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CONTENTS

Forthcoming Papers

ISSR, anthocyanin content and antioxidant activity analyses to characterize strawberry genotypes-Samir C Debnath and Elodie Ricard (USA).

The effect of high daytime temperatures on inhibition of flowering in 'Koroneiki' olives (*Olea europaea* L.) under chilling and non-chilling nighttime temperatures- Nasir S.A. Malik and Joe M. Bradford (USA).

Extracting within-experiment precision of horticultural experiments useful for meta-analysis-Guido Knapp, Bimal K. Sinha, and Dihua Xu (USA).

Suitability of autumnal hybrids of cauliflower (*Brassica olearacea* var. *Botrytis* L.) to spring production in a southern Mediterranean area-M. Sciortino and G. Iapichino (Italy).

Irrigation management in greenhouse tomato production on peat substrate-G.A. Peyvast and N. Mayer (Germany).

Efficacy and physical properties of ground, composted rice hulls as a component of soilless substrate for selected bedding plants-C. Song, Paul V. Nelson, Carl E. Niedziela Jr., and D. Keith Cassel (USA).

Extraction of anthocyanins from strawberries (*Fragaria* × *ananassa*) and analysis using HPLC/Q-TOF mass spectrometry-Qiong Zhang, Hong-qing Wang and Le-xin Jia (China).

A study on adaptation of tomato ecotypes from northern latitudes under southern Iran conditions-Naser Alemzadeh Ansari and Reza Mamghani (Iran).

Sucrose synthase and acid invertase activities in relation to the floral structures abortion in pepper (*Capsicum annuum* L.) grown under low night temperature-Néji Tarchoun, Salah Rezgui and Abdelaziz Mougo (Tunesia).

Low cost hydroponic devices and use of harvested water for vegetable and flower cultivation-A. Das, and D. Sing Majhi (India).

Effect of different preharvest treatments on yield and chemical quality of tomato-M. Melkamu, Tilahun Seyoum, Kebede Woldetsadik (Nigeria).

Starch degradation characteristics in relation to physiological and biochemical properties during growth and maturation of apple fruit-Manasikan Thammawong and Osamu Arakawa (Japan).

Hydraulic resistance and water relations of bell pepper as affected by fertigation regimes-S.O. Agele (Nigeria).

Use of plastic shades to regulate growth of korarima (*Aframomum corrorima* (Braun) P.C.M. Jansen)-S. Eyob and A. Tsegaye (Ethiopia).

Extraction and determination of α-solanine in eggplant fruits-Zhiwen Li, Baoli Zhou, Yuwen Ding and Xiang Liu (China).

Persian walnut (*Juglans regia* L.) grafting as influenced by different bench grafting methods and scion cultivars-Babak Dehghan, D. Kourosh Vahdati, Darab Hassani and Reza Rezaee (Iran).

Is CropSyst a useful practical tool for simulating growth and yield of processing tomato?- Onofri Andrea, Beccafichi Catia, Benincasa Paolo, Guiducci Marcello, Tei Francesco (Italy).

Water use and water use efficiency of drip-Irrigated tomato under deficit irrigation-Berhanu Kebebew and Ketema Tilahun (Ethiopia).

Effects of antibrowning agents on the shelf life of fresh-cut green jackfruit (*Artocarpus heterophyllus* **Lam.)-**Boodia Navindra, Ruggoo Arvind and Boodoo B. Hassina (Mauritius).

Digital color space properties of pixels for adaxial and abaxial surface of mango leaves- Ahmed Yahya Hammad, Farid Saad Eid Kassim and Mohamed Naguib Hassan Ismail (Egypt).

Evaluation of the FAO CROPWAT model for deficit-irrigation scheduling for onion crop in a semiarid region of Ethiopia-Samson Bekele and Ketema Tilahun (Ethiopia).

An evaluation of health of cactus collection availabel in botanical garden of Cluj-Napoca Romania-Gyorgy Feszt and Lucica Mihalte (Romania).

Performance of asparagus under the desert conditions of Arabian Peninsula: A pilot study-N. Kameswara Rao and Mohammed Shahid (UAE).

Effect of ripening concentrate on ripening and sensory properties of locally cultivated mangoes (*Mangifera indica* L.)-William Ofori Appaw , Ibok Oduro, William Otto Ellis (Ghana).

Horizontal and vertical soilless growing systems under Cyprus conditions-Damianos Neocleous, Charalambos Kaittanis, Nicos Seraphides and Polycarpos Polycarpou (Cyprus).

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Mild heat shocks to extend the shelf life of minimally processed lettuce

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Abstract

Changes in ascorbic acid contents, microbial population and sensory attributes of cut Romaine lettuce subjected to thermal shocks were investigated. Immersion of cut lettuce in the thermal baths produced reduction in the ascorbic acid contents between 190 and 300 g kg^{-1}, with the greater losses corresponding to the higher bath temperatures. However, the rate of ascorbic acid degradation during refrigerated storage was independent of the thermal treatment and all samples presented a sharp decrease during the first day of storage and a gradual decrease thereafter. Thermal shocks did not reduce the initial microbial population. During storage, an increment in microbial counts was observed, being more notorious in samples that had been exposed to the highest shock temperature (50 °C). The thermal treatment at 50 °C was the only one to delay the onset of midrib and edge browning up to four days of refrigerated storage. This midrib and edge browning was considered to the most relevant to the overall visual quality of the product.

Key words: Cut-lettuce, heat shock treatments, ascorbic acid, total microbial counts, sensory attributes.

Introduction

Minimal or fresh-cut processing of vegetables provides convenience to food services and retail customers, but may result in limited post cutting shelf life because of undesirable physiological changes (Cantwell *et al.*, 2001). Tissue disruption caused by cutting results in elevated respiration and transpiration, which can lead to rapid deterioration. In addition, cut tissues trigger chemical reactions and release nutrients that support the growth of the microflora present on raw produce. Browning of fresh fruits and vegetables reduce quality and is often the factor limiting shelf life and marketability (Saltveit, 1998). Enzymatic and non-enzymatic reactions with phenolic compounds produce brown pigments in plant tissue. Preventing browning in these tissues requires deactivation of the enzymes (*e.g.* polyphenoloxidase) responsible for browning, exclusion of oxygen, or application of chemical antioxidants.

Heat treatments have been demonstrated to be effective as a non-chemical means of improving postharvest quality for a variety of horticultural products. Moreover, the applications of mild heat shocks constitute an alternative for the preservation of organically cultivated crops for which the use of synthetic chemicals is objectionable. Heat treatments may affect ripening and protect against physiological disorders and have been used as an effective alternative treatment for decay control (Cantwell and Nie, 1996). Heat shock treatments prevented browning of minimally processed lettuce (Loaiza-Velarde *et al.*, 1997). Wounding lettuce leaves induces the synthesis of specific enzymes and the accumulation of specific phenolic compounds associated with tissue browning (Ke and Saltveit, 1989; Brech, 1995; Tomás-Barberán and Espin, 2001). Non-stressed Iceberg and Romaine lettuce leaves contain low levels of phenolic compounds. When wounded, phenylalanine ammonia-lyase (PAL), the first committed step in the synthesis of phenylpropanoid compounds, is synthesized *de novo*. Later, phenolic compounds are synthesized and accumulated, and tissue browning occurs (Kang and Salveit, 2003). A brief heat shock (90 s at 45 ºC) disrupts the wound-induced increase in PAL activity, delaying and diminishing the accumulation of phenolic compounds and tissue browning (Loaiza-Velarde *et al.*, 1997). Murata *et al.* (2004) showed that heat shock treatment is useful for prolonging the shelf life of cut lettuce, repressing the induction of PAL activity and phenolic accumulation during storage, and preventing tissue browning. Most published results on the use of thermal shocks to extend the shelf life of vegetables correspond to the use of chlorinated water. However, in processing organic vegetables, the use of chemical additives would be objectionable.

Moreira *et al.* (2005) found that immersion of whole Romaine lettuce leaves in unchlorinated water at 50 ºC for 120 s reduced browning. However, this beneficial effect was accompanied by undesired phenomena. This treatment produced important loses in the initial ascorbic acid contents and fastened its rate of degradation during refrigerated storage. In addition, it affected the texture of the leaves. Finally, although the treatment produced some initial reductions of the native microflora, it induced faster growth during storage and microbial counts eventually surpassed those of the controls.

Fresh cut, minimally processed lettuce, presents a shorter shelf life than whole leaves. This comes as a result of some concurring factors such as higher metabolic rates induced by physical stress and the liberation of cellular contents by membrane disruption. Our group have done work related to the development of technologies for the preservation of horticultural crops compatible with organic production methods, with low energy inputs and low environmental impact by the use of preservation factors of

natural origin. The purpose of the present work was to investigate if, during the shorter shelf life of processed lettuce, the beneficial effects of mild thermal shocks associated to reduction of browning and initial microbial populations make a contribution to the quality of the products before the adverse effects take over. Since mechanical processing also reduces ascorbic acid contents, the relevance of losses associated to thermal treatments was also analyzed.

Materials and methods

Sample preparation: Heads of Romaine lettuce (*Lactuca sativa*, type Cos, variety Logifolial) were harvested at optimal maturity. They were transported to the laboratory within 1 h of harvesting (in container at ca. 5 ºC), and were immediately subjected to preliminary operations and conditioning. Outer leaves were discarded and only photosynthetic leaves (green leaves) were included in the samples. Lettuce leaves were cut before the immersion in different bath treatments. Cuts, 1 cm wide stripes, were made perpendicular to the midrib with sharp stainless-steel knives. To apply the heat-shock, the following steps were carried out: lettuce leaves were dipped in water baths with gentle agitation (10 L volume capacity) for 2 min at four temperatures (20, 30, 40 and 50 °C), at a ratio 1: 10 w / v. The introduction of cold material in the warm water baths resulted in a temperature drop. The initial water temperature was correspondingly higher to account for this and reach the desired final temperature in the system. Bath temperatures were monitored with a Data Logger (Testo GMBH & Co. Testo- Str. 1, D-79853 Lenzkirtch, Germany). It was assumed that the leaves reached the bath temperature almost immediately because they were placed loose in the agitated baths, they are thin and have a thermal diffusivity similar to that of water.

Afterwards, lettuce leaves were dipped in a water bath at 5 °C for 30 s and then centrifuged for 30 s at 500 rpm to eliminate surface water. Samples treated in water at 20 °C were taken as the control samples. In each separate experimental run, for each temperature (20, 30, 40 and 50 ºC) and for each storage time (0, 2, 4, 6, 16, 20, 24, 28, 48, 72 and 96 h), three lettuce lots were prepared. Each lot consisted of cut lettuce leaves (100 g), placed in polyethylene bags (25cm. 20 cm, useful volume: 1.8 L) with an O_2 permeability of ca. 1000 $cm^3\ m^{-2}\ day^{-1}$, CO_2 permeability of ca. 5000 $cm^3\ m^{-2}\ day^{-1}$ and water vapor of ca. 6 g $m^{-2}\ day^{-1}$. During sample packing environmental temperature was 15 ºC. Samples were placed in boxes with overall dimensions of (0.4. 0.3. 0.3m), made of heavy-duty, 0.60 cm thick, transparent acrylic, with 97-99% relative humidity, and stored at 5-7 °C. The experimental design consisted in three independent runs. In each run, two or three lots were analyzed by duplicate or triplicate.

Determination of ascorbic acid: Ascorbic acid contents were determined by the titrimetric assay described by Roura *et al.* (2003). Ground lettuce leaves (20 g) were extracted with 100 mL of metaphosphoric acid solution (60 g kg^{-1}) for 3 min using a tissue homogenizer (Multiquick, MR 5550 CA, Braun) with a speed of 3500 to 7000 rpm. The homogenate was made up to 250 mL with 30 g kg^{-1} metaphosphoric acid, and filtered through Whatman # 42 filter paper. Temperature during ascorbic acid extraction was maintained at 0 ºC. Aliquots (5 mL each) of the filtrate were titrated with 2,6- dichloroindophenol. Ascorbic acid contents (mg 100 g^{-1}) were reported on a wet basis and were performed by triplicate on three lots from three separate experimental runs (Moreira *et al.*, 2003).

Microbiological studies: Lettuce leaves (25 g) were macerated in 90 mL PO_4K_3 buffer solution (0.1 mol L^{-1}), pH = 7.2, with a homogenize (Stomacher 400 Circulator Homogenize). Macerated lettuce was accomplished by spread plating. The enumeration and differentiation of mesophilic aerobic bacteria were performed on PCA (Plate Count Agar) and incubated at 35 ºC for 48 h (Moreira *et al.*, 2003). Microbial counts were performed in duplicate on two lots from three separate experimental runs.

Sensory evaluation: The ability of panelists (members of our laboratory with experience in sensory evaluation of leafy vegetables) to discriminate and reproduce results was tested in replicate test on fresh leaves treated with heat shocks and no treated. At each sampling time (0, 1, 2, 3 and 4d of storage) lettuce leaves were removed 20 min prior evaluation to let them reach room temperature. They were subjected to sensory evaluation in duplicate on three lots from three separate experimental runs.

The coded (3 digit) samples were presented one at a time in random order to the judges. Sensory sessions were conducted in an air- ventilated room under white light (daylight equivalent). The sensory attributes color (uniformity and intensity), midrib and edge browning, texture appearance and sensorial acceptability (overall visual quality) were scored on a five-point scale. A score of 5 points represented excellent quality; scores of 4, 3 and 2 represented, respectively, very good, good and poor qualities. Finally, a score of 1 represented very poor quality. Therefore, samples receiving scores less than 3 in any of the sensory attributes analyzed was considered to be unmarketable (Roura *et al.*, 2000).

Phenylalanine ammonia lyase (PAL) activity measurements: PAL activity was assayed following the methodology described by Ke and Saltveit (1989) and Pereyra *et al.* (2005). One unit of PAL activity is defined as the amount of PAL that produces 1 µmol of cinnamic acid in 1h under the specified conditions and is expressed as 1 µmol $g^{-1}\ h^{-1}$. PAL activity was measured during 24 h of storage and the assays were performed in triplicate on two lots from two separate experimental runs.

Statistical analysis: Differences among samples were tested by least significant differences variance analysis (Box *et al.*, 1978). Differences in slopes for ascorbic acid evolution in minimally processed lettuce subject to different heat temperature shocks were tested according to Volk (1980). Wherever differences are reported as significant, a 99.5 or 99.9% confidence level was used.

Results

Heat shock effects on ascorbic acid degradation and microbial growth: The initial ascorbic acid content of fresh whole lettuce leaves dipped in water baths for 2 min at 20 °C was 0.210 ± 0.029 (g kg^{-1} of fresh weight). The time elapsed between lettuce harvest and ascorbic acid determination in lettuce samples was one hour. Because of cutting lettuce leaves into 1 cm wide stripes, the ascorbic acid contents were reduced in the range of 100 to 150 g kg^{-1}, reaching a final ascorbic acid content value of 0.180 ± 0.026 (g kg^{-1} of fresh weight).

The exposure of cut lettuce to mild heat shocks by immersion in heated water for 120 s produced decreases in the initial ascorbic acid contents. Compared to control samples (cut lettuce immersed in a water bath at 20 °C), samples exposed to 30, 40 and 50 °C lost 190, 200 and 300 g kg^{-1} of ascorbic acid, respectively.

The evolution of ascorbic acid contents, represented as the ln of the ratio of ascorbic acid contents over the initial contents against storage time, is presented in Fig. 1. Apparently, there would be two periods for ascorbic acid degradation. There was a rapid decrease in the first 24 h of refrigerated storage. Thereafter, the ascorbic acid contents degraded at a lower rate. No significant differences were found among the slopes of the tendency lines for the different bath temperatures, neither during the first 24 h of storage nor thereafter. However, the slopes of the tendency lines before and after the first 24 h of storage were significantly different.

Fig. 2 presents the evolution of total microbial counts in fresh cut-lettuce dipped in water at 20, 30, 40 and 50 ºC during 4 days of storage. Total microbial counts in control samples were 6.90 ± 0.39 (log colony forming units [CFU]. g^{-1}, n= 13). Samples subjected to mild heat treatments at 30, 40 and 50 °C did not present reductions in the microbial populations respect to control samples (20 ºC).

During refrigerated storage, samples treated at different heat shock temperatures presented a same pattern for microbial populations evalution (Fig. 2). There was a sharp increase in the number of CFU g^{-1} during the first day of storage. During day 2 and 3, the microbial populations stabilized around 8.2 log CFU g^{-1}. Finally, between days 3 and 4 of storage, another increase in microbial

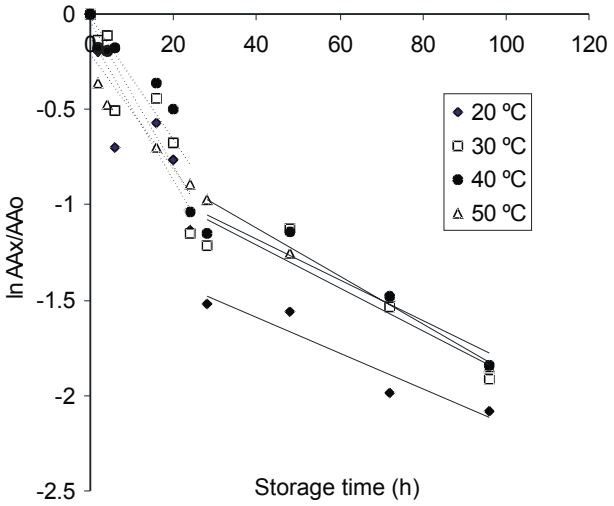

Fig. 1. Ascorbic acid degradation (ln AAx/AAo) in fresh cut-lettuce dipped in water at 20, 30, 40 and 50 ºC. Dotted lines correspond to tendency lines obtained during the first 24 h of storage with a slope average of –0.03475. Full lines correspond to tendency lines obtained during the period 24-96 h of storage, with a slope average of –0.0109. Each assay was performed by triplicate of 3 lots on 3 separate experimental runs.

Table 1. Sensory attributes (midrib and edge browning, color, texture and overall visual quality) in fresh cut-lettuce as influenced by heat shock temperatures and by refrigerated storage at 5 ºC for up to 4 d

Sensory Attributes*	Heat shock temperature (°C)	Storage time (days)				
		0	1	2	3	4
Midrib Browning	20	5.0±0.0a x *	4.7±0.4b x	4.2±0.4c x	3.5±0.7d x	3.4±0.3d x
	30	5.0±0.0a x	4.8±0.2b x	4.0±0.3b x	3.7±0.6d x	3.6±1.2d x
	40	5.0±0.0a x	4.9±0.2a x	4.7±0.1b y	4.0±0.5c y	4.0±0.8c y
	50	5.0±0.0a x	5.0±0.0a y	5.0±0.0a z	4.9±0.1a z	4.9±0.2a z
Edge Browning	20	5.0±0.0a x	4.5±0.2b x	4.0±0.5c x	3.1±0.7d x	2.5±0.6d x
	30	5.0±0.0a x	4.9±0.1b y	4.2±0.3c x	3.2±0.4d x	3.0±0.2d y
	40	5.0±0.0a x	5.0±0.0a y	4.5±0.2b y	3.5±0.5c y	2.9±0.3d y
	50	5.0±0.0a x	5.0±0.0a y	5.0±0.0a z	4.9±0.1a z	4.8±0.1a z
Texture	20	4.9±0.3[a] x	4.8±0.2a x	4.4±0.4b x	4.3±0.3b x	3.4±0.5c x
	30	4.8±0.2a x	4.7±0.2[a] x	4.6±0.2a x	4.3±0.6a x	3.5±0.7b x
	40	4.6±0.1[a] y	4.5±0.2[a] x	4.3±0.3a x	3.9±0.4b x	3.6±0.4b x
	50	4.4±0.1a z	4.4±0.1a y	4.1±0.4a x	3.8±0.2b x	3.1±0.5c x
Colour	20	5.0±0.0a x	4.4±0.2b x	4.1±0.2c x	3.7±0.4d x	3.1±0.5e x
	30	5.0±0.0a x	4.6±0.4b x	4.0±0.1c x	3.8±0.5c x	2.9±0.2d x
	40	5.0±0.0a x	4.4±0.2b x	4.3±0.2b x	3.9±0.4c x	3.0±0.1d x
	50	4.8±0.4a x	4.3±0.2b x	3.9±0.3c x	3.6±0.6c x	3.1±0.3d x
OVQ	20	5.0±0.0a x	4.5±0.3b x	3.8±0.5b x	3.2±0.4c x	2.2±0.5d x
	30	4.9±0.2a x	4.6±0.3b x	4.1±0.2c x	3.3±0.5d x	2.7±0.2e x
	40	4.9±0.1a x	4.6±0.2b x	4.4±0.2c y	3.7±0.3d y	2.9±0.1e y
	50	4.8±0.3a x	4.3±0.8b x	3.9±0.3c x	3.7±0.2c y	3.3±0.7d y

OVQ= overall visual quality

*Mean scores of three independent lots. Samples in each lot were run by duplicate.

Five point scale: 5 = very good and 1 = poor

a,b,c,d,e : Values within a same row with a different letter are significantly different ($P<0.05$).

x,y,z : Values within a same column with a different letter are significantly different ($P<0.05$).

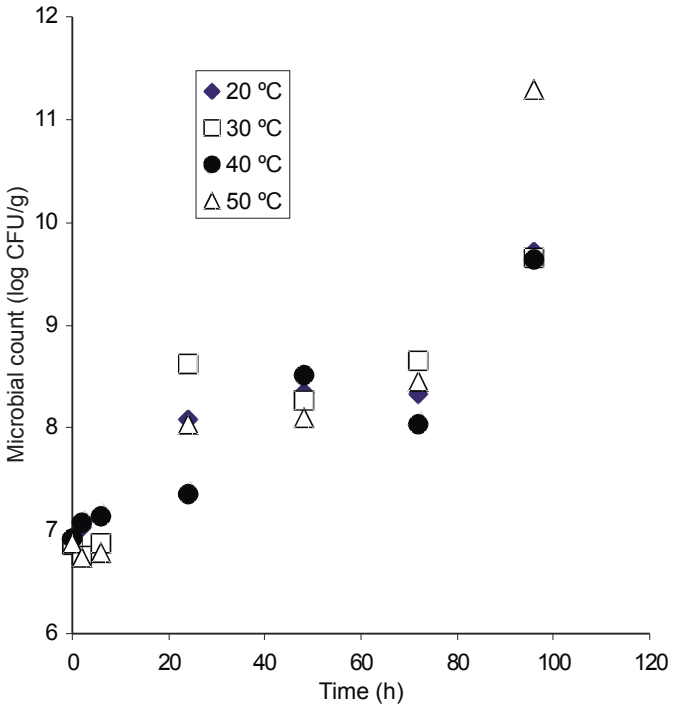

Fig. 2. Evalution of total microbial counts (CFU. g^{-1}) in fresh cut-lettuce dipped in water at 20, 30, 40 and 50 ºC during 4 days of storage. Each assay was performed in duplicate of 2 lots on 3 separate experimental runs.

counts was found. The largest increases in microbial populations corresponded to samples that had been exposed to the highest bath temperatures, that is 50 °C.

Sensory acceptability and PAL activity: Table 1 presents the sensory attributes (midrib and edge browning, color, texture appearance and overall visual quality) in fresh cut-lettuce as influenced by heat shock temperatures and by refrigerated storage at 5 ºC for up to 4 d. The application of the thermal shocks did not cause any noticeable changes in the sensory evaluations at the beginning of the storage period. Although the texture appearance of samples treated at 40 and 50 °C received lower scores, associated to a little softening of the tissues, the differences were not significant. The scores attributed to cut-lettuce color were similar in all the samples, independent of the temperature at which they had been exposed. Samples treated at 20 and 30 °C consistently obtained higher scores in texture appearance than those treated at 40 and 50 °C. Nevertheless, after 4 d of storage, this particular index did not reach rejection levels.

On the other hand, samples treated at 50 °C received significantly higher scores in midrib and edge browning throughout the 4 d of storage. The scores assigned to these samples for midrib and edge browning corresponded to excellent quality even after 4 d of storage. Since browning has an important impact on the overall visual quality (OVQ) of this product, samples treated at 50 °C were the only ones to receive scores of OVQ above 3 at day 4.

Fig. 3 represents the phenylalanine ammonia lyase (PAL) activity in midrib tissue of fresh cut-lettuce treated at 50 ºC compared with control cut-lettuce (20 ºC) during the first hours of storage. No significant increase in PAL activity was observed in the first 12 h of storage in both samples, so only PAL values obtained at storage times higher than 13 h were presented. Heat shock treatments at 50 ºC significantly decreased the rate of PAL activity

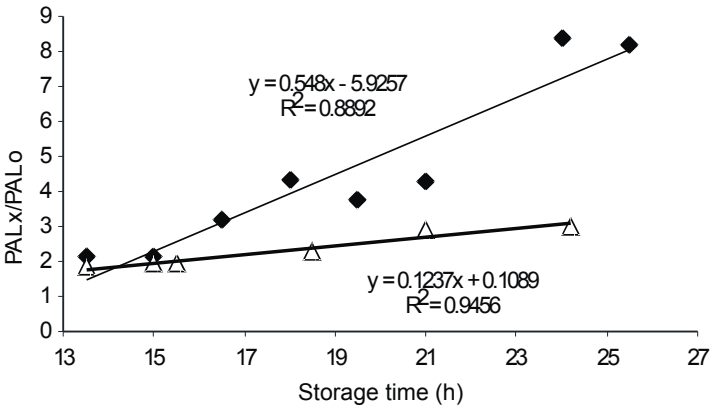

Fig. 3. PAL activity in midrib tissue of fresh cut-lettuce during the first 24 h of storage. Δ- Cut-lettuce subjected to heat-shock treatment at 50 ºC, ♦- Control cut-lettuce. Each assay was performed by triplicate of 2 lots on 2 separate experimental runs.

in the first day of storage (Fig. 3), being 4 times lower than in control samples.

