectare of six non-irrigated orchards was calculated to be $18,079.10.

In application of periodic income capitalization approach, first of all capitalization rate should be determined. In the study for determining appropriate capitalization rate, total net income obtained from the above mentioned six non-irrigated orchards divided by total sales price of these orchards (Mulayim, 2001), and a capitalization rate of 5.32 % has been calculated.

In this study, value of bare land of olive orchards over periodic net income was calculated to be $ 19,684.87. Further, tree values for between 10 and 80 ages were determined by using the periodic income capitalization approach. When periodic net income is used in calculation, past or future values approaches can be preferred. Past values approach was used for determining tree values in this study. Tree values per hectare varied between $ 9,189.86 and 16,768.13 according to tree ages (Table 2).

In this study, the planting space was found between the interval of 5x5 and 8x8 m. The most common and preferable planting space in olive orchards was 6x6 m. The number of trees per hectare was determined to be average 280 trees. Thus, if total value of trees in one hectare is divided by 280 trees, the value of one olive tree can be calculated which ranged between $32.82 and 59.89 according to ages of trees.

Discussion

For valuation of orchards, the sales comparison, the periodic income capitalization and the cost approaches can be used. However, the approach to be used varies, depending on the purpose of the appraisal (condemnation, taxation etc.), the period of establishment, obtainable data, and regulations of the country. Cost approach can be used only for valuation of olive trees (1-6 ages). However, value of olive trees (1-100 ages) can be also determined by the periodic income capitalization approach. But, the periodic income capitalization approach for valuation of trees is preferred more than the cost approach in valuation practices of orchards (Casler and White, 1982; Correra, 1997)

In determination of value of bare land of olive orchards, the sales comparison and the periodic income approaches can be used, in general. In valuation of trees; the sales comparison, the periodic income capitalization and the cost approaches can be used. Especially, if olive orchards are within the establishment period the cost approach for calculating value of trees can be preferred. But, if the establishment period is over the sales comparison and the periodic income capitalization approaches are better.

As stated above, the approach to be used in valuation of olive orchards differs depending on the regulations of the country. For example in valuation for condemnation; cost and sales comparison approaches are preferred in countries like Holland, Germany, and Italy; income capitalization approach is preferred in England, Scotland, Ireland, and Turkey; and that both approaches are used in USA and Canada. Besides, it should be stated that application of the same approach varies from one country to another (Correra, 1997; Gulten, 2000; Mulayim, 2001).

According to the results of this study, it was found out that value of bare land of olive orchards calculated over net income and a rate of 5.32% was higher then the sales comparison approach. This result can be evaluated as a contradiction. But, the value of land with tree per hectare of olive orchards by the sales comparison approach can be less than the value of bare land per hectare of olive orchards by the periodic income capitalization approach in valuation practices. There are many reasons for value loosenesses in Turkish land market. For example; avoiding the high property tax, economical crisis, agricultural and land policies, industrialization, economic affects of drought, immigration from villages to urban etc. The same results were determined also in some previous studies done in Turkey (Engindeniz, 2001; Engindeniz, 2003).

Basically, making calculations according to more than one

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approach is important in making comparisons and interpretation of results. But, different approaches may arrive to different results. Further, different approaches can be used according to aims of valuation and country's law. Accuracy of data to be used is as important as the selection of the approach to be used. For example, if the sales comparison approach is used, recent sales-and-purchase values should be determined; if the income capitalization approach is used, periodic net income and proper capitalization rate should be determined; and if the cost approach is used, the cost should be carefully determined. Therefore, advantages and disadvantages of every approach should be taken into consideration in determining the approach to be used.

Acknowledgement

This study was supported by General Directorate of State Hydraulic Woks. Several farmers helped in collection of data for this study. The help of the farmers and other colleagues who provided assistance is appreciated.

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