Discussion

The initial contents of ascorbic acid in fresh vegetables depend on various pre and post harvest factors such as climatic conditions, maturity at harvest, harvesting methods or exposure to sunlight (Lee and Kader, 2000). Since ascorbic acid is degraded during storage of these commodities, the time elapsed between harvest and analysis, that is the freshness of the sample, plays an important role in its initial levels. The narrow period (1 h) between lettuce harvest and ascorbic acid determination resulted in higher initial ascorbic acid content in the lettuce samples assayed. The values obtained were significantly higher than those previously obtained with the same lettuce variety (Moreira *et al.*, 2005; Roura *et al.*, 2003).

Ascorbic acid is lost during the different processing operations: some is lost in the cutting operation and some due to application of heat shocks. Cutting resulted in loss of ascorbic acid content in the range 100 to 150 g kg^{-1}. Kader (1992) reported that fresh vegetables lose ascorbic acid when they are severely cut or shredded. Cut-lettuce samples subjected to a heat shock at 50 ºC presented additional losses in ascorbic acid around 300 g kg^{-1}. Similarly, Moreira *et al.* (2005) reported that the immersion of whole lettuce leaves in hot water produced losses in ascorbic acid content.

During storage, ascorbic acid degradation in fresh cut-lettuce subjected to mild heat shocks presented two periods with different degradation rates: a rapid decrease in the first 24 h of refrigerated storage and thereafter, a period of lower rate (Fig. 1). This would indicate different mechanisms for ascorbic acid degradation during the first stages of refrigerated storage that could be attributed to residual stress factors and to leaching and oxidation of the ascorbic acid exposed by the cuts. The physical damage caused by preparation increases respiration and ethylene production within minutes, and associated increases occur in the rates of other biochemical reactions responsible for changes in color, flavor, texture and nutritional quality (Kader, 1992). In whole lettuce leaves, subjected to the same thermal treatments, the increase in ascorbic acid degradation rate during the first day of storage was not observed (Moreira *et al.*, 2005), indicating

that in cut-lettuce samples the first period of degradation would be attributed at stress factors accumulation due to cutting operation.

Although different ascorbic acid degradation rates before and after the first 24 h of storage were observed, no significant differences were found among the slopes of the tendency lines for the different bath temperatures. Murata *et al.* (2004) reported that exposure of cut lettuce to 50 °C for 90 s did not affect the ascorbic acid contents.

Microorganisms play an important role in the shelf life of fresh vegetables. Both, cutting and thermal treatments may affect the microbial populations that will proliferate in minimally processed lettuce. The initial microbial counts found in control cut-lettuce (20 ºC) were similar at those previously reported for whole lettuce leaves of the same variety (Moreira *et al.*, 2005; Roura *et al.*, 2003). Thermal treatment applications did not produce any initial microbial counts reductions in fresh cut-lettuce (Fig. 2). In contrast, initially microorganisms reductions were observed when whole lettuce leaves were subjected to mild heat shocks (Moreira *et al.*, 2005). Therefore, this different behavior would be due to the cutting operation. Fresh, whole vegetables usually have a hard, protective layer and waxy material on the outer surface. In minimally processed vegetables, the continuity of this protective layer is affected and some cells are ruptured. This would expose cellular material high in water contents, sugars, organic acids and other organic substances (King and Bolin, 1989). Damaged tissue would not only provide a portal for the entry of microorganisms but would also facilitate their access to nutrients for their metabolism. Furthermore, microorganism can establish itself inside broken cells or cells adjacent to broken tissue and although fresh-cuts are washed with sanitizing agents, microorganisms can survive when they are located within cells or areas not penetrated by the washing treatment (Watada *et al.*, 1996). Delaquis *et al.* (2004) reported reduction in the microbial populations of about 1 log cycle on fresh-cut iceberg lettuce treated for 1 min at 50 °C in chlorinated water. Li *et al.* (2001) also reported reductions in the populations of mesophilic aerobic bacteria in the range of 1.71 to 1.96 log cycles on lettuce treated for 90 s at 50 °C in chlorinated water. The presence of chlorine would be responsible for the differences with our results.

Independently of the bath temperature assayed, microbial counts in cut-lettuce increased gradually during storage presenting samples treated at 50 ºC the highest microbial counts (Fig. 2). On one side, this could be due to the availability of nutrients for microbial growth resulting from the physical disruption of tissue structures associated to the cutting operation. On the other side, during the first hours of storage there is a transient temperature until thermal equilibrium between the samples and the storage chamber is reached, that could favor bacterial growth. This would correspond to a period when the low storage temperature would hinder the proliferation of microorganisms. Moreira *et al.* (2005), found that high heat shocks temperatures also promoted faster microbial growth in whole lettuce. Li *et al.* (2001) also reported enhanced microbial growth during the storage of iceberg lettuce that had been treated at 50 °C.

Fresh leafy vegetables are very fragile foodstuffs that deteriorate rapidly. The sensory attributes that make deterioration most evident are changes in texture appearance (wilting) and color (enzymatic discoloration). Thermal treatment application on cut-lettuce did not produce changes in the sensorial acceptability determined by color, texture appearance and overall visual quality (Table 1). In a previous work, Moreira *et al.* (2005) reported similar results for whole lettuce leaves subjected to mild heat shocks.

Thermal treatment at 50 °C was the only effective treatment in delaying the onset of midrib and edge browning up to four days of refrigerated storage. This attribute was considered by the panelist as the most relevant in the overall visual quality of the product. Minimal processing generally increases the rates of metabolic processes that cause deterioration of fresh produce. Control of the wound response is the key to provide processed products of good quality (Kader, 1992). Apparently, the impact of wounding was reduced by the application of heat shock treatments at 50 °C, reducing wound-induced metabolic activity. Loaiza-Velarde *et al.* (1997) reported that treatments at 45 °C for 90 s disrupt the wound-induced increase in PAL activity, delays the accumulation of phenolic compounds and diminishes tissue browning. Salveit (2000) reported that treatment at 45 °C for 90 s was so persistent in fresh-cut lettuce that it did not show any browning, even after 15 d in air at 5 °C.

A significant decrease in PAL activity in cut-lettuce as a consequence of thermal treatment at 50 ºC was obtained (Fig. 3). The ability of heat-shock treatments to reduce wound-induced browning may be the result of the decreased synthesis of wound-induced PAL activity (Saltveit, 2000). Ke and Saltveit (1989) indicated that wound-induced PAL activity is evident in the first hours of storage and reaches a maximum after 1 day. Pereyra *et al.* (2005) working with the same lettuce variety (Romaine lettuce) found a high regression coefficient when the slopes of the tendency lines of PAL activity during the first day of storage where plotted against the slopes of the tendency lines of OVQ scores. They reported that changes in OVQ scores and in PAL activity during the first day of storage could be concurrent phenomena and therefore the initial rate of change in PAL activity could be used to predict the shelf life of minimally processed lettuce.

Cut lettuce treated at 50 °C received significantly higher scores in midrib and edge browning throughout the 4 d of storage. Since browning has an important impact on the overall visual quality (OVQ) of this product, samples treated at 50 °C were the only ones to receive scores of OVQ above 3 at day 4. Since fresh-cut lettuce presents a shorter shelf life than whole lettuce leaves and midrib and edge browning are enhanced by wounding, the retardation of discoloration disorders becomes the more important quality index in cut vegetables and thermal shocks at 50 °C could make a great contribution to extend the shelf life of minimally processed lettuce.

This work presented evidence for the potential application of mild heat shocks as a novel preservation technology for minimally processed vegetables compatible with organic and low input farming systems.

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Morphological changes in the apex of *Prunus persica* L. during floral transition and effects of gibberellin on flower bud differentiation

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Abstract

The aim of the research was to study the morphological and histochemical evolution of the bud meristems of 'Lavinia' nectarine cultivar. Moreover, the effectiveness of Release LC (a gibberellin chemical compound) in controlling the rate of flower bud differentiation was also evaluated. During a two-year period, the Release LC was applied in postharvest to avoid problems of possible chemical residues on marketable fruits. To determine the effect of treatment, several biological parameters such as initial flower and vegetative bud number, flower bud drop, evolution of the flower bud phenological stages, rate of bloom and fruit set were recorded. To establish the floral differentiation stage, the meristematic apices were collected before and after treatment and microscopically observed. The thin sections were analysed using histological (apex size, developmental stages of meristematic apex, co-axial stage), and histochemical (RNA fluorescent staining) techniques. In 'Lavinia' cv., the critical phase of the meristematic apex evolution occurred from May to June (60 and 90 days after full bloom): the presence of triple apices increased rapidly, the co-axial phase was achieved, the width and height of the meristematic dome increased markedly and the RNA appeared by a weak staining. As regards the flower bud differentiation control by exogenous treatments with Release LC, the different results obtained in our experiments indicate that the efficacy of treatment strictly depends on the growth stage of a meristematic apex.

Key words: Chemical thinning, flower bud differentiation, gibberellins, meristematic apex morphology, nectarine

Introduction

In many fruit species, the control of the crop load is an unavoidable operation in a modern orchard management system, to optimise the size and quality of fruits, prevents alternate bearing and balances the fruit-to-shoot ratio (Costa and Vizzotto, 2000). Thinning must be often performed every year and the hand thinning of fruitlets is the technique that ensures the best results, although the costs are very high and it takes between 100 to 500 h ha^{-1} depending on vigour and flower production, thinning intensity and season (Jimenez and Diaz, 2002). Consequently, more cheaper alternative means are still under evaluation. Chemical and bioregulator substances (Carbaryl, NAA, GAs and 6-BAP) are effective as fruit thinners in apple (Dennis, 2000; Fallahi and Willemsen, 2002), while no satisfactory results have been achieved in peach and nectarine (Southwick *et al.,* 1996; Costa and Vizzotto, 2000; Garcia-Pallas *et al.*, 2001). Chemical fruit thinning could create an uneven distribution of fruits in trees or preferentially remove large fruit at the expense of smaller fruit (Southwick *et al.*, 1996). Indirect thinning methods were applied for inhibiting flowering intensity by chemical applications during flower bud induction and, thus, reducing costs of hand thinning. Gibberellin seems able to reduce the number of potential floral buds, inhibiting the transition of meristematic apices from the vegetative to floral stage, when applied before floral differentiation (Oliveira and Browning, 1993; Gonzales-Rossia *et al.,* 2006). Investigations on *Prunus persica* [L.] Batsch., have indicated that GA treatments from 0 to 47 days after full bloom (Byers *et al.*, 1990) and from mid-June to early July (Southwick *et al.*, 1996) inhibit flower bud differentiation, reducing the subsequent crop load. However, conflicting results didn't allow generalising on application time and chemical concentration for the same cultivar (Taylor and Geisler-Taylor, 1998, Garcia-Pallas *et al.*, 2001). In addition, fruit load regulation through the chemical control of flower bud differentiation may increase the risk of total crop loss caused by accidental events (*i.e.* spring frost injury), which can occur during the ontogenetic cycle. Favourable responses have been shown in areas generally without adverse winter and spring weather stress (Southwick *et al.*, 1995).

Better understanding of chemical thinning action mechanism could be helpful to explore the evolution of a meristematic apex under morphological and physiological aspects. These aspects may be studied using classical histological techniques and by advanced cytochemical methods. In particular, cytochemical studies showed a relationship between the floral transition process (evocation) and RNA synthesis. During the evocation phase, responding to the floral stimulus, flowering genes are de-repressed and alternatively genes responsible for vegetative patterns of morphogenesis are eliminated (Evans, 1971). The new genetic order in the apical meristem leads to an increase in the RNA content, which is considered one of the earliest indicators of the evocation process (Bernier *et al.*, 1981; Buban and Faust, 1982) and involved in the transition from vegetative to reproductive phase (Wada *et al.*, 2002).

The aim of our research was to study the morphological and histochemical evolution of the meristematic apices and the effectiveness of Release LC (a gibberellin chemical compound)

in controlling the rate of flower bud differentiation on a nectarine cultivar. We chose a postharvest treatment to avoid problems of possible chemical residues on marketable fruits, considering the currently increasing pressure from consumers for fruits and vegetables cultivated according to low environmental impact systems.

Materials and methods

Plant material and experimental site: The experimental trials were carried out over two consecutive growing seasons (2002 and 2003) on mature (ten-years-old) 'Lavinia' nectarine peach trees located in a germplasm collection at the Department of Cultivation and Protection of Woody Species of Pisa-University (Italy; altitude 6 m, lat. 43.02 °N, long. 10.36 °E). The trees were grafted onto GF677, shaped as central leader and subjected to the usual cultural practices of the area (pruning, irrigation, and fertilisation). During the experimental trials minimum and maximum daily temperature and rainfall (Fig. 1) were acquired from the Department of Agronomy and Environmental Management (Pisa, University) and from the weather station of the 'Agenzia Regionale per lo Sviluppo e l'Innovazione nel settore Agricolo-forestale' (ARSIA-Florence).

Chemical Treatments: Release LC, a liquid formulation of gibberellic acid (4% w/w) was used in postharvest at the concentration of 80 mg L^{-1} resulted effective for peach (Costa and Vizzotto, 2000; Bartolini *et al.,* 2002). The chemical was sprayed to dripping point on the trees using a high pressure handgun, and Tween 20 (polyoxyethylene-20 sorbitan monolaurate, Sigma Chem. Co.) surfactant was added at 0.02% (v/v). The same trees were used every year and were treated the day after the fruit harvest: July 26th the first year and July 21st the second year. A non-sprayed control was included in the experiment. A randomised complete-block design with one-tree plots of four replications each was performed.

Biological and morphological observations under field conditions: One-year-old fruiting shoots (10/tree) of about 50 cm in length were tagged before treatment. Subsequently they were analysed from summer to spring, to evaluate the treatment effects in the current season and flowering and fruiting in the next season. The following biological and morphological parameters were recorded: a) initial flower and vegetative bud number; b) evolution of the flower bud phenological stages (Baggiolini, 1952); c) monthly count of the persisting flower buds for the evaluation of bud drop; d) rate of bloom and e) fruit set.

Histological and histochemical analysis: To establish the floral differentiation stage, the meristematic apices (25 for each sampling time) were periodically collected from the median portion of one-year-old fruiting shoots and microscopically observed. During the first year the apex collection covered a period of 40 days, from about 10 days before harvest to one month later (end of August). During the second year, the sampling of apices covered a longer period of 60 days from initial fruit-set to harvest time.

The meristematic apices were fixed in Carnoy (ethanol and glacial acetic acid 3: 1 v/v) and subsequently prepared for the anatomical and histochemical observations. The apices, after dehydration in a graded ethanol series, were embedded in histoplast and longitudinally sectioned (10 μm) with the cut parallel to the axis, using a Shandon microtome. The slides were then dewaxed, hydrated in an ethanol-water graded series and stained with Acridine Orange solution for 15 minutes (stock acridine orange 0.1% in the ratio 1/9 in Walpole's buffer at pH 4.2) for RNA localisation according to Bitonti *et al.* (2002). The slides were mounted with a synthetic mountant (Shandon) and examined using a Nikon epifluorescent microscope equipped with a 100W mercury lamp plus an excitation filter (B, type IF 420-490).

The width and height of the apical dome (Fig. 2a) were measured using a graduate ocular, on the same slides used for the histochemical analysis, and the pertinent structural features described. In particular, the co-axial stage was identified within the node, which consists of the achievement of lateral apices at the same level as the central one (Fig. 2b), as described by Giannino *et al.* (2003).

Representative selected sections was photographed with a digital camera (Olympus C-2000 z) equipped to the microscope.

Data analysis: Statistical analysis were conducted on treatment means using Student's *t*- test procedure, and analysis of variance (ANOVA) adopting the LSD multirange test for means separation (Statgraphics *Plus* software ver. 5; Manugistics, Inc., USA).

Results

First Year (summer 2002 – spring 2003)

Biological observations related to Release LC treatment: After the treatment with Release LC, applied the day after fruit harvest (July 26th), a particularly wet period occurred and in the following two months about 200 mm of rain were recorded (Fig. 1a). The treatment showed its efficacy in controlling the number of differentiated flower buds (Fig. 3a). In October, the treated trees had a number of flower buds significantly lower than untreated ones. From winter to spring, the differences were reduced due to a light increase of the flower bud drop recorded in the control trees. However, the flower bud drop was similar among the treatments and the highest percentages (about 27%) were found just before full blooming (March, 25th). After about 15 days from full bloom, a severe spring frost (-4°C) occurred (Fig. 1b), when the young fruits were at the phenological stage I (Baggiolini, 1952). After frost, it was possible to detect different fruit retention: while the fruits persisted on treated trees, on untreated trees all fruits dropped (Fig. 3b).

Morphological and histochemical observations: **Preharvest time (-10 days)**: At this time, in the first fifteen days of July, all sampled nodes were constituted by triple apices. The co-axial stage (lateral and central apices at the same level) were reached in about 50% of the examined samples. The meristematic dome measured about 133 μm in width and 77 μm in height (Table 1).

The histological analysis made it possible to distinguish different stages of floral differentiation (Fig. 4), according to Legave (1975). The buds vegetatively differentiated were about 30% while in 25% of the apices the receptacle appeared (stage B) and only the external layer of the tunica was noticed, because the other two layers were confused with the underlying cells. The remaining apices showed morphological features of undifferentiated buds (stage A) characterized by a conical shape, with three-layered tunica and recent foliar primordium according to the 'tunica-

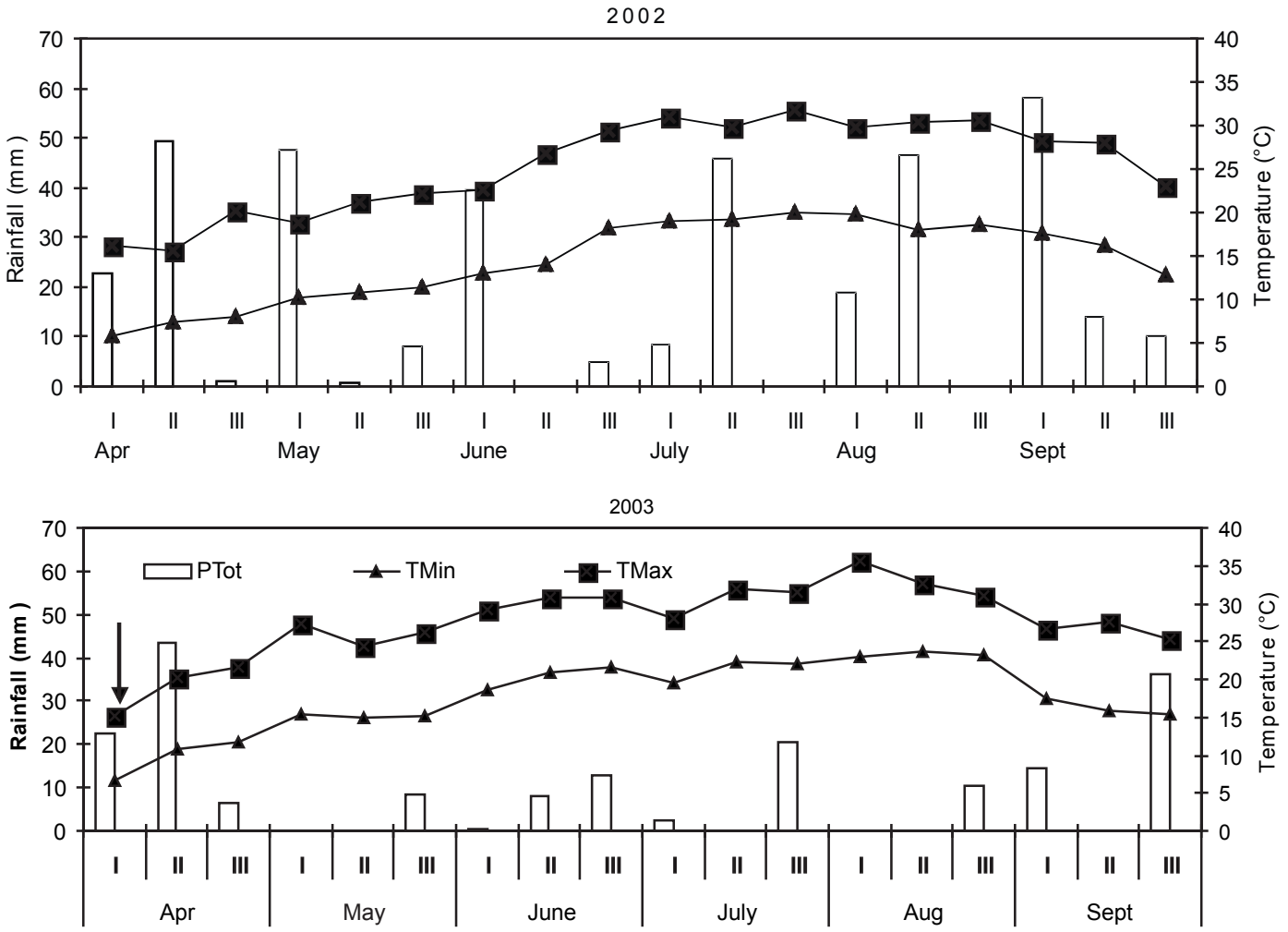

Fig. 1. Minimum and maximum temperatures (lines) and total rainfall (mm) (bars) recorded during the spring-summer season in 2002 (a) and 2003 (b) years.

corpus' model (Fig. 4). The external cellular layers of the tunica were characterised by very close cells with a dense cytoplasm and a scarcely visible nucleus.

Harvest time (July, 25th) the day before Release LC treatment: During few days (10) elapsed from the previous sampling time, the size of the meristematic dome increased both in width and height. The growth in height was particularly significant with values (about 140 µm), 80% greater compared to the previous value. Moreover, all the apices examined were at the coaxial phase. As regards the floral differentiation, the apices analyzed showed, in similar percentage (about 30%), vegetative, undifferentiated or differentiated (stage B) features (Table 1).

Postharvest (30 days after Release LC treatment): One month after fruit yield and Release LC treatment, a different evolution of apices between treated and untreated trees was observed (Table 1). In treated trees, 24% of the apices showed the anatomical characteristics of an undifferentiated stage (stage A), while 35% clearly showed morphological features of vegetative buds.

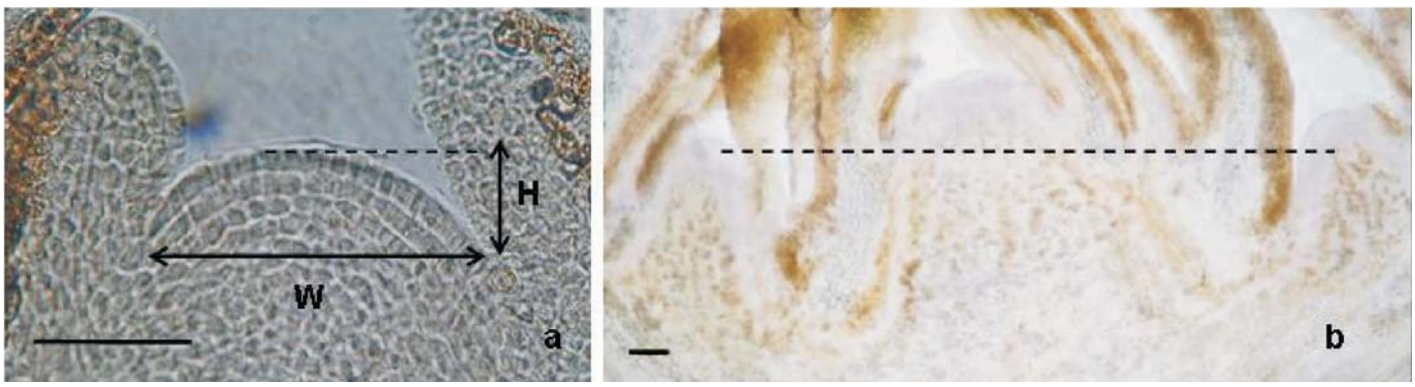

Fig. 2. Representation of measurements performed on longitudinal median sections of meristematic apices of 'Lavinia' peach cultivar. a) Height (H) and width (W) of meristematic dome (x 400; scale bar: 50 µm); b) Coaxial stage: achievement of lateral apices at same level as the central one (x 160; scale bar: 200 µm).

The remaining samples (41%) were at stages B and D-E of the flower differentiation. The untreated samples didn't show undifferentiated apices (stage A); most flower buds were at stage D-E, corresponding to the appearance of sepal, petal and stamen primordial; the remaining buds (36%) showed clear vegetative characteristics (Table 1).

Second Year (Summer 2003 – spring 2004)

Biological observation related to Release LC treatments: In this year, from July to September about 85 mm of rain was recorded and temperatures were particularly elevated mainly in the minimum daily values which never gone down below 20°C (Fig. 2b).

The Release LC treatment wasn't able to affect the differentiation process. From autumn to spring, in treated trees the number of differentiated flower buds per shoots was significantly higher

Table 1. Morphological characteristics of meristematic dome (Year 2002) and stages of floral differentiation in 'Lavinia' peach cultivar. The Release LC treatment was applied on the day after fruit harvest

Characteristic	- 10 days	Harvest time (July, 25th)	+ 30 days	
			Control	Release
(a) Meristematic dome				
Width (µm)	133.3 (±10.3)	166.7 (±15.3)*		
Height (µm)	76.7 (±16.3)	136.6 (±15.3)*		
Coaxial phase (%)	50	100		
(b) Differentiation stage (%)				
Vegetative buds (%)	30	32	36	35
Stage A	45	35	0	24
Stage B	25	33	0	25
Stage C	0	0	25	0
Stage D-E	0	0	39	16

Data represent the means values of 10 replicates (±SD). Between columns * denote significant difference by Student's *t* test ($P \leq 0.05$).

Table 2. Morphological characteristics of meristematic dome (Year 2003) (a): stages of floral differentiation (b) and type of node (c) in 'Lavinia' peach cultivar, recorded at May, June and July. (a) Width, height (µm) and coaxial phase (%) reached inside the triad apices. (b) Most advanced differentiation stage: undifferentiated apex (stage A), differentiated apex (stages B and C). (c) Type of node: percentage distribution of single, double and triple node related to the number of meristematic apices (Year 2002)

Characterstic	May, 20th	June, 10th	July, 20th
(a) Meristematic dome			
Width (µm)	84.0 (±11.4)b	116.7 (±5.8)a	122.67 (±21.5)a
Height (µm)	37.0 (±11.0)b	35.2 (±1.1)b	60.0 (±24.0)a
Coaxial phase (%)	27	86	100
(b) Differentiation Stage	A	B	B and C
(c) Type of node (%)			
Single	25	30	0
Double	65	30	20
Triple	10	40	80

Data represent the means values of 10 replicates (± SD). Between columns, values with different letters are significantly different at $P \leq 0.05$ (LSD test).

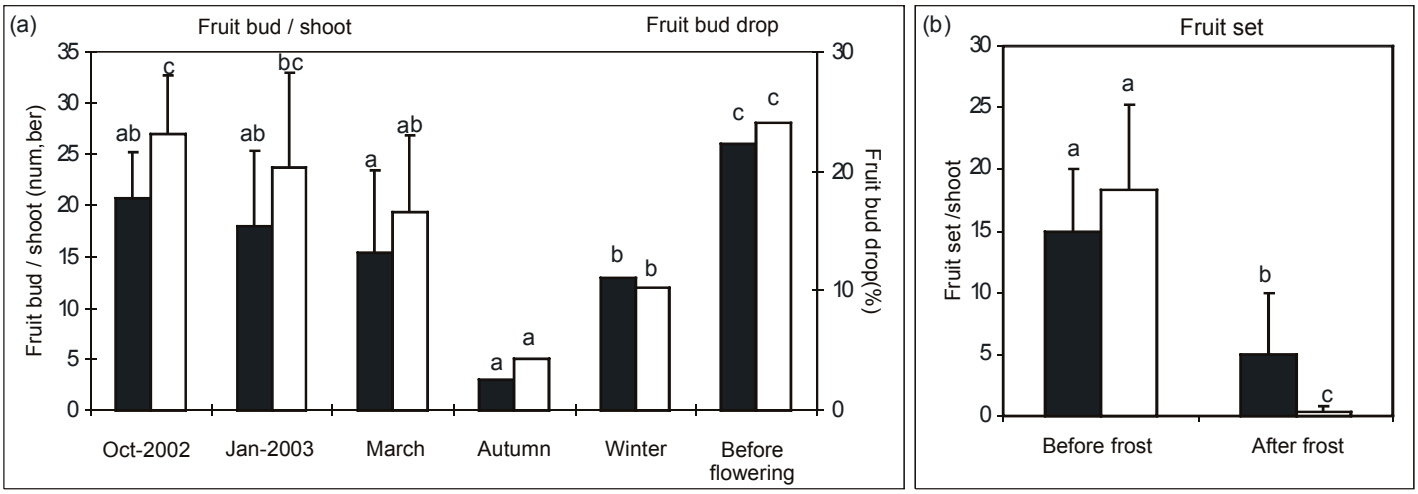

Fig. 3. 'Lavinia' peach cultivar: a) mean number (± SD) of flower buds/fruiting shoot (left axis) and percentage of flower buds drop (right axis) recorded from October 2002 to March 2003, in untreated (□) and treated (■) trees; b) mean number (± SD) of fruit set/fruiting shoot recorded before and after a spring frost (April) in untreated (□) and treated (■) trees. Means followed by different letters differ significantly at $P \leq 0.05$ (LSD test).

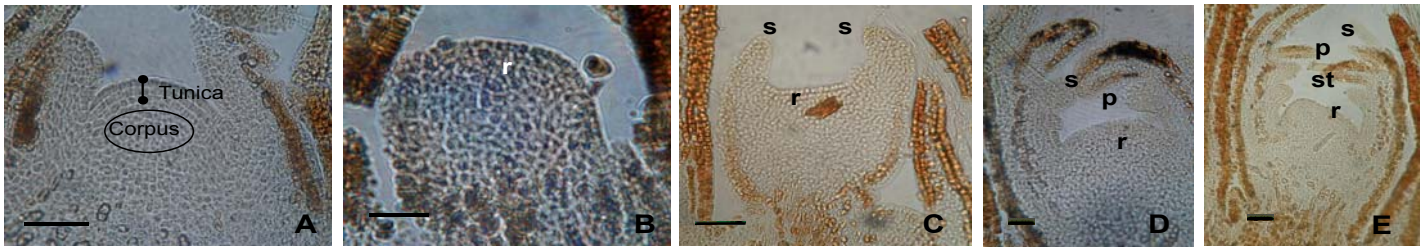

Fig. 4. Developmental stages observed in meristematic apex of 'Lavinia' peach cultivar. Stage A (x 400; scale bar: 50 µm): undifferentiated meristematic apex constituted by 'tunica' (external zone of the meristematic apex constituted by three layers of cells) and 'corpus' (under the tunica constituted by mother cells). Stages B – E: evolution of floral differentiation; Stage B (x 400; scale bar 50 µm): receptacle primordium arrangement; Stage C (x 200; scale bar: 200 µm): sepal primordia; Stage D (x 100; scale bar: 200 µm): petal primordia; Stage E (x 100; scale bar: 200 µm) stamen primordia (r: receptacle; s: sepal; p: petal; st: stamen).

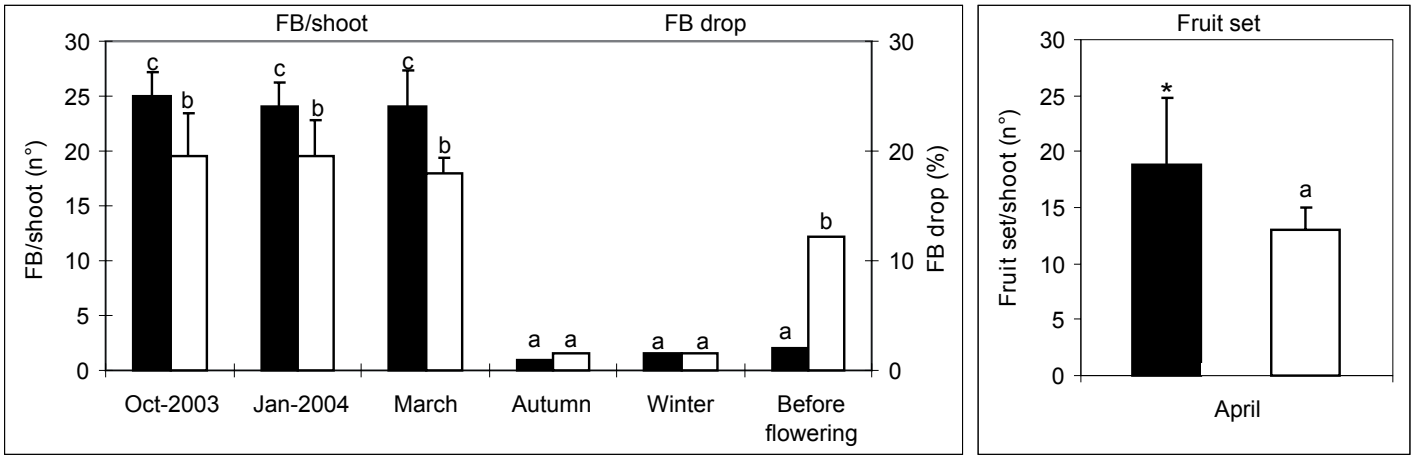

Fig. 5. 'Lavinia' peach cultivar: a) mean number (± SD) of flower buds/fruiting shoot (left axis) and percentage of flower buds drop (right axis) recorded from October 2003 to April 2004 in untreated (□) and treated (■) trees. Means followed by different letters differ significantly at $P \leq 0.05$ (LSD test); b) mean number (± SD) of fruit set/fruiting shoot recorded in April in untreated (□) and treated (■) trees. Asterisk (*) denote significant difference by Student's t test ($P \leq 0.05$).

than untreated trees (Fig. 5). This trend was also confirmed in terms of fruit set and treated trees had a highest number of fruits per shoot. In the treated trees, a negligible flower bud drop from autumn to spring was recorded and there was a larger tendency to retain fruits. On the other hand, in untreated trees a later flower bud drop occurred, just before flowering. We must remark that, on treated trees, a high percentage (more than 30%) of flowers showed double and triple pistils, abnormality observed in a very low rate on untreated trees (Fig. 6).

Morphological and histochemical observations

May, 20th (60 days after full bloom): At this time, corresponding to fruit enlargement, the meristematic dome measured 84 and 37 µm in width and height, respectively. The sampled nodes were mainly constituted by double apices (65%) and the co-axial phase was observed only in 27% of the apices. In meristematic apices, the cellular layers of the tunica were well visible, denoting an undifferentiated status (stage A) (Table 2). The histochemical procedure for RNA detection didn't give a positive reaction and no fluorescent signal was observed in the meristematic apices (Fig. 7a).

June, 10th (80 days after full bloom): The morphology of nodes showed several modifications in comparison to the observations of the previous month (Table 2). A significant increase in meristematic dome width was noticed, while the height dimension was unchanged. In particular, an increase of 30% in the number of triple apices was observed and the coaxial phase was achieved in most samples. The histological observations showed the loss of the tunica layers of the meristematic apices, denoting a prevalently presence of the differentiation stage B.

At meristematic apex level, it was possible to notice the appearance of a uniformly diffused fluorescent reaction related to the RNA localisation (Fig. 7b).

July, 20th (1 day before harvest time): The growth of the apices was underlined by a substantial increase in meristematic dome height, reaching about 2-fold higher values with respect to the previous records, while the width remained substantially unchanged (Table 2). Moreover, the sampled nodes showed triple apices which were mostly differentiated (stages B and C) and the coaxial phase was reached. The intensity of RNA staining fluorescent signal was very strong compared to the reactions observed on the previous dates. The localisation of the RNA

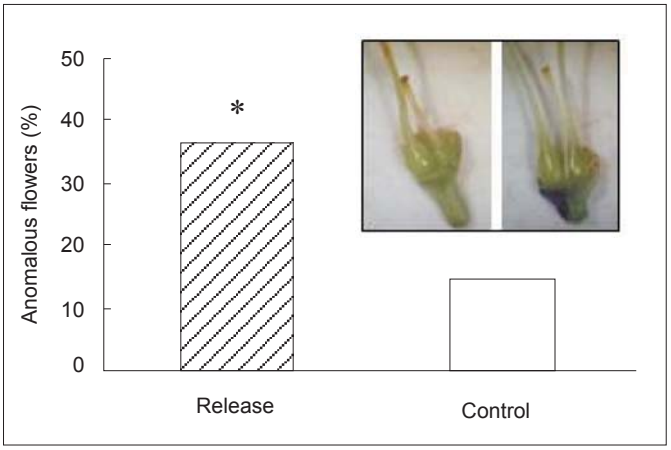

Fig 6. 'Lavinia' peach cultivar: percentage of anomalous flowers carrying double and triple pistils, recorded at blooming time (March, 20th 2003) on untreated (□) and treated (■) trees. Asterisk (*) denote significant difference by Student's t test ($P \leq 0.05$).

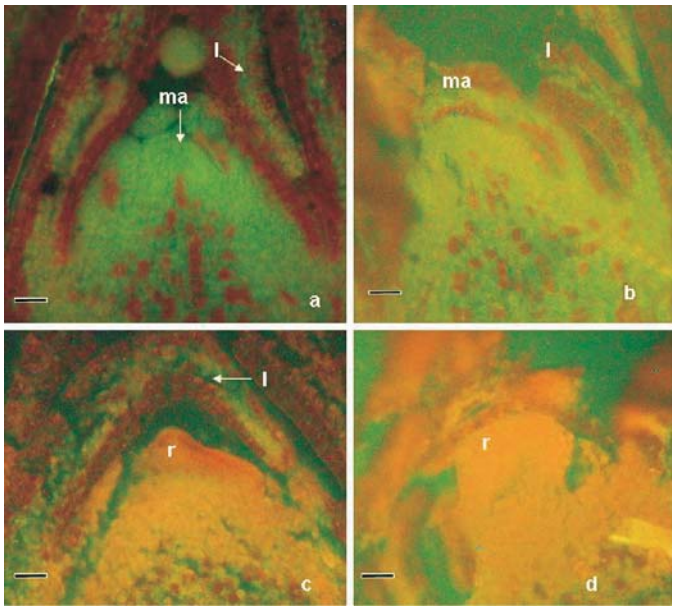

Fig. 7. 'Lavinia' peach cultivar: Fluorescence degree of RNA signal detected by acridine orange staining on meristematic lateral apices (x 200), sampled on May (a), June (b), July 3th, (c) and July 20th (d). (ma) meristematic apex; (r) receptacle primordium; (l) leaf primordium (Scale bars: 50 µm).

reaction was diffused on the apex and particularly concentrated on floral developing primordium (Fig. 7 c, d).

Discussion

The results obtained during a two year period permitted to identify the critical phase of the meristematic apex evolution in Lavinia cv., and to clarify the different results in the control of flower bud differentiation rate obtained by a postharvest application of Release LC.

The meristematic apex showed significant morphological and anatomical changes from May to June, 60 and 90 days after full bloom. In this period, the presence of triple apices increased rapidly, the co-axial phase was achieved, the meristematic dome growth markedly in width and height and the apex lost the tunica layering. This latter change has long been considered one of the classical anatomical parameters for defining flower apex induction (Tombesi, 1965; Martinez-Tellez *et al.*, 1982; Buban and Faust, 1982). The histochemical analysis carried out for the RNA localisation seems to confirm June as the month most involved in the differentiation process. In this period, we observed the first RNA appearance by a weak staining and the strongest signal was related to the morphological apex evolution, denoted by an increase in the meristematic dome dimensions and the attainment of the co-axial phase. These changes occurred in concomitance with the final peach fruit growth (data not shown). The increase in RNA detection in the meristematic apex is considered to be the first indicator of a transition period called 'floral evocation', responding to the floral stimulus (Evans, 1971; Bernier *et al.*, 1981; Pinney and Polito, 1990). In this phase, RNA and proteins are synthesised and mitotic activity increases when the stimulus triggering flower initiation reaches the apical meristem (Buban and Faust, 1982). In apple, during the transition period, a gene involved in floral differentiation began clearly to express itself (Wada *et al.*, 2002).

As regards the different effect of Release LC on the flower bud differentiation control, the results were supported by the morphological and histochemical analysis. In the first year, this chemical compound was able to reduce the initial number of differentiated flower buds: at the time of treatment, the apices were mostly still undifferentiated and, later, a slower evolution of floral differentiation was observed. A similar retardative effect, on overall flower bud development, was reported in peach after exogenous GA_3 applications (Corgan and Widmoyer, 1971; Basconsuelo *et al.*, 1995). It should be pointed out that following a late spring frost, the fruit yield of untreated trees was all injured, whereas some fruits persisted on plants treated the previous summer. The greater increase in cold hardiness wasn't due to a delay in bloom, as reported by Corgan and Widmoyer (1971) and Basconsuelo *et al.* (1995), but it could be related to a different physiological or biological status of the young fruits, hypothesis which need further and appropriate analysis. This late spring frost should affected the differentiation process of the current year which was faster. Indeed, at the same treatment time as the previous year, the apices were mainly at an irreversible stage, and thus the Release LC was ineffective in controlling the flower bud differentiation rate. Clanet and Borsani (1972) showed that GA treatments didn't induce any trouble in the differentiation process when chemical was sprayed at more advanced stage of bud differentiation (stage C). In addition, the drought conditions occurred during the summer season could have interacted with the chemical treatment, also inducing the appearance of anomalous flowers. In treated trees, high percentages of double and triple pistils were observed at blooming time in accordance with Garcia-Pallas *et al.*(2001) and Reinoso *et al.* (2002) which found a relationship between exogenous gibberellin and high floral anomalies. As consequence of strong competitions, these anomalies produce non-marketable fruits involving high costs to reduce the fruitlets to increase the final fruit size at maturity.

From a practical point of view, the postharvest application of the Release LC in 'Lavinia' cultivar not appeared an useful technique to control the flower bud differentiation rate. The different results of our experiments confirm that the response to chemical treatments may be mediated by numerous endogenous and exogenous factors. The efficacy of Release LC seems strictly depending on the growth stage of a meristematic apex. The sensitivity to this treatment proved to be during the 'transition phase' which is influenced by several physiological and environmental factors (Tromp, 2000). The high variability of the climatic conditions occurring between different years didn't allow to predict the efficacy of a chemical treatment, also within the same cultivar. The strategy of postharvest applications to avoid chemical contamination of marketable fruits fails, so as to use this approach like an indirect technique of fruit thinning in order to provide a reduction in costs.

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Effect of slow release multi-nutrient fertilizers on the yield and nutrient uptake in turmeric (*Curcuma longa* L.)

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Abstract

To evaluate the efficacy of slow release NPK fertilizers in turmeric, two field experiments were conducted on a sandy clay loam soil. These slow release NPK fertilizers are new products in the form of tablets, mixtures and coated formulations, which contains all the three major nutrients in them. Five slow release NPK fertilizer sources were tested in comparison with straight fertilizers at three NPK levels *viz.,* 75, 100 and 125 % of recommended dose in a randomized block design. The results clearly indicated that the wet rhizome yield significantly increased with increasing levels of NPK and when applied in the form of tablets. The N, P and K uptake both in shoot as well as rhizome of turmeric increased significantly up to 125 % of NPK level applied. The uptake was significantly higher in plots which received tablet form of slow release fertilizers than other fertilizer sources.

Key words: Nutrient tablets, fertilizer placement, slow release fertilizers

Introduction

Crop productivity measured in terms of response to fertilizers can only be sustained if soil fertility levels are maintained to match with crops' need and in proper proportions. To sustain the production system, it is essential that the nutrient demand of a crop to produce a target yield and the amount removed from the soil be perfectly matched. In this context nutrient recovery from applied fertilizers is primarily important which varies according to crop species, management practices, soil properties and environmental conditions and above all nutrient sources. The nitrogen use efficiency in cereal crops in developed and developing nations are 42 and 29 %, respectively (Raun and Johnson, 1999). Despite placement of phosphatic fertilizers near the seed or plant hill, the amount of phosphate present in the soil solution is very low in comparison with adsorbed P (by the reaction of complexation and fixation). Results of the Long Term Fertilizer Experiments in India revealed a declining trend in total K with the progress in the number of crops at a particular site as a result of the removal of K by the crops far exceeded the quantum of K fertilizer input. This situation demands for evolving strategies and policies to boost NPK supplies in a timely manner in adequate quantities and to increase fertilizer use efficiency.

One such strategy is the use of multi-nutrient slow release fertilizers (SRFs). Fertilizer and Chemicals Travancore Limited (FACT), Cochin, India has evolved such a strategy by bringing slow release NPK fertilizers, which are new formulations containing all the three major nutrients in a single source. Number of slow release-N formulations have been tested *viz.*, isobutylidene diurea and sulphur-coated urea on potatoes (Elkashif *et al.*, 1983), sulphur-coated urea on onion (Brown *et al.*, 1988). Slow release NPK fertilizers were tested on rice (Maheswari, 1997), sugarcane (Mathywathany, 1998) and slow release N sources on maize (Pal, 1996) and tomato (Senthilvalavan, 2000).

Intensively grown cash crops with high yields consume large amounts of nutrients. Turmeric, an important commercial as well as spice crop occupies about 1.6 lakh ha with a production of 6.54 lakh tonnes and sharing 15.06 % of total spices export of the country. Turmeric being a long duration and high yielding crop, it consumes greater amount of nutrients from the soil as well as from applied fertilizers for a prolonged period. Singh *et al.* (1992) reported that increasing rate of nitrogen and potassium application increased the accumulation of N, P and K in turmeric rhizome. Sadanandan and Hamza (1998) obtained positive effects of NPK fertilization on the nutrient uptake and yield of turmeric.

Though the above studies review the fertilization effects on turmeric growth and nutrient uptake, use of slow release fertilizers on this important spice crop has not been attempted so far. For such high fertilizer responsive crop, slow release fertilizers will be of immense use in increasing the yield and quality. With these background knowledge the present attempt was made to evaluate the effect of multi-nutrient slow release fertilizers on turmeric.

Materials and methods

Two field experiments were conducted in a farmer's field on a sandy clay loam soil (fine mixed calcareous isohyperthermic Udic Haplustalf) with turmeric (cv. Erode local) as test crop in a consecutive year. The pH of the experimental soil was 8.2 and electrical conductivity was 0.32 dS m^{-1} in 1: 2.5, soil water suspension. Organic carbon content was 0.963 %, $KMnO_4$-N was 235 kg ha^{-1}, Olsen-P was 24.2 kg ha^{-1} and NH_4OAc-K was 384 kg ha^{-1}.

Five slow release (SR) NPK sources tested for their efficacy in the present study were tablet 1 (contains urea formaldehyde, ammonium sulphate, amophos, rock phosphate, muriate of potash and clay), tablet 2 (contains phosphogypsum-urea, ammonium sulphate, amophos, rock phosphate, muriate of potash, clay and gypsum), mixture 1(mixture of contents of tablet 2), mixture 2 (mixture of contents of tablet 2 + neem cake) and coated FAP (coated amophos, urea and muriate of potash). In the first field

experiment these five SR NPK sources were tested in comparison with straight NPK fertilizers *viz.*, urea, single super phosphate and muriate of potash. These six fertilizer sources were applied at three NPK levels *viz.*, 75, 100 and 125 % of recommended dose (150: 60: 108 kg N, P_2O_5 and K_2O ha^{-1}, respectively). In the second experiment only four SR NPK sources were tested and the tablet 1 was excluded (because of high production cost and problem in handling as expressed by the manufacturer). Thus, there were totally eighteen treatments (six NPK sources each at three levels) in the first experiment and fifteen treatments (five NPK sources each at three levels) in the second experiment, which were replicated three times in a randomized block design.

Sowing of well matured, disease free turmeric rhizome (cv. Erode local, 10 months duration) was done by following a spacing of 45 x 15 cm. These slow release fertilizers were applied to their respective plots in two splits, one at 30th day after sowing (DAS) and another at 120th DAS. The required quantity of N, P_2O_5 and K_2O as per the levels *viz.*, 75, 100 and 125 % of recommended dose for each plant was satisfied with 4 tablets (in the case of tablets) and its equivalent quantity in the case of mixtures. In the case of straight fertilizers single super phosphate was applied basally on 30th DAS whereas urea and muriate of potash were applied in five equal splits at monthly interval starting from first month after sowing. All other routine cultural operations until the harvest of the crop were followed as per the recommendations (Anonymous, 2004).

Plant sampling was done by destructive method at different growth stages of turmeric crop *viz.,* 90th DAS, 180th DAS and at harvest. Five numbers of turmeric plants were uprooted from the sampling row of each experimental plot and the uprooted plants were cleaned, separated into shoot and rhizome and dried in a shade followed by drying in hot air oven at 60° C, ground using Wiley mill and stored in butter paper covers. The dry weight was recorded and the respective dry matter production was computed. At maturity the dried above ground portion (shoot) was removed 10 days before harvest leaving below ground portions so as to allow the rhizomes to mature. The rhizomes were harvested by manual digging and the wet rhizome yield was recorded.

The shoot and rhizome samples were analyzed for total N by microkjeldahl method (Humphries, 1956). In di-acid (nitric and perchloric in 9: 4 ratio) digest, total P was estimated by vanadomolybdo phosphoric yellow method (Jackson, 1973) and total K by flame photometry (Jackson, 1973). Using the data on nutrient concentration the respective nutrient uptake were computed. The data were statistically analysed as per the method described by Panse and Sukhatme (1967).

Results and discussion

Rhizome yield: Influence of different NPK sources and their levels on the wet rhizome yield is presented in Table 1. Wet rhizome yield in field experiment 1 varied from 28.10 t ha^{-1} with mixture 1 at 75 % NPK level to 41.21 t ha^{-1} with tablet 2 at 125 % NPK level. Yield increase with each successive level of NPK fertilizers was significant over its previous level. In field experiment 2 the highest wet rhizome yield was obtained with tablet 2 at 125 % NPK which is significantly superior to other sources and levels. The lowest yield (27.33 t ha^{-1}) was with mixture 1 at 75 % NPK level. The effect of tablet 1 and 2 in influencing rhizome yield was similar but significantly superior over other SR NPK sources as well as straight fertilizers. The other sources, *viz.*, mixture 1 & 2 and coated FAP were all on par with straight fertilizers in influencing the rhizome yield. Turmeric being a long duration crop it is essential that the fertilizers must be applied in optimal amounts and the release of nutrients from them must be steadily prolonged to match the nutrient needs of the crop over its growth period. The point, which deserves mention, is the comparable performance of SR NPK sources *viz.*, tablet 1 and tablet 2 at 75 % NPK level to that of straight fertilizer at 100 % NPK level. This trend would be a boon for the development of a fertilizer nutrient conservation package leading to a saving of 25 per cent of NPK input for this important commercial crop. The saving of 25 per cent NPK with the use of SR NPK fertilizers has already been realized in rice (Maheswari, 1997) and sugar cane (Mathywathany, 1998). Shankaraiah and Reddy (1988) have recorded similar yield increases for NPK fertilization in turmeric.

Nutrient uptake

The uptake of nutrients *viz.,* nitrogen, phosphorus and potassium were computed for both field experiments. Since there is no significant variation in the uptake pattern between two field

Table 1. Yield of wet rhizome (t ha^{-1}) in turmeric with different NPK sources (S)

Field Experiment I

Level (L)	Tablet 1	Tablet 2	Mixture 1	Mixture 2	Coated FAP	Straight Fertilizer	Mean
75 % NPK	31.67	32.62	28.10	28.81	29.05	28.81	29.84
100 % NPK	37.62	39.05	33.49	33.81	33.04	34.54	35.26
125 % NPK	39.64	41.21	36.67	36.19	36.43	37.62	37.96
Mean	36.31	37.62	32.75	32.94	32.84	33.66	34.35
	L	S	L×S				
SE (d)	0.62	0.88	1.52				
CD (*P*=0.05)	1.26	1.79	3.10				

Field Experiment II

Level (L)	Tablet 2	Mixture 1	Mixture 2	Coated FAP	Standard Fertilizer	Mean
75 % NPK	34.33	27.33	29.33	30.50	27.50	29.80
100 % NPK	40.33	33.17	35.00	34.00	32.33	34.97
125 % NPK	43.67	37.83	38.17	38.00	37.33	39.00
Mean	39.44	32.78	34.17	34.17	32.39	34.59
	L	S	L× S			
SE (d)	0.62	0.80	1.38			
CD (*P*=0.05)	1.27	1.64	NS			

Table 2. Nitrogen uptake (kg ha^{-1}) in turmeric shoots with different NPK sources (S)

Level (L)	Tablet 1	Tablet 2	Mixture 1	Mixture 2	Coated FAP	Straight Fertilizer	Mean
				90 DAS			
75 % NPK	18.0	20.9	11.6	13.8	19.3	13.5	16.2
100 % NPK	17.6	21.0	15.4	18.4	18.6	16.8	18.0
125 % NPK	23.6	22.7	17.4	16.0	18.8	19.1	19.6
Mean	19.8	21.5	14.8	16.1	18.9	16.5	17.9
	L	S	L×S				
SE (d)	0.8	1.2	2.0				
CD (*P*=0.05)	1.7	2.4	NS				
				180 DAS			
75 % NPK	30.3	32.3	25.7	27.5	32.9	31.5	30.0
100 % NPK	51.2	45.2	30.5	37.1	35.7	33.5	38.9
125 % NPK	51.9	53.9	34.4	34.2	38.7	36.2	41.6
Mean	44.5	43.8	30.2	32.9	35.8	33.7	36.8
	L	S	L×S				
SE (d)	0.9	1.2	2.1				
CD (*P*=0.05)	1.7	2.4	4.2				
				At Harvest			
75 % NPK	10.4	10.8	7.6	8.5	9.9	7.5	9.1
100 % NPK	11.4	10.2	8.4	10.0	11.8	8.5	10.3
125 % NPK	15.1	13.6	8.2	10.3	10.0	9.8	11.2
Mean	12.3	11.5	8.6	9.6	10.6	8.6	10.2
	L	S	L×S				
SE (d)	0.2	0.2	0.4				
CD (*P*=0.05)	0.3	0.5	0.8				

Table 3. Nitrogen uptake (kg ha^{-1}) in turmeric rhizome with different NPK sources (S)

Level (L)	Tablet 1	Tablet 2	Mixture 1	Mixture 2	Coated FAP	Straight Fertilizer	Mean
				90 DAS			
75 % NPK	8.7	9.5	5.6	7.0	9.9	6.9	7.9
100 % NPK	8.5	9.8	7.3	9.3	9.4	9.2	9.0
125 % NPK	12.3	11.9	8.4	8.3	10.7	9.4	10.1
Mean	9.8	10.4	7.1	8.2	10.0	8.5	9.0
	L	S	L×S				
SE (d)	0.4	0.5	0.9				
CD (*P*=0.05)	0.7	1.1	1.8				
				180 DAS			
75 % NPK	36.0	41.1	23.0	29.5	35.9	31.2	32.8
100 % NPK	53.2	54.1	32.8	35.8	41.6	38.5	42.7
125 % NPK	58.2	62.1	41.9	46.6	45.1	41.9	49.3
Mean	49.1	52.4	32.6	37.3	40.9	37.2	41.6
	L	S	L×S				
SE (d)	1.0	1.4	2.4				
CD (*P*=0.05)	1.9	2.8	4.8				
				At Harvest			
75 % NPK	69.0	63.0	46.6	53.6	47.7	56.5	56.1
100 % NPK	84.6	77.0	59.8	74.4	72.8	54.1	70.4
125 % NPK	85.2	90.2	72.0	72.2	68.2	63.1	75.2
Mean	79.6	76.7	59.5	66.7	62.9	57.9	67.2
	L	S	L×S				
SE (d)	1.3	1.8	3.2				
CD (*P*=0.05)	2.6	3.7	6.5				

experiments, the results of field experiment 1 are only presented and discussed hereunder.

Nitrogen uptake: Nitrogen (N) uptake in turmeric shoot at different growth stages increased significantly with increasing levels of NPK (Table 2). The highest N uptake was recorded at 180th DAS with tablet 2 (53.9 kg ha^{-1}) at 125 % NPK level and this was on par with tablet 1. The N uptake at harvest stage was low, however, there was significant influence by the sources and levels. N uptake in turmeric rhizome was significantly influenced by SR NPK sources at all levels and stages (Table 3). Highest N uptake was observed with tablet 2 (11.9, 62.1 and 90.2 kg ha^{-1} at 90th DAS, 180th DAS and harvest stage, respectively) at 125 % NPK level. This was followed by coated FAP and mixture 2. The lowest N uptake was recorded with mixture 1 at 75% NPK level. Unlike in shoots the N uptake in rhizome at harvest stage was higher and significant than the previous stages.

Similar increases in N uptake in turmeric due to NPK fertilization was reported by Sadanandan and Hamza (1998) and Selvakumari

Table 4. Phosphorus uptake (kg ha^{-1}) in turmeric shoots with different NPK sources (S)

Level (L)	Tablet 1	Tablet 2	Mixture 1	Mixture 2	Coated FAP	Straight Fertilizer	Mean
				90 DAS			
75 % NPK	2.3	2.3	1.4	1.8	2.3	1.8	2.0
100 % NPK	2.4	2.6	1.9	2.3	2.3	2.2	2.3
125 % NPK	3.0	3.2	1.9	2.4	2.6	2.4	2.6
Mean	2.5	2.7	1.7	2.2	2.4	2.2	2.3
	L	S	L×S				
SE (d)	0.11	0.12	0.23				
CD (*P*=0.05)	0.22	0.30	NS				
				180 DAS			
75 % NPK	2.9	3.0	2.6	2.6	2.7	2.5	2.7
100 % NPK	5.1	5.1	3.5	3.7	3.9	3.4	4.1
125 % NPK	4.9	5.0	3.5	3.9	4.4	3.5	4.2
Mean	4.3	4.4	3.2	3.4	3.7	3.1	3.7
	L	S	L×S				
SE (d)	0.08	0.11	0.19				
CD (*P*=0.05)	0.16	0.22	0.38				
				At Harvest			
75 % NPK	0.62	0.64	0.46	0.54	0.60	0.53	0.57
100 % NPK	0.63	0.74	0.52	0.65	0.64	0.59	0.63
125 % NPK	0.77	0.82	0.60	0.64	0.65	0.69	0.70
Mean	0.67	0.73	0.53	0.61	0.63	0.60	0.63
	L	S	L×S				
SE (d)	0.02	0.02	0.04				
CD (*P*=0.05)	0.03	0.05	NS				

Table 5. Phosphorus uptake (kg ha^{-1}) in turmeric rhizome with different NPK sources (S)

Level (L)	Tablet 1	Tablet 2	Mixture 1	Mixture 2	Coated FAP	Straight Fertilizer	Mean
				90 DAS			
75 % NPK	1.40	1.57	0.97	1.09	1.80	1.35	1.36
100 % NPK	1.41	1.62	1.27	1.45	1.57	1.52	1.48
125 % NPK	1.90	1.99	1.49	1.49	1.72	1.55	1.69
Mean	1.57	1.73	1.24	1.34	1.70	1.47	1.51
	L	S	L×S				
SE (d)	0.06	0.08	0.14				
CD (*P*=0.05)	0.12	0.17	NS				
				180 DAS			
75 % NPK	8.4	9.5	6.1	6.9	8.4	7.8	7.9
100 % NPK	13.4	15.3	8.5	9.5	12.1	11.5	11.7
125 % NPK	15.4	15.9	9.8	10.2	10.7	10.2	12.0
Mean	12.4	13.6	8.1	8.9	10.4	9.8	10.5
	L	S	L×S				
SE (d)	0.25	0.36	0.62				
CD (*P*=0.05)	0.51	0.72	1.25				
				At Harvest			
75 % NPK	17.4	18.5	14.7	16.1	14.9	16.3	16.3
100 % NPK	22.6	23.0	17.6	17.7	19.4	20.6	20.1
125 % NPK	25.0	25.2	20.7	22.1	21.5	21.3	22.6
Mean	21.7	22.2	17.6	18.6	18.6	19.4	19.7
	L	S	L×S				
SE (d)	0.37	0.53	0.90				
CD (*P*=0.05)	0.76	1.07	NS				

and Basker (1998). N uptake in turmeric shoot and rhizome generally increased with advancement of turmeric growth from 90th DAS and up to 180th DAS. At harvest stage, however, N uptake in turmeric shoot declined whereas in rhizome increased.

Among the SR NPK sources, tablet 1 and 2 recorded significantly higher level of N uptake in turmeric shoot and rhizome as compared to other sources. These tablets were applied by placement at a depth of 5 cm near the rhizosphere. Slow release of N coupled with reduced losses due to NH_3 volatilization and leaching have evidently enhanced N uptake from the tablets. Whereas in the case of mixtures and straight fertilizers, N uptake was of low magnitude which might be due to increased losses of N through NH_3 volatilization and leaching on their application with larger volume of soil on surface as compared to the tablets.

Phosphorus uptake: Phosphorus (P) uptake in turmeric shoot at 90th DAS ranged from 1.4 kg ha^{-1} with mixture 1 at 75 % NPK level to 3.2 kg ha^{-1} with tablet 2 at 125 % NPK (Table 4). P uptake at 180th DAS was maximum (5.1 kg ha^{-1}) with tablet 1 & 2 at 100

Table 6. Potassium uptake (kg ha^{-1}) in turmeric shoots with different NPK sources (S)

Level (L)	Tablet 1	Tablet 2	Mixture 1	Mixture 2	Coated FAP	Straight Fertilizer	Mean
				90 DAS			
75 % NPK	52.3	55.3	36.3	41.4	51.6	42.7	46.6
100 % NPK	52.7	56.7	43.5	47.3	56.8	51.0	51.3
125 % NPK	69.6	69.4	51.8	52.1	57.4	60.7	60.2
Mean	58.2	60.5	43.9	46.9	55.3	51.5	52.7
	L	S	L×S				
SE (d)	2.3	3.3	5.6				
CD (*P*=0.05)	4.7	6.6	NS				
				180 DAS			
75 % NPK	78.1	84.8	77.3	76.6	79.2	81.1	79.5
100 % NPK	136.9	125.7	93.0	91.4	93.4	93.7	105.7
125 % NPK	136.0	139.1	102.1	93.4	111.9	105.7	114.7
Mean	117.0	116.5	90.8	87.2	94.9	93.5	100.0
	L	S	L×S				
SE (d)	2.0	2.9	5.0				
CD (*P*=0.05)	4.1	5.8	10.1				
				At Harvest			
75 % NPK	35.4	36.0	30.9	32.3	33.6	28.7	32.8
100 % NPK	38.8	45.4	27.2	32.0	34.2	34.2	35.3
125 % NPK	44.3	45.7	35.0	35.2	39.5	38.0	39.6
Mean	39.5	42.4	31.0	33.1	35.7	33.6	35.9
	L	S	L×S				
SE (d)	0.43	0.60	1.05				
CD (*P*=0.05)	0.87	1.23	2.13				

Table 7. Potassium uptake (kg ha^{-1}) in turmeric rhizome with different NPK sources (S)

Level (L)	Tablet 1	Tablet 2	Mixture 1	Mixture 2	Coated FAP	Straight Fertilizer	Mean
				90 DAS			
75 % NPK	27.2	30.5	19.0	22.2	30.3	22.9	25.4
100 % NPK	28.4	32.3	25.4	28.0	30.7	30.2	29.2
125 % NPK	38.1	38.0	27.8	28.2	33.3	33.9	33.2
Mean	31.2	33.6	24.1	26.2	31.5	29.0	29.2
	L	S	L×S				
SE (d)	1.1	1.6	2.7				
CD (*P*=0.05)	2.3	3.2	NS				
				180 DAS			
75 % NPK	78.6	83.6	58.1	66.5	73.0	62.7	70.4
100 % NPK	129.1	132.0	73.1	82.3	82.0	94.1	98.8
125 % NPK	140.1	144.8	86.2	92.3	92.0	87.8	107.2
Mean	115.9	120.1	72.4	80.3	82.3	81.6	92.1
	L	S	L×S				
SE (d)	2.2	3.1	5.3				
CD (*P*=0.05)	4.4	6.3	10.8				
				At Harvest			
75 % NPK	133.9	131.1	107.6	127.4	117.8	123.2	123.5
100 % NPK	160.0	179.0	149.5	133.2	148.3	149.2	153.2
125 % NPK	197.6	200.2	179.5	169.8	177.2	171.1	182.6
Mean	163.9	170.1	145.5	143.5	147.8	147.8	153.1
	L	S	L×S				
SE (d)	2.9	4.2	7.1				
CD (*P*=0.05)	5.9	8.4	14.6				

% NPK level and minimum (2.5 kg ha^{-1}) with straight fertilizer. Similar trend was observed at harvest stage also. P uptake in turmeric rhizome ranged from 0.97 kg ha^{-1} with mixture 1 at 75 % NPK level to 1.99 kg/ha with tablet 2 at 125 % NPK level at 90th DAS (Table 5). However, the uptake with tablet 2 was on par with tablet 1 and coated FAP and significantly superior over mixtures and straight fertilizers. At harvest stage, the uptake at 125 % NPK level was significantly higher than 100 and 75 % NPK level.

Singh *et al.* (1992) have reported significant increase in P uptake in turmeric due to NPK application. Translocation of P from turmeric shoot to rhizome occurred in significant amounts with advancement of turmeric growth. Placement of tablet near the rhizosphere soil ensured a higher concentration of P in unit volume of soil solution in the immediate vicinity of turmeric roots. Thus, a higher concentration gradient was set up for the P from the tablets to diffuse faster to the turmeric roots as compared to the P from other sources. Similar beneficial effects of P placement were discussed by Prummel (1957), Reith (1959) and Ryan (1962). In the case of mixtures, coated FAP and

straight fertilizers, the volume of the experimental soil, which is calcareous, with which these fertilizer materials were in contact on application was large which evidently enhanced P reversion reactions at a faster rate resulting in the fixation of applied P in amounts of higher magnitude as compared to those from tablet form of SR NPK sources.

Potassium uptake: Potassium (K) uptake in turmeric shoot at various growth stages is presented in Table 6. Maximum K uptake was observed at 180th DAS with tablet 1 at 125 % NPK level. The uptake at 90th DAS ranged from 36.3 kg ha^{-1} with mixture 1 at 75% NPK level to 69.6 kg ha^{-1} with tablet 1 at 125% NPK level. At 180th DAS, the K uptake was enhanced significantly by all the sources at 125% NPK level and the maximum was recorded with tablets 1 and 2. At harvest stage, K uptake in shoots varied from 27.2 kg ha^{-1} with mixture 1 at 100% NPK level to 45.7 kg ha^{-1} with tablet 2 at 125% NPK level. K uptake in turmeric rhizome was also significantly influenced by SR NPK sources in all the stages (Table 7). At harvest stage, K uptake ranged from 107.6 kg ha^{-1} with mixture 1 at 75% NPK level to 200.2 kg ha^{-1} with tablet 2 at 125% NPK level.

With enhanced level of NPK, uptake of K in turmeric shoot and rhizome increased. Such positive influence of K fertilizer application on K uptake in turmeric was reported by Balashanmugam and Subramanian (1991) and Sadanandan and Hamza (1998). There was a significant translocation of K from turmeric shoot to rhizome with advancement of growth. In all the SR NPK sources, muriate of potash is the common K source component, which is water soluble. Muriate of potash is normally applied by broadcast and very rarely by banded application in soils of low K availability or with high K fixing capacity. Welch *et al*. (1966) found as much as four times increase in crop response to banded application over broadcast application of K. In the present study, K placed in the form of tablets at a depth of 5 cm near the rhizosphere enhanced K uptake significantly over the uptake from other SR NPK sources. Thus, the slow and steady release of K from tablets near the rhizosphere matched the crop uptake sparing not as much K for fixation by soil clays as from other SR NPK sources resulting in enhanced K uptake from the tablets by turmeric. The larger soil volume with which K from the mixtures, coated FAP and straight fertilizers had come in contact on application enhanced K fixation and thus K uptake from these sources was low as compared to the tablets.

Thus, the results of the present study clearly established that the wet rhizome yield increased significantly up to 125 % of NPK level. This implied that the presently followed recommendation of 150 kg N, 60 kg P_2O_5 and 108 kg K_2O per ha is sub-optimal and there exist a scope to redefine the fertilizer optima for turmeric. Until precise fertilizer optima is established through proper fertilizer optimization studies, the 125% of NPK level, *viz.*, 187.5 kg N, 75 kg P_2O_5 and 135 kg K_2O per ha may safely be used to harvest better yields than what is presently harvested. Also, the superiority of tablet form of SR NPK in influencing the yield and nutrient uptake in turmeric was clearly established in the present investigation.

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Effect of cultivar, root container size and temperature on days to flower and number of leaves before flowering in tomato

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Abstract

Seedlings of three tomato cultivars, 'Reika', 'Marryroad' and 'First Power' were grown in either pots or plug trays in order to clarify the interaction effect of cultivar and root container size on the number of days to flowering and the number of leaves preceding the first inflorescence under different temperature regimens. The number of days to flowering was greater in seedlings raised in plug trays than those in pots, regardless of temperature regimen. Flowering was delayed at 23/18°C compared with 30/25°C for seedlings raised in either pots or plug trays. The number of leaves preceding the first inflorescence was greater in seedlings raised in plug trays than those in pots, except for 'Marryroad' at 23/18°C. Regardless of root container size, the number of leaves preceding the first inflorescence was greater in 'First Power' than in 'Reika' and 'Marryroad' at 30/25°C. These results suggest the importance of cultivar choice for the production of tomato seedlings with a small number of leaves preceding the first inflorescence using plug trays in cool conditions.

Key words: *Lycopersicon esculentum* Mill., plug tray, root restriction, temperature

Introduction

Currently, some farmers harvest tomato fruits only from the first truss, or from the first to the third trusses, in order to transplant tomato seedlings at a high density in glasshouses several times a year. This cultivation method is effective for increasing yearly yield, although it requires a large number of transplants. The raising of seedlings using plastic plug trays reduces the cost of transplants because it requires a small amount of artificial growth media, and many operations such as seeding, water supply and transplantation can be mechanized. Therefore, seedlings raised in plug trays are commonly used in such a high-density cultivation method. However, transplants raised in summer exhibit spindly growth, especially transplants raised in plug trays, because of high temperatures and low light intensity caused by mutual shading.

A smaller number of leaves preceded the first inflorescence under low temperatures at the early stage of development, lead to an increase in early yield (Wittwer and Teubner, 1956; Calvert, 1957). The number of leaves preceding the first inflorescence in tomato seedlings usually increases in summer due to high temperatures (Oda *et al.*, 2005). Therefore, seedlings raised in summer come into flower later than those in other seasons, leading to reductions in early yields. To overcome this problem, Oda *et al.* (2005) proposed that tomato seedlings raised in plug trays in highlands should be transported to lowlands in the summer.

There are many studies about the effect of root container size on the growth and yield of tomatoes. Small container size was found to restrict growth and delay flowering in tomatoes (Knavel, 1965; Weston and Zandstra, 1986). However, little is known about the interaction effects of cultivar, temperature, and root container size on the number of leaves preceding the first inflorescence and the days to flowering. Here, we studied the number of leaves preceding the first inflorescence and the days to flowering when three different cultivars of tomato seedlings were raised in either pots or plug trays under different temperature conditions.

Materials and methods

Seeds of tomato plants (*Lycopersicon esculentum* Mill. cvs. 'Reika', 'Marryroad' and 'First Power') were sown in either plastic pots (15 cm in diameter) or plug trays with 128 cell packs in glasshouses on 27 October 2005. Temperatures in the glasshouses were maintained at 23/18°C or 30/25°C (day/night temperatures) and shifted between 5 a.m. and 6 a.m. and between 6 p.m. and 7 p.m. Photoperiods were not controlled. Plastic pots and cell packs were filled with 1,000 mL and 22 mL of a growth medium (Soil Mix, Sakata, Yokohama, Japan), respectively. Plug trays with 8 × 16 cell packs were 52.5 × 26.5 × 4.5 cm in size.

Seedlings that had been raised in plug trays were periodically removed from cells at transplanting stage, ten seedlings of each cultivar were transplanted into 15 cm pots. 'Reika', 'Marryroad' and 'First Power' seedlings raised in plug trays attained the transplanting stage 29, 25, and 26 days after sowing (DAS) at 23/18°C, respectively. At 30/25°C, the corresponding values were 20, 20, and 22 DAS, respectively. At transplanting, an additional five seedlings of each cultivar were randomly sampled from the plug trays. Seeds of the three cultivars were also sown directly into 15 pots each, and seedlings from five pots were sampled when those raised in the plug trays were transplanted into pots. Ten seedlings raised in either pots or plug trays were harvested at anthesis of the first flower in the first inflorescence. Fresh mass and seedling height were measured at transplanting. At flowering, the number of leaves preceding the first inflorescence, fresh mass and seedling height were measured. Shoot apices were observed under a stereoscopic microscope to check flower

initiation in seedlings raised in pots and plug trays when the latter seedlings were transplanted into pots. Because of limited glasshouse facilities, temperature treatments were not replicated. Therefore, data were subjected to analyses of variance in each temperature regimen, and mean separation was performed by the least significant difference (LSD) ($P<0.05$).

Results

At transplanting, seedlings raised in plug trays were much smaller than those raised in pots, regardless of cultivar and temperature regimen (Tables 1 and 2). At 23/18°C, 'Reika' seedlings were heavier and taller than 'Marryroad' and 'First Power' seedlings when they were raised in pots, but there were no significant differences among cultivars when they were raised in plug trays. At 30/25°C, there were no significant differences in the fresh mass of seedlings among cultivars raised in pots or plug trays. On the other hand, 'First Power' seedlings raised in pots were taller than 'Reika' and 'Marryroad' seedlings at 30/25°C.

Table 1. Effect of root container size and temperature on the fresh mass (g) of seedlings raised in pot and plug trays at the transplanting stage [z]

Cultivar	23/18°C		30/25°C	
	Pot	Plug tray	Pot	Plug tray
'Reika'	10.0 c [y]	0.7 a	3.5	0.7
'Marryroad'	2.8 ab	0.6 a	2.9	0.7
'First Power'	3.5 b	0.6 a	3.6	0.8
Significance				
Cultivar	**		NS	
Rooting volume	**		**	
Interaction	**		NS	

[z] Transplanting stages in 'Reika', 'Marryroad', and 'First Power' at 23/18°C were 29, 25, and 26 DAS, respectively. At 30/25°C, the corresponding values were 20, 20, and 22 DAS.

[y] Separation of means within a temperature regimen of 23/18°C by the LSD procedure ($P = 0.05$).

NS- not significant, ** - significant at $P \leq 0.01$.

At both temperature regimens, seedlings raised in pots initiated flower buds at the time when seedlings raised in plug trays were transplanted into pots (25-29 DAS at 23/18°C and 20-22 DAS at 30/25°C). When seedlings were raised in plug trays at 23/18°C, one, three and five out of five seedlings initiated flower buds in 'Reika', 'Marryroad' and 'First Power', respectively. When seedlings were raised in plug trays at 30/25°C, flower initiation was not observed in any seedling at transplanting.

The number of days to flowering was greater in seedlings raised in plug trays than in those from pots, and it decreased with an increase in temperature, regardless of cultivar (Table 3). The number of days to flowering was not significantly different among the cultivars at 23/18°C when seedlings were raised in pots, while the number of days to flowering was more in 'Reika' and less in 'Marryroad' when seedlings were raised in plug trays. At 30/25°C, the number of days to flowering was greater in 'First Power' than in the other two cultivars for seedlings raised in either pots or plug trays.

The number of leaves preceding the first inflorescence was not significantly different among seedlings raised in pots and plug trays in 'Marryroad' (Table 4). On the other hand, the number of leaves preceding the first inflorescence was significantly greater in seedlings raised in plug trays than in those from pots in 'Reika' and 'First Power'. At 30/25°C, the number of leaves preceding the first inflorescence was significantly greater in 'First Power' than in the other two cultivars for seedlings raised in either pots or plug trays.

Table 2. Effect of root container size and temperature on plant height (cm) at the transplanting stage for seedlings raised in plug trays [z]

Cultivar	23/18°C		30/25°C	
	Pot	Plug tray	Pot	Plug tray
'Reika'	87.4 c [y]	21.4 a	57.1 c	31.4 ab
'Marryroad'	32.0 ab	17.3 a	44.3 bc	28.8 a
'First Power'	48.6 b	22.4 a	77.5 d	34.8 ab
Significance				
Cultivar	**		**	
Rooting volume	**		**	
Interaction	**		**	

[z] Transplanting stages in 'Reika', 'Marryroad', and 'First Power' at 23/18°C were 29, 25, and 26 DAS, respectively. At 30/25°C, the corresponding values were 20, 20, and 22 DAS.

[y] Separation of means within a temperature regimen of 23/18°C by the LSD procedure ($P = 0.05$).

** - significant at $P \leq 0.01$.

Table 3. Effect of root container size and temperature on the number of days to flowering

Cultivar	23/18°C		30/25°C	
	Pot	Plug tray	Pot	Plug tray
'Reika'	45.7 a [z]	63.0 e	35.2	47.7
'Marryroad'	46.1 a	55.7 c	35.9	46.4
'First Power'	48.5 a	59.9 d	39.7	51.9
Significance				
Cultivar	**		**	
Rooting volume	**		**	
Interaction	**		NS	

[z] Separation of means within a temperature regimen of 23/18°C by the LSD procedure ($P = 0.05$).

NS- not significant, ** - significant at $P \leq 0.01$.

Table 4. Effect of root container size and temperature on the number of leaves preceding the first inflorescence

Cultivar	23/18°C		30/25°C	
	Pot	Plug tray	Pot	Plug tray
'Reika'	7.1 a [z]	9.1 b	7.5	9.4
'Marryroad'	7.4 a	7.5 a	7.6	9.4
'First Power'	7.5 a	8.7 b	9.3	12.0
Significance				
Cultivar	**		**	
Rooting volume	**		**	
Interaction	**		NS	

[z] Separation of means within a temperature regimen of 23/18°C by the LSD procedure ($P = 0.05$).

NS- not significant, ** - significant at $P \leq 0.01$.

Discussion

Root restriction stress due to limited rooting zone volume causes reductions in leaf area, dry mass of leaves, stems and roots, plant height and node numbers (Hameed *et al.*, 1987; Peterson *et al.*, 1991). Regarding reproductive development, Ruff *et al.* (1987) reported that plants grown in small containers flowered about three days later than those grown in large containers. On the other hand, Carmi (1986) found no delay in flowering in root-restricted cotton plants. The present study showed that the root restriction stress delayed anthesis regardless of temperature regimen, in accordance with the result of Ruff *et al.* (1987).

The number of leaves preceding the first inflorescence is determined by two processes, *i.e.*, leaf production rates during the vegetative stage and the time of flower initiation (Dieleman and

Heuvelink, 1992). In the present study, the effect of root container size on the number of leaves preceding the first inflorescence differed among cultivars at 23/18°C; root restriction stress increased the number of leaves preceding the first inflorescence in 'Reika' and 'First Power' but not in 'Marryroad' (Table 4). In relation to this different response to root restriction stress among cultivars, it is noteworthy that 'Reika' seedlings could be easily plucked out from cell packs three to four days later than 'Marryroad' and 'First Power' seedlings, and 'First Power' seedlings could be plucked out from plug trays one day later than 'Marryroad' seedlings. Judging from slower root development and an increased number of leaves preceding the first inflorescence, it is likely that 'Reika' is most sensitive to root restriction stress, while 'Marryroad' seedlings are affected to only a minor degree. It is not clear whether root restriction stress affects flower initiation through a suppressive influence on root development or if it affects flower initiation independently from root development. However, the results of the present study suggest that the number of leaves preceding the first inflorescence would increase in cultivars with slower root development if seedlings were raised in plug trays at 23/18°C. At 30/25°C, root development was faster than at 23/18°C. It is possible that fast root development causes root restriction stress to seedlings much earlier at 30/25°C than at 23/18°C, resulting in an increase in the number of leaves preceding the first inflorescence in all cultivars.

The retardation of flower initiation in 'Reika' when raised in plug trays at 23/18°C could account for the increased number of leaves preceding the first flower in this cultivar compared with 'Marryroad'. However, this explanation would not be applicable for 'First Power', because there was no difference in the number of leaves preceding the first inflorescence between 'First Power' and 'Reika', although flower initiation occurred earlier in 'First Power' than in 'Reika'.

Temperature does not affect the time to flower initiation at high light intensity, while high temperature delays flower initiation at low light intensity (Calvert, 1959). On the other hand, the rate of leaf production increases with an increase in temperature. As a result, the stimulating effect of high temperature on the number of leaves preceding the inflorescence was more evident as the light intensity decreased (Calvert, 1959; Hussey, 1963; Uzun, 2006). In the present study, the number of leaves preceding the first inflorescence increased with an increase in temperature in 'First Power', but not in 'Reika' and 'Marryroad', when they were raised in pots (Table 4). This suggests that the effect of temperature on the number of leaves preceding the first inflorescence differs among cultivars; 'First Power' is more sensitive to high temperature than 'Reika' and 'Marryroad'. The higher sensitivity of 'First Power' to high temperature is also inferred from an increase in plant height of pot-raised seedlings at 30/25°C (Table 2).

When plants are crowded, mutual shading may occur. In the present study, plant height at transplanting was greater in 'First Power' than in 'Reika' and 'Marryroad' when seedlings were raised in pots at 30/25°C, but there were no significant differences in plant height among cultivars when raised in plug trays. Therefore, an increase in the number of leaves preceding the first inflorescence in 'First Power' can be ascribed to the light intensity the seedlings received.

Oda *et al.* (2005) reported that high temperature delayed the number of days to flowering. In the present study, however, the number of days to flowering decreased with an increase in temperature (Table 3). Flower development after flower initiation is influenced by temperature; high temperatures hasten flower development (Calvert, 1957). In the present study, seedlings were grown under different temperature regimens until flowering, while seedlings were raised under different temperature regimens for only 20 days in the experiment of Oda *et al.* (2005). It is possible that the effect of temperature on the number of days to flowering is changed by the duration of temperature treatment.

In conclusion, seedling response to root restriction stress and high temperatures differed among cultivars, although it is unclear how root restriction stress delays flower initiation. Furthermore, the results of the present study suggest that cultivars that can develop roots rapidly in plug trays and at low-temperature conditions should be chosen if farmers want to reduce the number of leaves preceding the first inflorescence and the number of days to flowering by raising tomato seedlings in cool conditions.

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A simple and rapid extraction method to determine osmolar concentration of soluble carbohydrates from rose petals

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Abstract

To establish a simple and rapid extraction method for soluble carbohydrate for determination of osmolar concentration in petals by HPLC analysis, a method using a centrifugal filter device with microwave heating was developed. Rose 'Sonia' petals were placed in a centrifugal filter device and heated in a microwave oven to boiling. The centrifugal filter device was centrifuged with the petals at 12,000 g for 10 min. The resulting leached solution was subjected to HPLC analysis. No significant difference in soluble carbohydrate composition was observed between the solution obtained from this method and that obtained from a conventional extraction method in which tissues are homogenized using hot ethanol solution. Changes in soluble carbohydrate concentration with flower opening in 'Rote Rose' roses were investigated using the new method. The osmolar concentrations of glucose and fructose in the petals increased during flower opening. This increase was roughly comparable to the increase in osmotic pressure in the petals. The results suggest that the method using the centrifugal filter device with microwave heating is a simple and rapid way to determine osmolar concentration of soluble carbohydrates of rose petals.

Key words: Centrifugal filter device, extraction method, microwave heating, osmotic pressure, petal, rose, soluble carbohydrates.

Introduction

Soluble carbohydrates have an osmotic role and also provide substrates for respiration and cell wall synthesis. Since cut flowers are usually placed under conditions below the light compensation point for photosynthesis, they can assimilate little carbon by photosynthesis, and thereby lack reserve carbohydrates. Application of soluble carbohydrates, such as sucrose and glucose, extends the vase life of many cut flowers (Halevy and Mayak, 1979; Pun and Ichimura, 2004). For flower opening, large amounts of soluble carbohydrates are required and application of sugars promotes flower opening and pigmentation of petals (Halevy and Mayak, 1979; Ichimura, 1998).

In rose petals and flowers, the starch level decreased whereas glucose and fructose levels increased with flower opening (Evans and Reid, 1988; van Doorn *et al.*, 1991). Similarly, in *Gladiolus* flowers, glucose and fructose concentrations increasd with flower opening (Yamane *et al.,* 1991). In daylily flowers, fructan concentration decreased, which is accompanied with increased in glucose and fructose concentrations with flower opening (Bieleski, 1993). The increase in monosaccharide concentrations is associated with an increase in osmotic pressure, which may lead to cell expansion of petal cells. To clarify to what degree soluble carbohydrates contribute for maintaining osmotic pressure, osmolar concentrations of carbohydrate should be determined.

High performance liquid chromatography (HPLC) is often used for determination of soluble carbohydrate content since it is easy to perform and yield reproducible results. To extract soluble carbohydrates, petals have been traditionally immersed in hot ethanol solution, and then homogenized. The homogenate is centrifuged and the resulting supernatant is concentrated to remove ethanol, then used for HPLC analysis (Koyama *et al.*, 1995; Ichimura and Hisamatsu, 1999; Ogiwara *et al.*, 1999; Doi *et al.*, 2000). These procedures are laborious and time-consuming. Furthermore, the value obtained in this method represents carbohydrate amount per fresh weight including insoluble materials. Since osmolar concentration of soluble carbohydrates is expressed as moles of carbohydrates per volume of cell solution, osmolar concentrations of carbohydrates cannot be determined correctly by this method.

Previously, we developed a simple and rapid extraction method to determine concentration of soluble carbohydrate in petals using a centrifugal filter device (Norikoshi *et al.*, 2006). In this method, tissues are frozen in liquid nitrogen, after which they are centrifuged. The resulting leached fluid was used as a soluble carbohydrate sample for HPLC analysis. However, sucrose concentration decreased over time whereas glucose and fructose concentrations increased even when the samples were kept at low temperature. This change may have been due to invertase activity. To inactivate invertase activity, microwave heating prior to carbohydrate extraction is effective in various plants including persimmon, strawberry and grape berry fruits (Zheng and Sugiura, 1990; Ogiwara *et al.*, 1999; Fujiwara *et al.*, 1999ab).

In the present study, we developed a simple and rapid extraction method to determine soluble carbohydrate concentration from rose petals. We further investigated changes in osmolar concentration of soluble carbohydrate and osmotic pressure in rose petals during flower opening.

Materials and methods

Plant material: Cut 'Sonia' rose flowers were obtained from a grower of Namekata city, Ibaraki prefecture, whereas 'Rote Rose' rose flowers were from a grower of Ishioka city, Ibaraki

prefecture. For investigation of flower opening, 'Rote Rose' flowers at the following 3 stages were used in experiments. Stage 1, petals did not reflex; stage 2, outer petals started to reflex; stage 3, outer petals almost completely reflexed.

New method: Petals (200mg FW) were cut into pieces of about 5 mm^2 and placed in a centrifugal filter device (Ultrafree MC, 0.45 μm, Millipore, Medford, MA, USA). The filter device with sample was transferred into a microwave oven (ER-VS12, Toshiba, Tokyo, Japan) and heated at 500 W for about 45 sec to boiling. Then, the filter device was centrifuged at 12,000 × g. Unless otherwise stated, centrifugation time was 10 min. The leached fluid was used for HPLC analysis and measurement of osmotic pressure.

Conventional extraction method: The conventional method was conducted as previously described (Ichimura and Hisamatsu, 1999). The petals (200 mg FW) were immersed in 5 mL of 80% ethanol at 75°C for 30 min. The sample was then homogenized and centrifuged at 3,000 × g for 10 min. The pellet was twice re-extracted with 2.5 mL of 80% ethanol and the three supernatants were combined and dried *in vacuo* below 50°C. The residue was dissolved in 1 ml of distilled water and used for HPLC analysis.

Determination of soluble carbohydrate: Carbohydrates were separated using an HPLC system (Jasco, Tokyo, Japan) equipped with a refractive index detector on a Shodex SUGAR SP0810 column (Showa denko, Tokyo, Japan). The column was kept at 80°C and eluted with water at flow rate of 0.8 mL min^{-1}. The identity of each peak was confirmed using authentic carbohydrates. Peak area was determined by integration and the amount of each carbohydrate in the sample was calculated.

Effects of storing time: To evaluate stability of carbohydrate concentration of the sample, the extracted solution was stored at 4°C for 3 h or at -30°C for 1 or 7 days.

Measurement of osmotic pressure: The osmotic pressure of resulting solution was measured with a vapor pressure osmometer (VAPRO5520, WESCO, Logan, UT, USA) according to the instruction manual. Osmotic pressure due to total soluble carbohydrate was calculated as described by Thorpe *et al.* (1993).

Results

Changes in recovery of cell fluids obtained from rose petals: Fig. 1 shows recovery of cell fluids obtained from rose petals during different durations of centrifugation. About 110 μL fluid was recovered by centrifugation for 10 min. The amount of recovered fluid was slightly increased after further centrifugation.

Comparison of carbohydrate composition with conventional and new methods: Glucose, fructose, sucrose, methyl glucoside and *myo*-inositol were detected in samples obtained from the new method. To confirm the validity of this method, we compared carbohydrate composition obtained by the new method with that obtained by a conventional method because the units obtained from the conventional method are mg per fresh weight, which differ from the units obtained from the new method. No significant difference in amount of constitutive carbohydrates was observed between the two methods (Table 1).

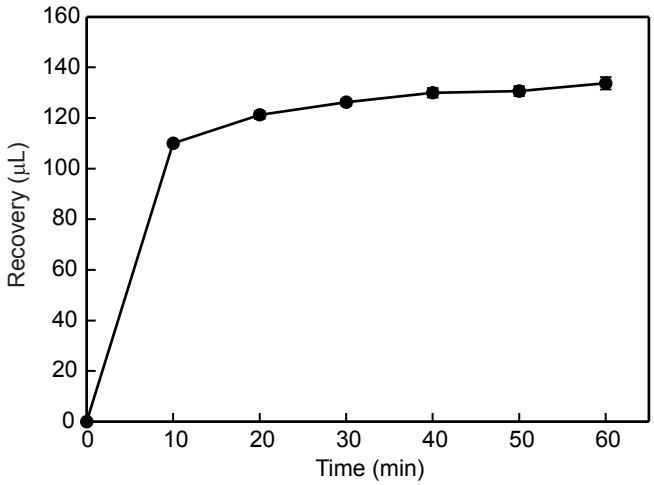

Fig. 1. Recovery of cell fluids obtained from rose petals during different durations of centrifugation

Sample stability: To clarify to what degree the extracted samples are stable, rose samples were stored at 4°C for 3 h. The carbohydrate concentration did not change significantly during this period (Table 2). When the samples were stored at -30°C for 1 and 7 days, the carbohydrate concentration also did not change significantly except for xylose stored for 1 day (Table 3).

Changes in soluble carbohydrate concentrations and osmotic pressure during flower opening in roses: To evaluate the contribution of soluble carbohydrate to osmotic pressure, changes in soluble carbohydrate concentrations and osmotic pressure

Table 1. Carbohydrate compositions obtained by conventional and new method from 'Sonia' rose petals

Method	Carbohydrate composition (%)[z]					
	Glucose	Fructose	Sucrose	Methyl glucoside	*myo*-Inositol	Xylose
Conventional	34.2	45.9	11.0	3.5	3.2	2.3
New	33.2	44.1	12.4	3.9	3.4	3.0
Significance[y]	NS	NS	NS	NS	NS	NS

[z] Mean of 4 independent experiments.
[y] NS indicates non-significant at $P<0.05$ by *t*-test. Statistical analysis was performed on data after arcsine transformation.

Table 2. Effects of storage periods on soluble carbohydrate contents of 'Sonia' rose flowers

Storage time (h)	Carbohydrate concentration (mM)[z]					
	Glucose	Fructose	Sucrose	Methyl glucoside	*myo*-Inositol	Xylose
0	55.3	129.3	9.8	7.6	11.9	9.4
3	53.2	133.0	9.6	7.1	10.8	9.3
Significance[y]	NS	NS	NS	NS	NS	NS

[z] Mean of 4 independent experiments.
[y] NS indicates non-significant at $P<0.05$ by *t*-test.

Table 3. Effects of storage periods on soluble carbohydrate contents of 'Sonia' rose flowers

Storage time (day)	Carbohydrate concentration (mM)[z]					
	Glucose	Fructose	Sucrose	Methyl glucoside	*myo*-Inositol	Xylose
0	18.2	30.6	2.2	1.2	1.3	3.7
1	17.4[y]	30.4	2.3	1.1	1.4	5.0*
7	19.6	33.2	2.5	1.0	1.3	3.5

[z] Mean of 4 independent experiments.
[y] All values except for xylose stored for 1 day (*) were not significant at $P\leq0.05$ compared with initial values (0 time) by Dunnett's test.

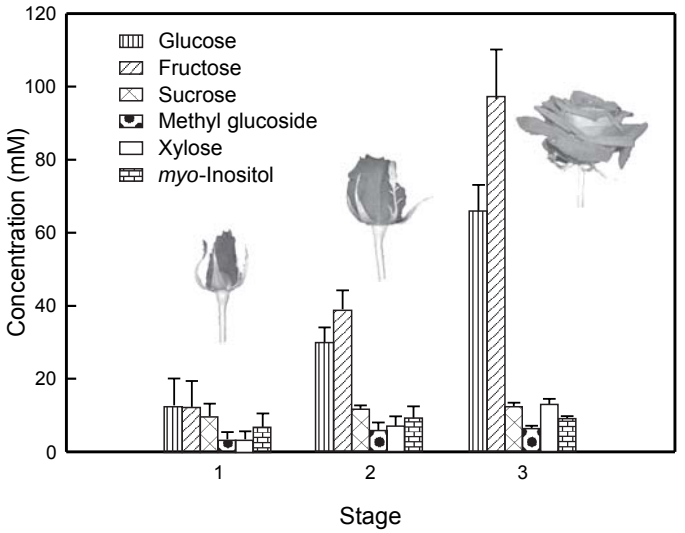

Fig. 2. Concentration of differnt soluble carbohydrate at different flower opening stages.

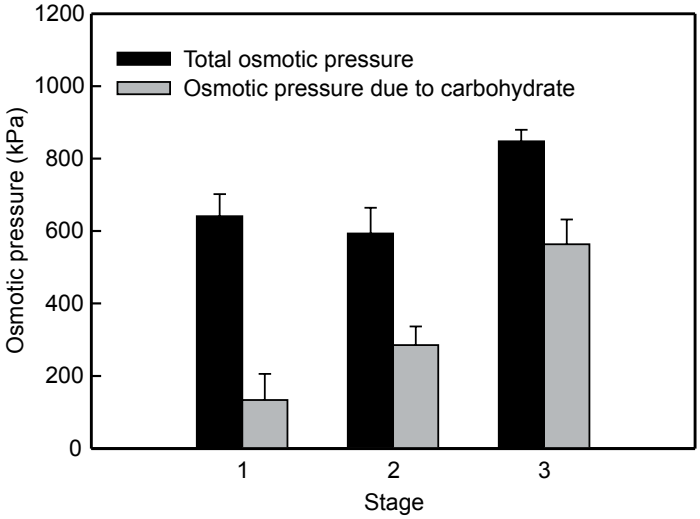

Fig. 3. Total osmotic pressure and osmotic pressure due to carbohydrate at different flower opening stages.

during flower opening in roses was investigated using solutions obtained by the new method. Glucose and fructose concentrations in the petals increased whereas other carbohydrate concentrations remained more or less constant during flower opening (Fig. 2). Osmotic pressure in the petals was almost constant from stage 1 to 2 and increased thereafter (Fig. 3). Osmotic pressure due to total soluble carbohydrate increased during flower opening. From stage 2 to 3, total osmotic pressure and osmotic pressure due to soluble carbohydrates increased by 254 and 279 kPa, respectively.

Discussion

Previously, we reported that sucrose concentration in extracts obtained from frozen rose petals by centrifugation decreased with time (Norikoshi *et al.*, 2006). As found in many plants, invertase activity in rose petals is relatively high (Ho and Nichols, 1977). Thus, this decrease may be due to invertase activity. In the present study, petals were heated in a microwave oven to inactivate enzyme activities prior to extraction of soluble carbohydrates, since microwave heating also apparently inhibits invertase activity (Zheng and Sugiura, 1990; Ogiwara *et al.*, 1999; Fujiwara *et al.*, 1999ab). Sugar concentrations did not change during the storage period (Tables 2 and 3). Thus, invertase activity in the samples in this method may be inactivated by microwave irradiation, resulting in high stability of the carbohydrate samples.

In rose petals, glucose, fructose, sucrose, methyl glucoside, xylose and *myo*-inositol have been reported as the carbohydrate constituents (Ichimura *et al.*, 1997). In the present study, these carbohydrates were detected in samples prepared by the new method. Little difference in the carbohydrate composition was observed between the new method and the conventional method (Table 1). It takes less than 90 min from preparation of plant sample to the start of HPLC. In contrast, it takes more than 1 day by the conventional method. Furthermore, apparatuses, such as centrifugal evaporators and homogenizers, are not used in the new method. Thus, the new method appears to be simple and rapid for extraction of soluble carbohydrates of petals.

In the present study, we measured the osmotic pressure of the cell fluid obtained by centrifugation. For measurement of osmotic pressure, in general, cell solution is obtained from plant materials by degrading their membranes (Nonami, 2001). To degrade membranes, tissues are generally frozen (Evans and Reid, 1988). In our previous paper, rose petals were frozen in liquid nitrogen and cell solution was collected from them by centrifugation. However, the soluble carbohydrate concentrations of the resulting solution changed with time (Norikoshi *et al.*, 2006). This suggests that osmotic pressure of the solution from frozen tissues changes with time. In contrast, carbohydrate concentrations did not change when tissues had been subjected to microwave heating (Table 2 and 3). Thus, we concluded that the solution obtained by the method using microwave heating is suitable for the measurement of osmotic pressure.

We investigated changes in soluble carbohydrate concentration and osmotic pressure during flower opening to confirm whether soluble carbohydrate contributes to increase in osmotic pressure. In our study, glucose and fructose concentrations increased during rose flower opening (Fig. 2). The increase in total osmotic pressure and osmotic pressure due to soluble carbohydrate in the petals from stage 2 to 3 was 254 and 279 kPa, respectively (Fig. 3). Thus, as reported in daylily flowers (Bieleski, 1993), an increase in glucose and fructose concentrations largely contributes to the increase in osmotic pressure, which may lead to expansion of petal cells. Although soluble carbohydrate concentration increased from stage 1 to 2, osmotic pressure did not increase (Fig. 3). Other than soluble carbohydrate, inorganic ions and organic acid act as osmotica (Cram, 1976). These compounds contribute to changes in osmotic pressure of rose petals.

In conclusion, we developed a new method to determine the osmolar concentrations of soluble carbohydrates using rose petals. Carbohydrate composition was almost the same as those determined by the conventional method. This method is simple and rapid and the extracted samples are stable. The solutions appear to be suitable for measurement of osmotic pressure. The method could be used to clarify to what degree soluble carbohydrate contributes to maintaining osmotic pressure in petals of floricultural plants.

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Effect of root zone cooling on flower development and fruit set of 'Satohnishiki' sweet cherry

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Abstract

The effects of root zone cooling on flower development and fruit set of 'Satohnishiki' sweet cherry were studied. Soil temperature in pots of the tree was maintained at approximately 11°C from bud burst until petal fall, then at about 15°C until harvest by circulating cooled water through a tube coiling the pots. Root cooling did not appreciably affect flower size, pollen germination and pollen tube elongation in pistils. However, the treatment prolonged ovule longevity and markedly increased the fruit set rate. These results suggest the possibility of applying root cooling to improve the fruit set of sweet cherries grown in warm regions.

Key words: Fruit set rate, ovule development, *Prunus avium*, soil cooling

Introduction

Recently, attempts have been made to produce sweet cherries in southwestern Japan to harvest the fruits earlier than in the northern major production areas and to supply local markets. In this region, however, severe yield fluctuation on account of poor fruit set is a serious problem that prevents stable production (Beppu and Kataoka, 2006).

Under controlled conditions, it was demonstrated that the fruit set rate in the 'Satohnishiki' cultivar decreased markedly mainly due to the rapid degeneration of the nucelli and embryo sacs after anthesis when the trees were exposed to high temperatures, above 20°C, from one month before anthesis to petal fall (Beppu *et al.*, 1997). In south-western Japan, temperatures during bud development are high. For example, in Takamatsu City, Kagawa Prefecture, the daily high temperature in April exceeded 20°C for an average 16 days during the past 10 years. These facts imply that cooling the whole tree is a possible method for improving fruit set of sweet cherries grown in warm regions. Unfortunately, the costs involved to cool the entire tree are prohibitive for commercial applications.

However, it has been documented that the poor flower bearing of satsuma mandarin due to high temperature in early forcing culture can be improved by cooling only the root zone (Poerwanto *et al.*, 1989), and this method has already been put to practical use in Japan (Hirose, 2003). Likewise, root zone cooling in sweet cherries grown in warm regions might improve the flower development and the fruit set.

In this study, we examined the effect of root zone cooling in spring on flower development and the fruit set of sweet cherry grown in a warm region.

Materials and methods

Five-year-old 'Satohnishiki' sweet cherry (*Prunus avium* L.) trees, grafted on 'Aobazakura' (*Prunus lannesiana* Wils.), a common rootstock for sweet cherries in Japan, were grown in 7-liter pots filled with a granite soil: bark compost (2 : 1 ; v/v) at the research field of Kagawa University. Four pots of the tree were individually coiled with a vinyl tube, and covered with thermal insulation and aluminium coated polyvinyl chloride film. A filled 100 L water tank was placed below ground level. Water in the tank was cooled with a circulating cooler and pumped to the tube coiling the pots with a portable electric pump. The cooler was set at temperatures of 10°C from April 1 (bud burst) to April 27 (petal fall), then 15°C till May 29 (harvest). Four untreated trees were used as controls. When the soil moisture tension reached 10 kPa, 1 L of water was supplied. Soil temperatures in the pots were measured with thermocouples placed at a depth of 10 cm.

At anthesis, six flowers per tree were collected, the weight of the flower, and the lengths of the peduncles, petals, and pistils were recorded. From these flowers, pollen grains were collected to estimate the germination rate on a medium containing 15% sucrose, 5 ppm boron and 1% agar at 20°C. To evaluate ovule development, six flowers per tree were collected at anthesis and three days after anthesis. The ovaries were fixed in FAA solution and after dehydration, embedded in paraffin wax, and sliced into 16 µm thick longitudinal serial sections which were stained with Mayer's acid-hæmalun. The lengths and widths of ovary, ovule and nucellus were measured with a micrometer under a light microscope, and the developmental stages of the embryo sac and nucellus were observed. Ovules were classified into two groups: normal or abnormal (degenerated embryo sac or nucellus); the developmental stages of the normal ovules were further classified into six categories: no embryo sac, embryo sac mother cell, two-nucleate stage, four-nucleate stage, and eight-nucleate stage with fused or unfused polar nuclei.

To promote the fruit set, all flowers were hand-pollinated at anthesis with the 'Takasago' pollen which, *in vitro*, had more than a 60% germination rate. The rate of the fruit set was recorded at harvest. To observe the pollen tube growth in the pistils, pistils from hand-pollinated flowers were collected three days after anthesis and preserved in FAA solution. Observations were made using a fluorescence microscope.

Results and discussion

The actual ranges of soil temperature in the pots are shown in Fig. 1. Soil temperature in the root cooling treatment was kept approximately at the above-mentioned set temperatures. The average soil temperature in the treatment, from the onset of the treatment until April 27, was 11.4°C, which was 5.0°C lower than that of the control. It was 15.3°C until May 29, 4.6°C lower than the control. Root cooling lowered the average daily maximum soil temperature during the treatment period by 11.6°C.

Most of the control and root cooling-treated trees began blooming on April 15. Root cooling had no appreciable effect on the size of the flower parts at anthesis (Table 1). Pollen germination rates on the medium were almost 50% in treated and untreated trees. The pollen tubes reached the locules three days after pollination with or without cooling treatment (Data not shown). In a previous study, it was demonstrated that overall temperature did not affect pollen germination or pollen tube growth in pistils (Beppu *et al.*, 1997). Likewise, root zone temperature does not seem to have an influence.

On the other hand, root zone temperature clearly affected the ovule development and fruit set. At anthesis, many embryo sacs were at the four-nucleate stage or earlier, whereas three days after anthesis, the frequencies of ovules with an eight-nucleate stage of embryo sac or with a degenerate embryo sac or nucellus increased dramatically (Table 2). The percentage of embryo sacs at eight-nucleate stage in the root cooling treatment was considerably higher than that in the control. Conversely, the rate of ovules with a degenerated embryo sac or nucellus was lower in the treatment. These results suggest that root cooling prolongs ovule longevity, as the low temperature of the whole-tree does (Beppu *et al.*, 1997). Ovule development is often influenced by plant hormones, especially gibberellin (Komatsu, 1987; Stösser and Anvari, 1982; Takagi, 1980). In a previous study, it was indicated that early ovule degeneration under high temperature is induced by the increased level of endogenous gibberellin (Beppu *et al.*, 2001; 2005). In general, endogenous gibberellins are synthesized in the root apices and young leaves and translocated to other organs (Sembdner *et al.*, 1980). Environmental conditions, such as temperature,

Table 1. Effect of root cooling on flower development in 'Satohnishiki' sweet cherry

Treatment	Flower weight (mg)	Length (mm)		
		Peduncle	Petal	Pistil
Control	160.9±10.0[z]	15.9±1.6	13.3±0.5	12.7±0.5
Root cooling	172.2±5.8	14.8±1.5	13.2±0.3	12.9±0.6

[z] SE

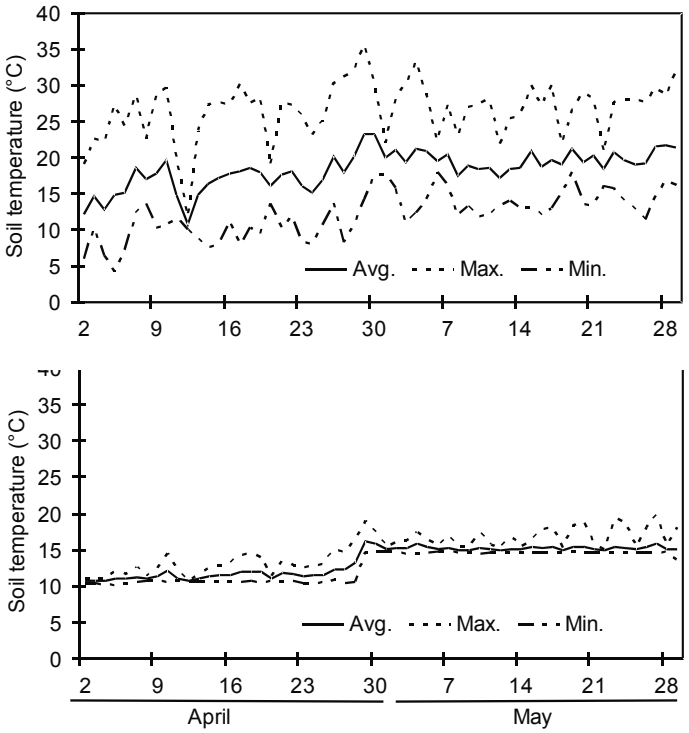

Fig. 1. Daily average, maximum and minimum soil temperatures in the control (above) and in the root cooling treatment (below).

affect both gibberellin synthesis and translocation. In satsuma mandarins, the endogenous gibberellin level in leaves decreased when the root and shoot temperature were lowered as low as 15°C (Poerwanto and Inoue, 1990). In grapevines, the activity of gibberellin-like substances in shoots decreased when the root temperature was reduced to as low as 13°C (Kubota *et al.*, 1986). Likewise, in this experiment, the endogenous gibberellin level in above-ground parts may be reduced by root cooling, resulting in prolonged ovule longevity.

Root cooling markedly increased the fruit set rate such that it was 31% compared to 14% in the control (Fig. 2). This may be due to prolonged ovule longevity because of low root zone temperature.

On the other hand, not only phytohormons but also tree nutrition, such as non-structural carbohydrates, are involved in ovule development and fruit set of sweet cherries (Beppu *et al.*, 2003a). High temperatures reduce the carbohydrate accumulation as a result of increase of respiration rate (Beppu *et al.*, 2003b). In apples, it was reported that the root respiration rate per fresh weight was almost of the same level as the shoot, and that it increased in the warm season (Proctor *et al.*, 1976). Thus, in this

Table 2. Effect of root cooling on ovule development in 'Satohnishiki'sweet cherry

Days after anthesis	Treatment	Stage of development							Degenerated embryo sac or nucellus
		Number of embryo sac	Embryo sac mother cell	Two-nucleate	Four-nucleate	Eight-nuleate			
						Unfused polar nuclei	Fused polar nuclei	Total	
0	Control	43.4[z]	14.7	9.4	9.4	6.0	1.8	7.7	15.5
	Root cooling	30.8	10.4	14.8	15.6	13.5	0.0	13.5	14.9
3	Control	8.9	0.0	5.4	0.0	7.1	10.9	18.0	67.7
	Root cooling	6.1	0.0	2.5	3.6	4.3	27.5	31.8	56.1

[z] Percentage of ovules with embryo sac or nucellus at different stages of development

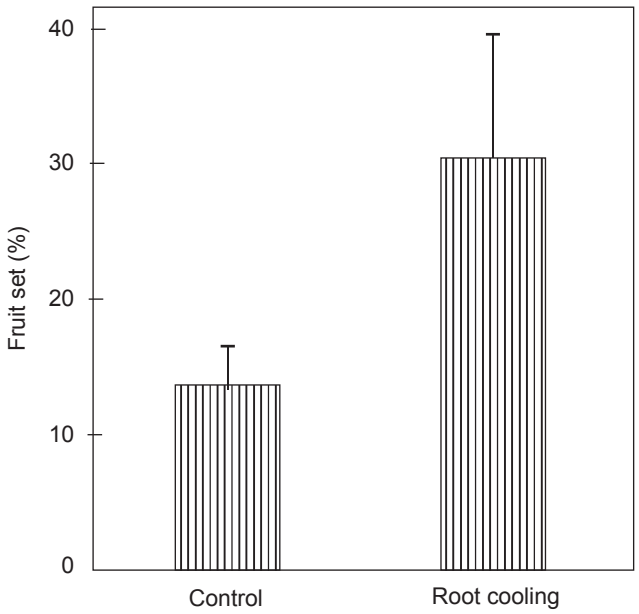

Fig. 2. Effect of root cooling on fruit set in 'Satohnishiki' sweet cherry. Bars indicate SE values

experiment, root cooling could have reduced the carbohydrate consumption through respiration in the root, which might have resulted in a greater supply of the carbohydrate to the reproductive organs resulting in an improvement of the ovule development and fruit set.

Our results indicate that root zone cooling prolongs ovule longevity and consequently increases fruit set of sweet cherries grown in a warm region. For practical application in industry, one should examine a root cooling system in which cooled water circulates through tubes buried in the soil of an orchard, such as the kind of systems already applied in a commercial context in the early forcing culture of the satsuma mandarins (Hirose, 2003).

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Effects of different preharvest treatments on yield and chemical quality of tomato

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Abstract

Field experiment was conducted to study effects of preharvest treatment of ComCat® spray, organic manure, NP fertilization and the combinations of ComCat® with the two forms of fertilizers on yield and chemical quality of tomato (*Lycopersicon esculentum* Mill.). Total and marketable yields were significantly influenced by the preharvest treatements. The result showed that the use of ComCat® and its combination with organic manure gave the highest total yield of 58.5 and 55.8 t ha^{-1}, respectively. At harvest, 94 and 93% of tomatoes subjected to preharvest ComCat® and ComCat® plus organic manure treatment were marketable, respectively. The chemical quality parameters tested such as total soluble solids, pH, titratable acidity, ascorbic acid, reducing sugar and total sugar were significantly ($P<0.01$) affected by the preharvest treatments. The study clearly demonstrated the importance of integerated agro-technology in order to simultaneously improve the yield and quality of tomatoes.

Key words: ComCat®, manure, NP fertilization, yield, quality

Introduction

Postharvest qualities of tomatoes partly depend up on preharvest factors such as cultural practices, genetic makeup and environmental conditions (Hobson, 1988). Cultural practices such as nutrient, water supply and harvesting methods are claimed to be factors influencing quality of tomato before and after harvest (Fischer and Richter, 1986; Watkins and Pritts, 2001). Recent research findings also indicated the possibility of screening natural plants as original untouched wild species, for their bio-stimulatory activity (Hüster, 2001; Schenabel *et. al.,* 2001). As a result, ComCat® was developed as a natural product with its plant strengthening properties and the ability to improve growth and yield in different agricultural crops. ComCat® is natural biocatalysts, which is extracted from seeds of plants and mainly consists of amino acids, gibberellins, cytokinins, auxin (indole-3-acitic acid), brassinosteroids, natural metabolites, pathogen-resistance-proteins with defense reactions, terpenoids, flavonoids, vitamins, inhibitors, other signal molecules, biocatalysts and cofactors (Schenabel *et al*., 2001). On the other hand, organic manure application is common practice in Ethiopia to improve tomato yield. However, the effect of preharvest treatments on chemical quality of tomatoes at harvest is not yet extensively investigated.

Quality management starts in the field and continues until produce reaches the end user. The response of fruit and vegetables during storage to postharvest factors also, in part, depends on preharvest practices like use of natural plant extract such as ComCat®, fertilizers, manure and environmental factors. Understanding and managing the various roles that preharvest factors play on quality is very important in order to achieve maximum harvest and postharvest quality of any crop. Mostly, preharvest conditions are of overriding importance in determining storage behavior. In some cases, their effects can be greater than the effects of adjustment of storage environment. To date, preharvest treatment recommendations for fruits and vegetables have been established primarily for higher productivity and not for improving quality, nutritive value and shelf life. Therefore, the present study was designed to investigate the effect of farmyard manure, NP fertilizers and ComCat® on the growth, yield and chemical quality of tomatoes at harvest.

Materials and methods

Site description: Field experiment was conducted at Haramaya University farm located in Dire Dawa, Ethiopia, during September to January, 2004/2005. The research site is located at an altitude of 1197 m above sea level and lies at 9° 6' N and 41° 8' E. The station lies in the semi-arid belt of the eastern rift valley escarpment with mean annual rainfall of 520 mm and mean maximum and minimum temperatures ranging from 28.1 to 34.6 and 14.5 to 21.6°C, respectively (Belay, 2002).

Experimental materials and design: Fresh market tomato variety, Marglobe, was raised in glass house for about two weeks pricked and grown on nursery bed for another three weeks. The seedlings were transplanted to plots consisting of six rows 0.75 m apart, with 90 plants per plot and spaced 0.5 m apart in the row. The spacing between plots in each replication and adjacent replications was 2 and 1.5 m, respectively.

Field treatments consisted of recommended rate of NP fertilization (92 kg of P_2O_5 and 95 kg N ha^{-1}), farmyard manure (20 tons ha^{-1}), ComCat® (100 g ha^{-1}), NP and manure each in combination with ComCat®. The source for NP fertilizer was diammonium phosphate (DAP) and urea. Manure, DAP and half of the nitrogen fertilizer were incorporated to the experimental plots before planting while the rest of the nitrogen was applied two weeks

after the establishment of seedlings. ComCat® was applied at 100 g ha^{-1} in 350 L and sprayed twice during the growth period. First spray was just prior to transplanting of seedlings while the second was carried out before flowering as recommended by Hüster (2001). Other agronomic practices (weeding, irrigation, staking, etc.) were applied uniformly as needed to all plots. Plots were irrigated every other day for the first two weeks and then at weekly interval. Fungicides (Ridomil+ MZ 63% and Mancozeb 3.5 kg ha^{-1}) were used to control leaf diseases and cypermethrin (100 g ha^{-1}) was used to control insect pests; and were sprayed at seven days interval from transplanting to 20 days before first harvest. The experiment was laid out in randomized complete block design with three replications.

Data collection: The following data were recorded from the central four rows of ten randomly selected plants per plot. The total numbers of leaves was counted at weekly intervals starting from crop emergence till 50% of the plants got bloomed. The heights (cm) of plants were measured from the ground level to the highest point at blooming stage. The number of primary and secondary branches of each plant was recorded. Mean height (cm) of primary lateral shoots of each plant of each treatment at blooming stage was recorded. The average length of three leaves (cm) from the upper, middle and lower part of the plant was measured at blooming stage. The average size of three leaves (cm) at the widest point from the upper, middle and lower part of the plant was measured at blooming stage. Days to 50% flowering was recorded when approximately 50% of the flower clusters on the plant had some flowers that were in bloom. Days to maturity was recorded when approximately 70% of the plants had attained physiological maturity. Number of cluster per plant was counted at physiological maturity. Number of fruit per cluster was counted at physiological maturity.

Yield assessment: Tomato fruit, which were handpicked at the green mature, were selected from each treatment of the middle four rows. Harvesting was carried out once a week. The total fruit yield, marketable fruit yield, and fruit number per plant, were determined immediately after each harvest while fruit volume, fruit size and juice content was determined at the third harvest. Harvesting for yield comparison was done eight times roughly at weekly interval. Dropped fruits were not considered.

Total number/weight of fruits is the sum total number/weight of fruits of successive harvests.

Marketable and unmarketable fruit number and weight: At each harvest, fruits were categorized as marketable and unmarketable fruits of each treatment. Fruits, which were cracked, damaged by insect, diseases, birds and sunburn, etc. were considered as unmarketable while fruits, which were free of damage, were considered as marketable.

Fruit size: Diagonal section of the fruit measured by caliper.

Fruit volume: Ten randomly selected fruits from ten plants in a plot were taken and floated in a water jar and their displacement was recorded. Average fruit volume was taken by subtracting the initial water level in the jar from the final and by the number of fruit immersed.

Fruit juice content: The juice content of tomato was extracted using a juice extractor (60001 X, 31Je35 6X-00777, BauJhar-93, Hesteller). The intact tomato weight was recorded prior to juice extraction. After extraction, extracted juice was measured using a graduated glass cylinder and expressed in milliliter of juice per kilogram of fruit weight (mL kg^{-1}).

Chemical Analysis

Total soluble solids: The total soluble solids (TSS) was determined using an aliquot of juice extracted using a juice extractor. A bench top 60/70 ABBE (A90067, Bellingham & Stanley Ltd, England) refractometer with a range of 0 to 32 °Brix and resolutions of 0.2 °Brix was used to determine TSS by placing 1 to 2 drops of clear juice on the prism.

Ascorbic acid: The ascorbic acid content (AA) was determined by the 2, 6-dichlorophenol indophenol method (AOAC, 1970). An aliquot of 10 mL tomato juice extract was diluted to 50 mL with 3% metaphosphoric acid in a 50 mL volumetric flask. The aliquot was then centrifuged for 15 min and titrated with the standard dye to a pink end point (persisting for 15 sec). The ascorbic acid content was calculated from the titration value, dye factor, dilution and volume of the sample.

pH and titratable acidity: Tomato juice was extracted from the sample with a juice extractor (60001 X, 31Je35 6X-00777, BauJhar-93, Hesteller) and clear juice was used for the analysis of titratable acidity (TA). The titrable acidity expressed as percent citric acid, was obtained by titrating 10 ml of juice to pH 8.2 with 0.1N NaOH. The pH value of the juice was measured with a pH meter.

Sugar analysis: Reducing sugar (RS) and total sugar (TS) were estimated by using calorimetric method as described by Seyoum (2002). Liquidized fresh tomato tissue (10 g) was added to 15 mL of 80% ethanol, mixed and heated in a boiling water bath for sufficient time until the ethanol odor went off. After extraction, 1 mL of saturated lead acetate ($Pb(CH_3COO)_2.3H_2O$) and 1.5 mL of saturated sodium hypophosphate (Na_2HPO_4) were added and the contents were mixed by gentle shaking. After filtration, the extract was made up to 50 mL with distilled water. An aliquot of 1 mLextract was diluted to 25 mL with 1 mL copper reagent in a test tube and heated for 20 min in a boiling water bath. After heating, the contents were cooled under running tap water without shaking. Arsenomolybdate color reagent (1 mL) was added, mixed, made up to 10 mL with distilled water and left for about 10 minutes to allow color development, after which the absorbance was determined by Jenway model 6100 spectrophotometer at 540 nm. For total sugar determination, sugar was first hydrolyzed with 1N HCl acid by heating at 70°C for 30 min. After hydrolysis, total sugar was determined following the same procedure employed for the reducing sugar. A blank was prepared using distilled water.

Data analysis: Difference between the treatments were determined by analysis of variance (ANOVA) for factorial experiment in randamized complete block design (RCBD) using MSTAT-C software (MSTAT, Michigan University, East Lansing) and comparison of the treatment mean by Duncan's Multiple range test.

Results and discussion

Growth of plant: One month after transplanting, there was a relatively poor stand of seedlings in manure, ComCat® + manure

and control plots while the application of NP fertilizer enhanced early growth. The reason for poor stand of seedlings could be attributed to competition of decaying microorganisms for nutrients and slow availability of nutrient during early stage. The higher initial growth in NP treated plants could also be the addition of NP fertilizer that dissolves rapidly to meet the immediate nutrient demand of the plant. Initial growth in terms of leaf number was significantly ($P \leq 0.01$) higher for ComCat®, ComCat® + NP and NP treatments (Table 1). For the leaf counting, well established and strong seedlings were observed in the case of ComCat® treated tomato plants. This could be due to the ability of ComCat® to enhance better root development that could enable plants absorb water and nutrients (Hüster, 2001; Schnabel *et al.*, 2001) and resistance to disease and environmental stress (Pretorius *et al.,* 2003; Hüster, 2001; Schnabel *et al.*, 2001).

The preharvest treatments significantly ($P<0.01$) affected number of leaves (Table 1). During the second count, the number of leaves of tomato plants subjected to NP, ComCat® + NP and ComCat® + manure treatments were significantly ($P<0.01$) higher compared with the number of leaves of tomato plants subjected to farmyard manure and control treatments. This result clearly showed that nitrogen enhances the vegetative growth during the early developmental stage of tomato plants.

Manure application resulted in significantly ($P<0.05$) longer tomato plants compared to control (Table 2). This might be because of the ability of manure in creating suitable plant growing environment by improving moisture and nutrient status of the soil. Hader (1986) reported that organic fertilizers compensate both the deficit and the excess of elements in the soil, which can take place with mineral fertilization. ComCat® had no significant effect on the plant height when compared to the control plants. Similar finding was reported by Hüster (2001). There was no difference in plant height among the manure, ComCat® + manure, ComCat® + NP and ComCat® treatments. However, manure treated tomato plants were relatively taller followed by ComCat® + manure, ComCat® + NP and ComCat® treated plants.

Application of NP and ComCat® + NP fertilizer resulted in significantly ($P<0.01$) higher number of primary lateral branches per plant compared to ComCat®, ComCat® + manure, manure and control tomato plants. The application of ComCat®, manure and their combinations had no significant ($P>0.01$) difference on the number of primary lateral shoot. Although vegetative growth (plant height and number of lateral shoots) was enhanced by the application of inorganic fertilizer and ComCat® + NP treatments, ComCat® had no significant effect on the vegetative growth but the branches were observed to be stronger. This might indicate that nitrogen stimulates excessive vine growth while ComCat® does not have such an effect. Hüster (2001) reported the simulating effect of nitrogen on vegetative growth of beet root and cauliflower while such property is absent in ComCat® treated plants.

Leaves were significantly ($P<0.01$) longer in ComCat® treated tomato plants compared to manure, NP, ComCat® + NP, ComCat® + manure and control tomato plants (Table 2). Similarly, the

Table 1. Weekly count of tomato plant leaf number starting from establishment

Treatment	Week						
	1	2	3	4	5	6	7
CC	36.90a	88.80bc	102.07bcd	205.70b	288.90ab	313.03bc	355.67c
M	-	69.57cd	83.00cd	197.73b	246.60b	326.80bc	340.77c
NP	33.63a	96.40ab	110.77bc	235.27ab	338.03a	384.63ab	434.33b
CC + M	-	100.50ab	133.00b	241.63ab	293.03ab	326.80bc	356.97c
CC + NP	42.13a	115.57a	216.87a	282.67a	313.57ab	452.70a	571.50a
Control	-	50.80d	65.70d	107.37c	251.83b	275.23c	298.40c
CV (%)	32.4	12.32	18.83	14.73	13.92	14.03	9.62
LSD	11.07	19.48	40.62	56.73	73.09	86.32	68.75
SE ±	3.51	6.18	12.89	18.01	23.19	27.39	21.82
Significance	*	**	**	**	NS	*	**

- indicates that leaf count on week one was not done since plants of respective treatments did not establish well. Means within a column followed by the same letter (s) are not significantly different according to Duncan's multiple range test $P=0.05$) where NS, *, ** indicate nonsignificant or significant difference at $P<0.05$ or 0.01, respectively; CC, ComCat®; M, manure; NP, nitrogen and phosphorus; CC + M, ComCat® + manure; CC + NP, ComCat® + nitrogen and phosphorus; C, control.

Table 2. Effects of ComCat®, manure, nitrogen and phosphorous, and their combinations on growth components of fresh market marglobe tomato cultivar

Treatment	LL (cm)	LW (cm)	PLH (cm)	LSN (cm)	LSH (cm)	SLSN (cm)	SLSH (cm)	DYF (days)	DYM (days)
CC	5.42a	12.48a	52.47ab	5.86b	20.52a	2.57a	7.20a	40.67c	76.33c
M	4.78b	11.42ab	59.70a	6.00b	24.88a	2.27a	7.97a	41.67c	78.33bc
NP	4.53b	10.70bc	49.90ab	7.63a	25.50a	2.37a	11.86a	52.00b	89.67ab
CC + M	4.57b	11.24b	57.13ab	6.03b	23.12a	2.70a	10.75a	47.00bc	79.33abc
CC + NP	3.41c	9.14d	54.80ab	8.43a	21.85a	3.34a	9.18a	60.33a	91.00a
C	3.54c	9.87cd	45.17b	4.93b	21.75a	3.47a	10.44a	40.67c	75.67c
Significance	**	**	*	**	NS	NS	NS	**	*
SE ±	0.18	0.39	4.08	0.39	3.38	0.74	1.94	2.21	3.77

Means within a column followed by the same letter (s) are not significantly different according to Duncan's multiple range test $P<0.05$) where NS, *, ** indicate nonsignificant or significant difference at $P=0.05$ or 0.01, respectively, by LSD, respectively. LL, leaf length; LW, leaf width; PLH, plant height; LSN, lateral shoot number; LSH, lateral shoot height; SLSN, secondary lateral shoot number; SLSH, secondary lateral shoot height; DYF, days to flowering; DYM, days to maturity; CC, ComCat®; M, manure; NP, nitrogen and phosphorus; CC + M, ComCat® + manure; CC+NP, ComCat® + nitrogen and phosphorus; C, control.

application of manure, NP and ComCat® + manure resulted in significantly ($P<0.01$) longer leaves when compared to ComCat® + NP and the control tomato plants.

ComCat® significantly ($P<0.01$) increased both leaf length and width when compared with the other treatments. As a result, large and broad leaves were observed in ComCat® treated tomato plants. In addition, the leaves were deep green in colour which is in agreement with the previous findings by Pretorius *et al.* (2003). The vegetative growth of tomato in terms of the height of primary shoots, number and height of secondary shoots did not show significant variation among the treatments tested (Table 2).

Application of ComCat® + NP treatment took significantly ($P<0.01$) longer time (60 days) for 50% of flower clusters to bloom compared to the rest of the treatments (Table 2). NP fertilizer application took significantly ($P<0.01$) longer time (52 days) to bloom compared to ComCat®, manure and control tomatoes. This seems to indicate that excess nitrogen resulted in excessive lateral shoot growth that probably has impaired reproductive development by decreasing sink strength of inflorescences relative to vegetative tissues. This result is in agreement with the findings of Dieleman and Heuvelink (1992) who reported delayed flowering due to over fertilization. ComCat® (41 days), manure (42 days), ComCat® + manure (47 days) and control plants resulted in early flowering.

ComCat® + NP took significantly ($P<0.05$) longer time (91 days) for 70% of the fruits to get matured compared to ComCat®, manure and control tomato plants (Table 2). Similarly, NP treatment took significantly ($P<0.05$) longer time (90 days) compared to ComCat® and control tomato plants. As indicated earlier, ComCat®, manure and control treated tomato plants bloomed earlier.

Yield and fruit characterstics: The results of preharvest treatments on yield and yield related traits of tomato are presented in Table 3. ComCat® treated tomato plants had significantly ($P<0.01$) higher number of clusters per plant (17.3) compared with manure (14.7), ComCat® + NP (13.3) and control (9.6) tomatoes. NP and ComCat® + manure treated plants had statistically similar amount of cluster number per plant with that of ComCat® treated tomato plants. ComCat® + manure treatment had significantly ($P<0.05$) higher number of fruit per cluster compared to ComCat® + NP and control tomatoes. The maximum cluster number associated with ComCat® treated plants could be due to the activity of ComCat® in accelerating flower bud formation and increasing plant self defense mechanism and resistance (Hüster, 2001; Pretorius *et al.*, 2003). ComCat® improves better development that enables plants to adapt better and utilize the available soil water as well as nutrient.

ComCat® treatment significantly ($P<0.05$) increased fruit size compared to NP and ComCat® + NP treated tomato plants. Manure and ComCat® + manure treated tomato plants significantly ($P<0.05$) increased fruit size than ComCat® + NP treated tomato plants. Lower fruit size was obtained from tomatoes treated with NP, ComCat® + NP and control treatments. This result indicates that the addition of NP resulted in significant ($P<0.05$) reduction in fruit size. This may have been due to higher nitrogen levels promoting the development of more clusters per plant, which resulted in a greater fruit load per plant and smaller fruit size (Brecht *et al.,* 1976).

ComCat® stimulates higher sugar production which is the building blocks for cellulose and fruiting bodies (Seyoum, 2002). One of the physical expressions of theses response is better flowering and greater fruit biomass that can lead to increased yield in fruit and vegetables (Hüster, 2001; Schenabel *et al.,* 2001).

An overview of total, marketable and unmarketable fruit number and yield response of tomato plant to different preharvest treatments is presented in Table 4. ComCat® treated tomatoes had significantly ($P<0.05$) higher total fruit yield compared to NP, ComCat® + NP and control tomatoes. Similarly, ComCat® + manure treated tomatoes had significantly ($P<0.05$) higher total fruit yield than ComCat® + NP and control.

The total and marketable fruit number obtained from ComCat® + NP treated tomatoes was significantly ($P<0.05$) lower compared to other treatments (Table 4). The total and marketable fruit number obtained from that of the tomatoes grown using manure and NP fertilizers were not significantly ($P<0.05$) different from control tomatoes. Significantly ($P<0.05$) higher number and yield of unmarketable fruit was obtained from NP fertilized tomatoes, followed by ComCat® + NP treated tomatoes.

Table 3. The effects of ComCat®, manure, nitrogen and phosphorous fertilizer on the yield components of fresh market Marglobe tomato cultivar

Treatment	CN (No)	F/CL (No)	FRS (cm^3)	FV (cm^3)	FJ (ml kg^{-1})
CC	17.30^{a}	2.89^{ab}	7.61^{a}	0.26^{a}	680.50^{bcd}
M	14.70^{bc}	2.26^{cd}	7.03^{ab}	0.23^{a}	963.90^{a}
NP	16.50^{ab}	3.32^{a}	6.39^{bc}	0.14^{a}	864.70^{ab}
CC + M	16.37^{ab}	2.70^{bc}	7.33^{ab}	0.23^{a}	624.30^{cd}
CC + NP	13.27^{c}	2.05^{d}	6.01^{c}	0.12^{a}	545.30^{d}
Control	9.57^{d}	2.23^{d}	6.78^{abc}	0.15^{a}	770.10^{abc}
Significance	**	*	*	**	*
SE ±	0.65	0.14	0.31	0.01	31.00

Means within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test $P=0.05$) where NS, *, ** indicate nonsignificant or significant difference at $P<0.05$ or 0.01, respectively. CN, cluster number; F/CL, fruit per cluster; FRS, fruit size; FV, fruit volume; FJ, fruit juice; CC, ComCat®; M, manure; NP, nitrogen and phosphorus; CC + M, ComCat® + manure; CC + NP, ComCat® + nitrogen and phosphorus; C, control.

Table 4. The effects of ComCat®, manure, and nitrogen and phosphorus fertilization on the marketable, unmarketable, total fruit number and weight of fresh market tomato

Treatments	Number of fruit per plot			Fruit yield (ton ha^{-1})		
	M	UM	Total	M	UM	Total yield
CC	938.67^{a}	61.67^{c}	1000.34^{a}	55.00^{a}	3.52^{d}	58.53^{a}
M	689.33^{b}	58.33^{c}	747.66^{b}	41.65^{bc}	4.79^{bc}	46.44^{abc}
NP	615.67^{b}	122.00^{a}	737.67^{b}	36.69^{c}	6.13^{a}	42.82^{bc}
CC +M	987.33^{a}	65.67^{c}	1053.00^{a}	52.01^{ab}	3.76^{cd}	55.77^{ab}
CC+NP	445.00^{c}	95.33^{b}	540.33^{c}	13.74^{d}	4.99^{ab}	18.73^{d}
C	623.30^{b}	69.00^{c}	$692.^{b}$	33.48^{c}	4.19^{bcd}	37.67^{c}
Significance	*	*	*	*	*	*
SE±	31.268	6.9926	30.397	3.9781	0.3856	4.0848

Means within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test $P=0.05$) where * indicate significant difference at $P<0.05$. M, marketable yield or number; U, unmarketable yield or number; T, total yield or number; ComCat, M, manure NP, nitrogen&phosphorus CC + M, ComCat + manure, C + NP; ComCat + nitrogen and phosporus, C; control.

The higher marketable fruit yield of tomato treated with ComCat® compared to NP, manure, ComCat® + NP and control tomatoes, is in agreement with the findings of Schnabel *et al.* (2001) who reported the yield increase by 16-19% due to ComCat® treatment in different crops including tomato. In addition, Hüster (2001) reported that ComCat® reduced the occurrence of disease by more than 40% which could be, in part, the reason why ComCat® performed better than others.

For the increase in yield of ComCat® + manure treatment, it seems that the ComCat® enabled better nutrient uptake during the earlier growth stage and the slow release of nitrogen from manure might have contributed to the nutrient demand of the plant in the later stage of growth.

The highest unmarketable fruit yield was obtained from NP while the least was from ComCat® treated tomatoes. The unmarketable tomato fruit yield obtained from manure treated tomatoes was significantly ($P<0.05$) lower than NP treated tomato fruit. Most of the fruit grouped as unmarketable were bird attack, crack, soft rot and irregular shape. Blossom end rot appeared due to preharvest ComCat® + NP fertilizer treatment. This could be due to the excess N that might have fostered Ca^{++} deficiency, which is responsible for the cause of blossom end rot (Shaykewich *et al.*, 1971).

The difference in marketable fruit yields from manure and inorganic fertilizer was not significant ($P>0.05$); however, higher yield was obtained from manure than inorganic fertilizer treated tomato plants. In support of this study, Cacek and Lagner (1986) reported the less danger of over-fertilization by adding decomposed organic material. Application of manure and inorganic fertilizer did not show significant ($P<0.05$) variation in both total and marketable fruit yield compared to control. However, contrary to this result, Winsor (1970) reported yield increase in tomato due to nitrogen application. Some of the possible reasons for the comparable performance of control with fertilizer application under the present study could be attributed to the inherent fertility of the soil and uniform irrigation. The application of ComCat® + NP fertilizer depressed the performance of the crop, even when compared to the control treatment. It is reported that ComCat® is applied additional to normal fertilizers (Schenabel *et al.*, 2001). However, contrary to their findings, ComCat® + NP highly reduced yield in the present study. This could also be due to the cumulative effect of nitrogen in the soil and the additional N application, which leads to excessive vegetative growth. In this condition the plant may grow well, but be late yielding or low yielding because vegetative growth is favoured over reproductive growth (Wudiri and Henderson, 1985).

In general, both ComCat® and ComCat® + manure treatments had an enhancing effect on the yield of tomatoes where 39.1 and 35.62% more marketable yield increase was shown than in the control tomatoes, respectively. Hüster (2001) reported yield increase due to ComCat® in cabbage, cauliflower, beetroot and other cereal crops (wheat and maize).

Application of manure resulted in 19.6% increment in marketable yield while application of inorganic fertilizer resulted in only 8.74% increment when compared to the control. Even though there has been much controversy over manure versus inorganic fertilizer on yield increment, in the present study, manure outperformed by 10.86%.

Table 5. The effects of preharvest treatments on the chemical quality atributes of green mature tomato fruits

Treatment	Chemical composition at harvest					
	TSS	pH	TA	AA	RS	TS
CC	4.867^{a}	3.839^{c}	1.254^{a}	11.72^{bc}	0.6912^{bc}	1.841^{c}
M	4.533^{ab}	3.981^{b}	1.209^{a}	14.92^{a}	0.7094^{b}	2.027^{b}
NP	4.333^{bc}	4.017^{b}	1.376^{a}	12.97^{b}	0.7732^{ab}	1.633^{d}
CC + M	4.333^{bc}	3.929^{bc}	0.4463^{bc}	12.36^{b}	0.6212^{cd}	1.125^{e}
CC + NP	4.067^{c}	4.038^{b}	0.5887^{b}	10.88^{c}	0.5546^{d}	1.156^{e}
C	4.667^{ab}	4.213^{a}	0.37^{c}	13.00^{b}	0.7998^{a}	2.623^{a}
Significance	**	**	**	**	**	**
SE±	0.063	0.005	0.007	0.561	0.0211	0.005

Means within a column followed by the same letter (s) are not significantly different according to Duncan's multiple range test $P=0.05$) where NS, *, ** indicate nonsignificant or significant difference at $P<0.05$ or 0.01, respectively, by LSD. TSS, total soluble solid; TA, titratable acidity; AA, ascorbic acid; RS, reducing sugar; TS, total sugar; CC, ComCat; M, manure; NP, nitrogen and phosphorus; CC + M, ComCat + manure; CC + NP, ComCat + nitrogen and phosphorus; C, control.

The high performance of ComCat® over other treatments in both total and marketable yield could be due to the larger and broader leaves produced by ComCat® and might have increased the photosynthetic effeicency. Similarly, the increase in plant height and primay lateral shoot number in ComCat® + NP and NP fertilized tomato plants did not lead to increase in total and marketable yield. This indicates that stimulation of early growth could compete with fruit set and development and is not desirable for obtaining acceptable yields. ComCat® does not have an early vegetative growth stimulating effect, as is well known for early nitrogen fertilization (Hüster, 2001).

Chemical quality: At harvest, the green mature tomatoes subjected to preharvest ComCat® treated contained significantly ($P<0.01$) higher TSS compared with NP, ComCat® + NP and ComCat® + manure treated tomato fruits (Table 5). However, it did not show significant difference ($P>0.01$) when it is compared with the control and manure treated tomatoes. Seyoum (2002) reported that ComCat® increased the biosynthesis of polysaccharide carbohydrates while efficiently utilizing free sugars for physiological processes during growth and development. ComCat® is also known to increase the chlorophyll content and hence increase the production of total available carbohydrates (Seyoum, 2002).

At harvest, manure treatment showed an increase in TSS content of tomatoes than NP treatment, although it was not significant at $P>0.01$ (Table 5). This increase in the TSS content of manure treated tomatoes could be due to higher photosynthetic efficiency by the relatively larger and broader leaves and increase of fruit sink strength. This result contradicts with findings of DeEll (2003) who reported higher TSS concentration at harvest and after storage due to conventional fertilizer application in apple. However, the result is in agreement with the findings of Raupp (1996) who reported the positive effect of manure on TSS content of vegetables. Mccollum *et al.* (2004) found slight difference in soluble solids or acidity between conventional grown and organically grown fruit. Among the preharvest treatments, only ComCat® + NP treatment significantly ($P<0.01$) decreased the TSS content of tomato at harvest compared with the control. The rate of assimilate export from leaves and rate of import by fruit might be lower as vegetative growth was favored than reproductive growth in this treatment.

Preharvest treatment significantly ($P<0.01$) reduced the pH value of tomato fruits (Table 5). Moreover, preharvest treatments significantly affected the TA of tomato fruits at harvest (Table 5). Significantly ($P<0.01$) higher titratable acid content of tomato fruit was found in NP, manure and ComCat® treated tomato fruits compared to ComCat® combined with the fertilizers and control tomatoes. The increase in TA of tomato where manure and NP fertilizer applied is in accordance with the result reported by Hegde and Srinivas (1990) where acidity increased with increasing fertilizer. Contrary to this, DeEll (2003) reported no difference in TA content of apple due to fertilizer application. The high TA of ComCat® treated tomato than in control tomatoes is in agreement with report of Seyoum (2002). ComCat® + NP treated tomato also showed significantly ($P<0.01$) higher TA than in the controls, however, ComCat® + manure treated tomato had statistically similar TA content with that of ComCat® + NP and control. The least TA value was observed in the control tomatoes and ComCat® + manure treated tomatoes.

At harvest, the application of manure had a positive effect on the accumulation of AA content in tomatoes. Raupp (1996) indicated the positive effect of manure on the content of dry matter, sugar and AA in vegetables. Earlier studies by Cacek and Lagner (1986) also showed the positive effect of organic fertilizer on the nutritional value of vegetables. ComCat® + NP treatment significantly ($P<0.01$) lowered AA content of tomato fruits compared to the control. The lower AA content in the preharvest NP and ComCat® + NP treatments could be due to the effect of N fertilization. Likewise, Lisiewska and Kmiecik (1996) reported a decrease in AA content of fruits and vegetables with increasing amounts of nitrogen fertilizer.

Field study on the effect of preharvest treatment of ComCat®, ComCat® + manure, ComCat® + NP, NP and manure were conducted on Marglobe tomato. Vigorous growth was observed in ComCat® treated tomato plants. Seedling established earlier in ComCat®, ComCat® + NP and NP treated tomato plants whereas establishment was delayed in manure, ComCat® + manure and control tomato plants. The yield of fresh tomato was significantly ($P<0.05$) influenced by treatments. The highest yield was obtained from foliar application of ComCat® and ComCat® + manure treatments. Supplementing the recommended NP fertilizers with ComCat® reduced both marketable and total yield. Supplementing ComCat® with manure fertilizer slightly improved yield over ComCat® alone. In general, both ComCat® and ComCat® + manure treatments had an enhancing effect on the yield of tomatoes where 39.1 and 35.62% yield increase was shown than the control tomatoes, respectively. The difference in marketable fruit yields from manure and NP fertilizer was not significant ($P>0.05$); however, higher yield was obtained from manure than inorganic fertilizer treated tomato plants. Application of manure resulted in 19.6% increment in marketable yield while application of inorganic fertilizer resulted in only 9% increment compared to the control. Manure outperformed by about 10.6% compared to NP fertilizer.

In summary, the yield of tomatoes was improved under semi-arid conditions of the experimental area through the use of preharvest ComCat® and ComCat® + manure treatment. Generally, this study clearly demonstrated the importance of integerated agrotechnology in order to improve yield while improving or maintaining quality after harvest. The preharvest treatments had influenced the quality of tomatoes at harvest. ComCat® treated tomato fruits had lower pH, AA, reducing sugar and total sugar, and higher TA and TSS content. ComCat® treatment combined with manure and NP fertilizers had shown lower pH, TSS, TA, AA, RS and TS. Manure treated tomato fruits had higher TSS, TA, AA, TS and RS. NP fertilizer application resulted in higher TA.

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Effects of 1-methylcyclopropene on the postharvest life of Eksotika papaya

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Abstract

Papaya is a climacteric fruit, naturally fragile and cannot resist low temperature. Thus, prolonging the postharvest life of papaya fruit for long distance transportation is highly desirable to increase its commercialisation. 1-Methylcyclopropene (1-MCP) has been widely used to delay ripening and senescence of horticultural produces. The objective of this study was to determine characteristics of 'Eksotika' papaya treated with 1-MCP and optimum concentration of 1-MCP in prolonging postharvest life of papaya. Papayas were treated with 0, 10, 20, 30, 40 and 50 µL L^{-1} of 1-MCP for 7 days at 21˚C/90% relative humidity (RH). Then, the fruits were allowed to ripen at 26˚C/70% RH. 1-MCP did not affect L*, C*, soluble solids concentration, titratable acidity, pH, vitamin C and weight loss of papaya. The h^o and firmness of papaya treated with 30 µL L^{-1} of 1-MCP showed significant high values as compared to other concentrations. Similarly, visual quality evaluation also showed that fruits treated with 30 µL L^{-1} of 1-MCP retained green colour for 9 days and by day 13, no disease infection and shriveling was found in these fruits as compared to other concentrations. There is potential to prolong postharvest life of Eksotika papaya using 1-MCP.

Key words: Papaya, *Carica papaya*, 1-MCP, colour, firmness, weight loss, visual quality

Introduction

Papaya (*Carica papaya* L.) is extensively grown in tropical regions of the world. Malaysia is the world's next largest exporter of papaya after Mexico with 25% of the world market share (Rabu and Mat Lin, 2005). Papaya is a climacteric fruit, naturally fragile due to its thin skin and cannot resist low temperature. Thus, it ripens and perishes rapidly. Normally fruits are harvested and placed to ripen at the recommended harvest stage (one yellow stripe) and it atains 60-70% yellow colouration within 4-6 days under ambient tropical conditions (25-28^{o}C) (Rohani *et al.*, 1993).

For the maximum marketing period of papaya for export purpose, the fruit are transferred to low storage temperature (10-12^{o}C) and can be stored for 14-21 days if harvested at one yellow stripe stage. Temperature below this range causes chilling injury and rapid deterioration in fruit quality. Postharvest life would be significantly reduced if fruits are harvested at advanced stages of ripening. Therefore, increasing postharvest life period of papaya has become a main issue for long distance transportation and commercialisation purposes. Moreover, when the postharvest life is prolonged, exporters who formerly use air freight can switch to sea transportation which is four times cheaper and also enable the fruit to be exported to distant countries like Saudi Arabia, United Arab Emirates, Australia and Japan (Rohani *et al.*, 1993).

One of the new technologies that can extend the postharvest life of papaya is use of 1-methylcyclopropene (1-MCP). 1-MCP is an inhibitor of ethylene perception that can markedly affect ripening or senescence processes of many horticulture products (Watkins, 2006). In general, 1-MCP delays ripening and senescence and reduces ethylene production, respiration, colour change and softening. This compound is used at low rates, has a non-toxic mode of action and is active at very low concentrations. The objective of this study was to determine characteristics of 'Eksotika' papaya after 1-MCP treatment and optimum concentration of 1-MCP in prolonging postharvest life of papaya.

Materials and methods

Plant materials: 'Eksotika' papayas at maturity stage 2 were obtained from Exotic Star (M) Sdn. Bhd., Kajang, Selangor, Malaysia. Fruits weighing 500-650 g, well-formed, uniform in size and colour, defect- and disease-free, were selected. Eight fruits were then packed into six cartons of 32 x 21 x 23 cm.

1-MCP application and storage: Papayas were treated with Ansip-F® (Lytone Enterprise, Inc. Taiwan R.O.C.) tablet, containing 0.009% 1-MCP active ingredient. 1-MCP concentrations used were 0, 10, 20, 30, 40 and 50 µL L^{-1}. The tablet form of Ansip-F was crushed and weighed for 0, 3.5, 7.0, 10.5, 14.0 and 17.5 mg according to the treatments of 0, 10, 20, 30, 40 and 50 µL L^{-1}, respectively. The crushed powder was held by cotton and later it was moisten with 5 mL 40^{o}C distilled water and placed in the middle of carton with eight papaya fruits surrounding. The carton was then wrapped and tied up in a 0.035 mm thick polyethylene bag and stored for 7 days in a chamber at 21^{o}C/90% relative humidity (RH). After 7 days, the cartons were ventilated and fruit were allowed to ripen in a room at 26^{o}C/70% RH. Two fruits were analysed at interval of 2 days for skin colour, flesh firmness, soluble solids concentration (SSC), titratable acidity (TA), pH, vitamin C content, weight loss and visual quality for 13 days.

Determination of skin colour: Skin colour was determined using a Minolta CR-300 Chroma Meter (Minolta Corp., Osaka, Japan) and results were expressed as lightness (L*), chroma (C*) and

hue (h°). The L* value ranges from 0 (black) to 100 (white). The h° is an angle in a colour wheel of 360°, with 0, 90, 180 and 270° representing the red, yellow, green and blue hues, respectively, while C* is the intensity or purity of the hue. Measurements were carried out at stem end, mid region and floral end of papaya.

Determination of flesh firmness: Flesh firmness was evaluated using a computer controlled Instron 5543 Material Testing Machine (Instron® Ltd., High Wycomb, UK). Tissues were subjected to a puncture test at a constant speed of 20 mm min^{-1}, using a 5 mm diameter plunger probe. Three measurements were taken from stem end, mid region and floral end of a fruit for each day and penetration force was expressed in newtons.

Determination of SSC: Ten g of fruit was macerated and the tissue was homogenised with 40 ml of distilled water by using a kitchen blender. The mixture was filtered with cotton wool. A drop of the filtrate was then placed on the prism glass of refractometer (Model N1, Atago Co., Ltd., Tokyo, Japan) to obtain the %SSC. The readings were corrected for temperature compensation at 27°C.

Determination of TA and pH: The remainder of the juice from the SSC determination was used to measure TA by titrating with 0.1 mol L^{-1} NaOH using 1% phenolphthalein as indicator. The results were calculated as a percentage citric acid [(ml NaOH x 0.1 mol L^{-1}/weight of sample titrated) x 0.064 x 100]. The pH of the juice was measured using a glass electrode pH meter model Crison Micro pH 2000 (Crison Instruments, S.A., Barcelona, Spain). The pH meter was calibrated with buffer at pH 4.0 and 7.0 before being used.

Determination of vitamin C content: Ten g of papaya flesh was well homogenised with 3% cold metaphosphoric acid using a kitchen blender. The volume was made up to 100 ml and filtered with cotton wool. Then 5 ml of the aliquot was titrated with 2,6-dichlorophenol-indophenol solution to a pink colour. The vitamin C content was determined according to Ranganna (1977).

Determination of weight loss: Weight loss was determined by weight difference at days 7, 9, 11 and 13 compared with day 0, and expressed as percent (fresh weight basis). Fruits were weighed using a weighing scale.

Evaluation of visual quality: Visual quality of papaya fruits was evaluated based on the overall appearances of fruit such as skin colour, shriveling and disease from day 0 until 13.

Statistical analysis: The experimental design was a randomised complete block design with 4 replications. Data was analysed by using the analysis of variance (SAS Institute, Cary, NC) and means were separated by DMRT. Correlation analysis by using Pearson's correlation matrix was also performed.

Results and discussion

Skin colour: After a week of storage at 21°C/RH 90%, 1-MCP treated fruit tended to be greener than control fruit when measured with a chroma meter and the values of L*, C* and h° are presented in Table 1. Exposure to 1-MCP did not lead to significant differences in L* values of Eksotika papaya fruits (Table 1). However, L* values of Eksotika papaya fruits were significantly affected by days after exposure to 1-MCP. As days after 1-MCP treatment progressed, the L* values of Eksotika papaya fruits increased (Table 1) which means fruits had lighter skin as days progressed. This result is totally different from guava cv. 'Media China' that the L* values decreased after 7 days storage treated with 1-MCP at 25°C (Mercado-Silva *et al.*, 1998) and 'Hass' avocados after 7 weeks storage with exposure to 100 nL L^{-1} 1-MCP for 6 h (Woolf *et al.*, 2005).

Table 1. 1-MCP concentration and day after 1-MCP treatment effects on skin colour (L*, C* and h °) of 'Eksotika' papaya fruit. Fruits were treated with 1-MCP for 7 days at 21°C/90%RH and thereafter allowed to ripen at 26°C/70% RH until day 13

Factor	L*	C*	h°
Concentration (C), µL L^{-1}			
0	55.66 a[z]	36.84 a	115.37 f
10	53.98 a	34.24 a	126.78 c
20	55.44 a	36.56 a	136.41 b
30	55.65 a	35.72 a	141.23 a
40	55.40 a	36.15 a	118.83 e
50	54.73 a	34.99 a	121.47 d
Day 0 (before treatment)	48.81 e	25.58 e	166.06 a
Day after treatment (D)			
7	51.14 d	29.73 d	156.13 b
9	54.43 c	34.83 c	122.86 c
11	59.51 b	41.99 b	107.60 d
13	61.73 a	46.62 a	80.76 e
Interaction			
C x D	NS	*	**

NS, *, **Non significant (P>0.05) or significant (P≤0.05) or highly significant (P≤0.01), respectively.

[z]Mean separation within columns by DMRT at P=0.05.

The C* values refers to the vividness of colour and the C* values of Eksotika papaya fruits were not significantly affected by 1-MCP concentrations (Table 1). However, it is significantly affected by days after exposure to 1-MCP with significant increase as days progressed (Table 1). The h° values of Eksotika papaya fruits were significantly affected by concentrations and days after exposure to 1-MCP (Table 1). The fruit treated with 30 µL L^{-1} of 1-MCP showed the most significant h° values compared to other concentrations which reflect green in colour chart. Higher h° values indicate that papaya fruits had a greater maintenance in green colour and application of 1-MCP delayed colour changes in the skin. The h° values of fruits treated with 40 and 50 µL L^{-1} of 1-MCP was significantly lower than those treated with 30 µL L^{-1} of 1-MCP. This indicated that the fruits were more yellow than those treated with 30 µL L^{-1}.

The C* and h° values of Eksotika papaya fruits skin were significantly affected by the interactions between concentration x day after 1-MCP treatment (Table 1). The h° values of fruit skin decreased as days after 1-MCP treatment progressed indicating green skin turned to yellow (Fig. 1). After 9 days of 1-MCP treatment, skin of fruit treated with 30 µL L^{-1} of 1-MCP showed significant higher h° values and this trend continued to day 11. The values of h° and L* of Eksotika papaya fruit was negatively correlated (Table 2) indicating that the increase in the h° values towards green colour was associated with a decrease in the L* values of Eksotika papaya fruit. Lower L* values indicated that Eksotika papaya fruits were darker in colour when the fruit skin was green.

Flesh firmness: There were significant differences in flesh firmness as affected by different concentrations of 1-MCP as

Table 2. Correlation coefficients (r) for) skin colour (L*, C* and h°), flesh firmness (Firm), soluble solids concentration (SSC), titratable acidity (TA), pH, vitamin C (VC) and weight loss (WL) of 'Eksotika' papaya fruit

	L*	C*	h°	Firm	SSC	TA	pH	VC
C*	0.90**	--						
h°	-0.73**	-0.76**	--					
Firm	-0.78**	-0.81**	0.89**	--				
SSC	0.18*	0.25**	-0.19*	-0.22*	--			
TA	0.02	-0.07	0.07	0.04	-0.10	--		
pH	-0.03	-0.06	-0.02	-0.08	-0.17*	0.03	--	
VC	0.39**	0.40**	-0.24*	-0.25**	0.15*	0.13*	0.12	--
WL	0.75**	0.76**	-0.83**	-0.87**	0.15*	-0.06	0.12	0.25*

For correlation coefficients, n = 240.

L* = lightness, C* = chroma and h° = hue angle.

*, ** significant and highly significant at $P \leq 0.05$.

well as days after 1-MCP treatment (Table 3). Similarly, there were highly significant interaction between 1-MCP concentration x day after 1-MCP treatment (Table 3). The flesh firmness of Eksotika papaya fruits treated with 30 µL L^{-1} of 1-MCP was significantly higher compared to other treatments while it was significantly lower in fruits treated with 40 µL L^{-1} of 1-MCP than other treatments. This result was different from 'Hass' avocados where fruit treated with 500 nL L^{-1} 1-MCP were firmer than fruit treated with 100 nL L^{-1} and all 1-MCP treated fruits were firmer than control fruit (Woolf *et al.*, 2005). For 'Pedro Sato' guava, the fruits that remained firm and did not reach full ripening were those treated with 900 nL L^{-1} compared to those treated with 100 and 300 nL L^{-1} (Bassetto *et al.*, 2005). This indicates that avocado and guava fruits treated with higher concentrations of 1-MCP were firmer than fruit treated with lower concentration of 1-MCP. However, in Eksotika papaya, the degree of firmness did not follow the same trend.

Fig. 2 shows the interaction effect on flesh firmness between day after 1-MCP treatment x 1-MCP concentration. The firmness of flesh treated with 30 µL L^{-1} 1-MCP was significantly higher compared to other treatments as days progressed from 7 to 11. Fruits treated with 20 µL L^{-1} showed significantly lower firmness than fruits treated with 30 µL L^{-1} but higher than other treatments as days after treatment progressed from 7 to 11. The firmness of papaya fruit was negatively correlated with L* and C* values but positively correlated with h° values (Table 2). This indicated that the firmness of fruit reduced as colour of fruit changed from green to yellow.

SSC: 1-MCP had no significant effect on the SSC of Eksotika papaya fruits (Table 3). However, there was a significant increase in SSC as days after 1-MCP treatment progressed from 11 to 13. There was no significant effect on SSC Eksotika papaya fruits between concentration x day after 1-MCP treatment (Table 3).

Similar findings were found in oranges (Porat *et al.*, 1999), apricots and plums (Dong *et al.*, 2002), custard apple and mango (Hofman *et al.*, 2001) and apples (Rupasinghe *et al.*, 2000; DeEll *et al.*, 2002) where SSC were unaffected by 1-MCP. The significant increase in SSC as days after 1-MCP treatment progressed from 11 to 13 indicated the fruit become much sweeter and more acceptable with increase in sugar content (Table 3). Chan (1979) confirmed that sucrose is the main sugar for papaya which consisted of 80% of total soluble sugars in fully ripened fruit. The SSC and flesh firmness of Eksotika papaya is negatively

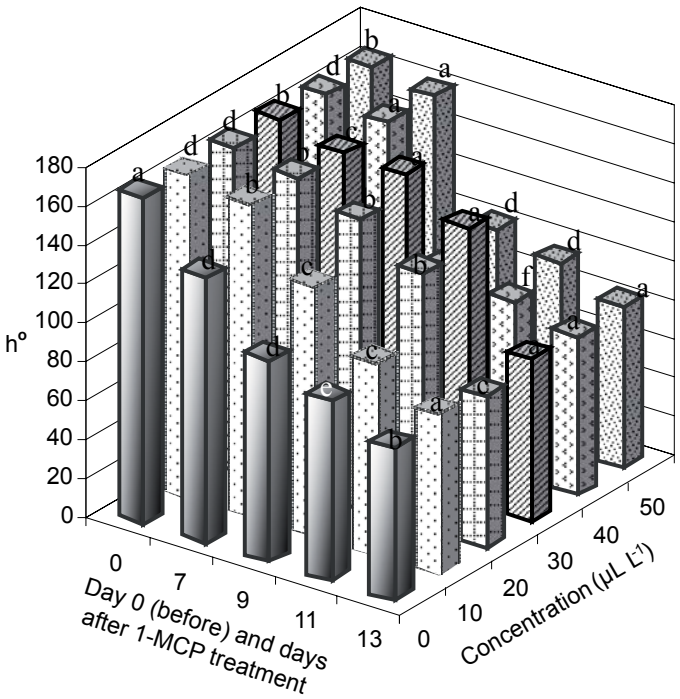

Fig. 1. Effects of day after 1-MCP treatment x concentration on skin colour (h°) of 'Eksotika' papaya fruit. Means separation pertaining to day after 1-MCP treatment by DMRT at P=0.05.

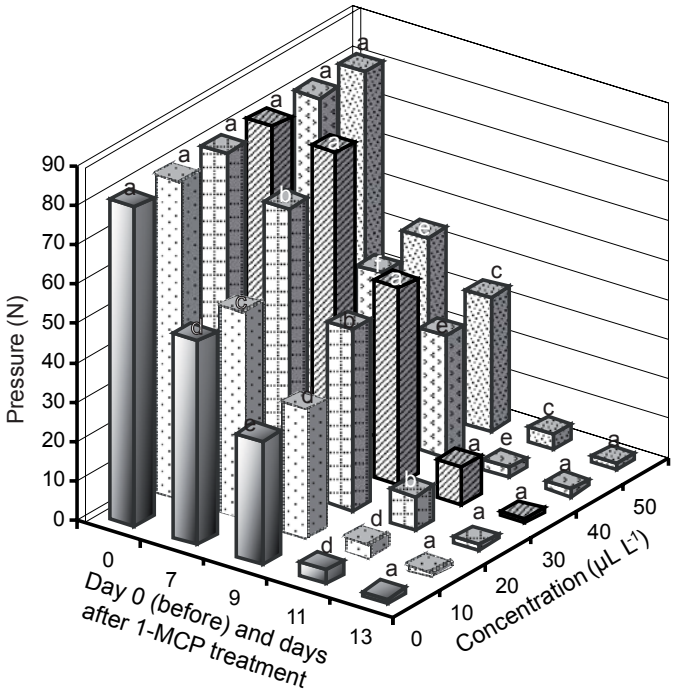

Fig. 2. Effects of day after 1-MCP treatment x concentration on flesh firmness of 'Eksotika' papaya fruit. Means separation pertaining to days after 1-MCP treatment by DMRT at P=0.05.

correlated (Table 2) because fruit became sweet and flesh softened when ripe.

TA: There was no significant difference in TA of Eksotika papaya when treated with six different concentrations of 1-MCP (Table 3). However, there was significant effect on TA of the Eksotika papaya fruits as days after 1-MCP treatment progressed. Initially the TA of fruit decreased, then increased and leveled off as days after treatment progressed (Table 3). Lazan *et al.* (1989) reported that the TA of Eksotika papaya fruit increased during ripening.

Table 3. Main and interaction effects between 1-MCP concentration and day after 1-MCP treatment on flesh firmness, soluble solids concentration (SSC), titratable acidity (TA), pH, vitamin C and weight loss of 'Eksotika' papaya fruit. Fruits were treated with 1-MCP for 7 days at 21°C/90%RH and thereafter allowed to ripen at 26°C/70% RH until day 13

Factor	Firmness (N)	SSC (%)	TA (%)	pH	Vitamin C (mg 100 g^{-1})	Weight loss (%)
Concentration (C), µL L^{-1}						
0	33.74 d[z]	6.24 a	0.40 a	5.39 b	24.91 a	4.14 a
10	34.27 c	5.79 a	0.39 a	5.62 a	23.30 a	4.06 a
20	41.88 b	6.34 a	0.37 a	5.60 a	26.23 a	4.19 a
30	44.24 a	4.78 a	0.31 a	5.68 a	23.33 a	4.43 a
40	31.77 f	4.76 a	0.31 a	5.68 a	24.82 a	4.15 a
50	33.08 e	4.85 a	0.34 a	5.68 a	23.74 a	4.03 a
Day 0 (before treatment)	81.23 a	4.33 b	0.34 ab	5.40 d	20.66 c	0.00 e
Day after treatment (D)						
7	56.99 b	5.42 b	0.45 a	5.78 a	24.02 bc	2.02 d
9	37.54 c	4.57 b	0.28 b	5.69 ab	22.29 bc	3.80 c
11	5.30 d	5.34 b	0.36 ab	5.61 bc	26.55 ab	5.98 b
13	1.41 e	7.64 a	0.33 b	5.55 c	28.42 a	8.65 a
Interaction						
C x D	**	NS	NS	*	NS	NS

NS, *, **Non significant ($P>0.05$) or significant ($P\leq0.05$) or highly significant ($P\leq0.01$), respectively.

[z]Mean separation within columns by DMRT at $P=0.05$.

Citric acid is the major organic acid in Eksotika papaya and the level decreases slightly during ripening while malic acid levels remained unchanged whereas that of succinic acid increases steadily. Cis-aconitic, oxalic and fumaric acids are present in relatively low amounts in Eksotika papaya.

The effect of 1-MCP on TA is mixed, with some crops being affected and others not. 1-MCP did not affect TA in apricots (Dong *et al.*, 2002), 'Red Chief' apples (Mir *et al.*, 2001), 'Fortune', 'Angeleno' and 'President' plums (Menniti *et al.*, 2004) and 'Shamouti' oranges (Porat *et al.*, 1999). In contrast, higher TA values in 1-MCP treated fruit was found in 'Law Rome', 'Delicious', 'Empire' and 'McIntosh' apples (Watkins *et al.*, 2000), 'Elberta' peach (Fan *et al.*, 2002) and Pedro Sato (Bassetto *et al.*, 2005). Fan *et al.* (2000, 2002) also found lower acidity loss during storage in pears and plums treated with 1-MCP. In addition, 1-MCP alone did not affect fruit TA but in combination with wax, TA was higher on mamey sapote fruit (Ergun *et al.*, 2005).

pH: There was a significant increase in pH of Eksotika papaya fruits treated with 1-MCP as compared to control (Table 3). However, no significant difference was observed as the concentration of 1-MCP increased. As days after 1-MCP treatment progressed, pH of the fruit increased significantly from day 0 to 7, then decreased significantly (Table 3).

pH is a measure of solution acidity, in terms of hydrogen ions (H^+) and also buffering capacity of the extracted juice (Wills *et al.*, 1998). Most fruit pH increased throughout maturation was due to metabolic processes in the fruits that resulted in the decrease of organic acids (Coseteng and Lee, 1987). Organic acids are important source of respiratory energy in plant cell (Ulrich, 1970) and respiratory oxidation, tricarboxylic cycle, carboxylations and decarboxylations are the main pathway for metabolism of organic acids. In this study, there was no significant correlation between pH and TA (Table 2), indicating pH of Eksotika papaya was not correlated with its organic acids.

Vitamin C content: The concentrations of 1-MCP used did not affect the vitamin C content of Eksotika papaya (Table 3). However, there was a significant difference in vitamin C content of Eksotika papaya as days after 1-MCP treatment progressed with highest content on day 13. There was no significant interaction between 1-MCP concentration x day after 1-MCP treatment on the vitamin C content of Eksotika papaya fruit (Table 3).

In Pedro Sato guava fruit treated with 100, 300 or 900 nL L^{-1} 1-MCP at room temperature, vitamin C content was not influenced by 1-MCP (Bassetto *et al.*, 2005). The vitamin C content of Eksotika papaya fruits had a significant negative correlation with h^o and flesh firmness (Table 2). As the h^o values and flesh firmness decreases, vitamin C content increases. Thus the fruit had higher vitamin C content during ripening as the flesh of Eksotika papaya fruit softened and skin turned yellow. On the other hand, vitamin C content of Eksotika papaya fruits is positively correlated to the SSC (Table 2) indicating when fruit has high SSC, the vitamin C content is also high.

Weight loss: Weight loss was not significantly affected by different concentration of 1-MCP treatments (Table 3). However, there was a significant effect on weight loss of the Eksotika papaya fruits as day after 1-MCP treatment progressed. In contrast, there were no significant interaction between 1-MCP concentration x day after 1-MCP treatment (Table 3). No difference in weight loss was found in Eksotika papaya fruit between control and 1-MCP treated fruits at different concentrations of 1-MCP (Table 3). Manenoi *et al.* (2007) observed no difference in weight loss (7.4-8.8%) between the 1-MCP treated and non-treated papaya fruit.

Treatment of 1-MCP did not affect weight loss in oranges (Porat *et al.*, 1999), it delayed weight loss in avocado (Jeong *et al.*, 2002) and in 'Rendaiji' persimmon fruits treated with 1-MCP had less weight loss as compared to 1-MCP non-treated fruit (Ortiz *et al.*, 2005). Bassetto *et al.* (2005) reported that 1-MCP treated Pedro Sato guava fruit showed greater weight loss as compared to control fruit, probably due to the longer storage period. Total of 8.65% of weight loss occurred as fruits stored for 7 days at 21°C/90% RH (Table 3). The weight loss showed an increasing trend by 1.78, 2.18 and 2.67% as days after 1-MCP treatment progressed from 7 to 9, 9 to 11 and 11 to 13 day, respectively.

Weight loss of Eksotika papaya was negatively correlated with firmness and h^o and positively correlated with L* and C* (Table 2). This indicated that weight loss occurred rapidly as fruit ripened and developed yellow and soft flesh.

Visual quality appearances: 1-MCP had a significant effect on papaya visual quality appearances depending on concentration being used and days after treatment as days progressed. The application of 1-MCP significantly delayed degreening as the fruits exhibited retention of green colour for 7 days at 21°C/90% RH especially those treated with 20 and 30 µL L^{-1} of 1-MCP than control fruit which showed about 20% yellowness on the surface of fruit. On day 9, the skin colour of fruit changed obviously from green to yellow except fruits treated with 20, 30 and 50 µL L^{-1} 1-MCP which showed little colour changes. On day 11, control and 10 µL L^{-1} 1-MCP treated fruits showed 90% yellow, while 40 and 50 µL L^{-1} fruit showed 70% yellow. Fruit treated with 20 and 30 µL L^{-1} 1-MCP only showed 50% yellow. By day 13, disease infection and shriveling was found in control, 10, 40 and 50 µL L^{-1} 1-MCP treated fruits. Fruit of 20 and 30 µL L^{-1} 1-MCP treated show 100 and 90% yellow, respectively, without disease infection and shriveling.

According to Hofman (2001), 1-MCP treated papayas tended to have the same or slightly less severe disease rating at the end of shelf-life and disease was the primary reason for shelf-life termination of treated fruit. In this study, all fruits except 20 and 30 µL L^{-1} 1-MCP treated fruits were infected by disease and shriveled at the end of postharvest life. Therefore, with higher concentration 1-MCP it is quite difficult to produce better quality of Eksotika papaya fruit. 1-MCP may inhibit a beneficial metabolic response or stimulate an undesirable characteristic, possibly relating to a natural defense mechanism (Ku *et al.*, 1999). Lower phenolic content in 1-MCP treated strawberries was considered to account for the increased disease incidence (Jiang *et al.*, 2001). Diaz *et al.* (2002) also found that treatment with 1-MCP increased susceptibility of tomatoes to *Botrytis cinerea*.

The results from this study demonstrated that the effectiveness of 1-MCP to extend postharvest life of papayas varied significantly according to the concentration of 1-MCP used. The use of 30 µL L^{-1} 1-MCP was most effective in prolonging green life of Eksotika papaya fruit which remained green until day 9 after 1-MCP treatment. The current knowledge is still inadequate to enable us to draw a clear conclusion on whether 1-MCP is more effective in inhibiting the ripening of fruit when it is used with and without any combination with other postharvest treatments. This therefore leads to a greater potential for continued research on 1-MCP in extending the postharvest life of Eksotika papaya fruits.

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Municipal solid waste compost increased yield and decreased nitrate amount of broccoli

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Abstract

A study of the nitrate uptake of Broccoli (*Brassica oleraceae* var. *italica*), grown with different amount of municipal solid waste compost (MSWC) was conducted in 2006 on open field at the University of Guilan, Rasht, Iran. The experiment was arranged in complete randomized block design with four treatments (0, 25, 50 and 100 Mg. ha^{-1} MSWC) and four replications. The results revealed that under prevailing local conditions, total yield of the broccoli was higher when fertilized with MSWC and low when compost was not applied to the planted area. The plants with the highest compost application (100 Mg ha^{-1}) gave significantly highest yield at 37.03 Mg ha^{-1} which was statistically different from other treatments. The significant differences were found also on marketable yield with an exception at lowest compost treatment. The lowest amount of nitrate (0.197 % in DM) in broccoli edible part was with application of 50 Mg. ha^{-1} compost.

Key words: Broccoli, municipal solid waste compost, nitrate, yield, quality.

Introduction

Nitrate occurs in many foods and drinking water, but vegetables are by far the main sources. Excessive use of nitrogen (N) fertilizers contributes to the accumulation of nitrate (NO_3^-) in soil and vegetable crops. The toxicity of NO_3^- in humans is a result of the reduction of NO_3^- to nitrite. By reacting with hemoglobin, NO_3^- forms methemoglobin, a substance that does not bind and transport oxygen to tissues. In many developing countries, the major increases in N use by agriculture during the last decades have inevitably been associated with large rises in N losses as NO_3^- in drainage water and higher NO_3^- content in arable and horticultural plants (Hudak, 2000; Nolan, 2001). Fertilizer consumption in developing countries is expected to continue to increase at a rate of about 2.6% per annum (Singh *et al.*, 1995).

Land application of wastes is becoming increasingly more popular as a means of disposal, treatment, nutrient cycling, irrigation, and groundwater recharge in many areas of the world (Cameron *et al.*, 1996). The nutrient contents in most effluents and wastes can be employed as fertilizers for agricultural production, if properly used. The use of composts in agricultural soils is a widespread practice and the positive effects on soil and vegetables are known from numerous studies (Stopes *et al.*, 1989; Thy and Buntha, 2005; Peyvast and Abbassi, 2006). Vegetable production would thus seem to be the logical target for studies to demonstrate the advantages of the different kinds of organic fertilizers (Peyvast *et al.*, 2007, 2008a, 2008b). Their recycling into soils with low amount of organic matter, which is widespread in these regions, could benefit soil structure and long term fertility, and is also an alternative to inorganic fertilizers in the growing organic vegetable production business (Peyvast *et al.*, 2007). If appropriate fertilizers are not applied, physiological disorders are apt to occur (Takahashi, 1981). The overall result has been a decline of fertility, imbalance in soil nutrients and low crop yields. For these reasons, most farmers like to use chemical fertilizers because they are easy to transport, are used efficiently for growth of the plants and give high yields, but it has been observed that with succeeding crops, the quantity of chemical fertilizer has to be increased apparently because of declining soil fertility. In contrast, organic fertilizer have beneficial effects on soil structure and nutrient availability, help to maintain yield and quality of the product and are less costly than chemical fertilizers (Thy and Buntha, 2005). Intensive production for crops such as spinach, parsley, lettuce, green and broad been occurs in North of Iran, which is a major supplier of these vegetables nationwide (Peyvast, 2006). The mild climate and the high input of fertilizers allow the production of two or three crops per year. Traditional practice in Iran is to use cattle manure although the quantity is not usually enough for the available crop areas. Alternative ways to improve the availability of organic manure seems to use compost from waste which is now available from a new compost manufactory in Guilan Province located in North of Iran. However, there have been few attempts to study the effect of composts on nitrate accumulation by vegetables.

The concentration of nitrates in the edible parts of vegetables are regulated by the European Commission Regulation No 563/2002 which has set upper limits in order to protect consumers from potential toxicological risks following the consumption of nitrate-rich foods (Maynard *et al.*, 1976; Walker, 1990; Bruning Fann and Kaneene, 1993).

The main objective of this research is to study the effect of municipal solid waste compost (MSWC) on the yield and nitrates accumulation in broccoli to estimate beneficial effect from this type of organic fertilizer.

Materials and methods

The study was conducted to assess the yield and nitrate content of broccoli (*Brassica oleracea* var. *Italica*) grown in the field during 2006 at the University of Guilan in Rasht, Iran. Four

different levels (0, 25, 50, and 100 Mg ha^{-1}) of municipal solid waste compost purchased from Bazyaft Zobaleh Co., Rasht, Iran and were supplied to the soil before planting. The physical and chemical characteristics of MSWC were measured (Table 1). Soil analyses prior starting the experiment also is shown in Table 1. The sandy loam soil used in these experiments was well drained with a total Kjeldhal N content of 1.80 g kg^{-1} (Table 2). Seeds of cv. `Embassy` (Asgrow Seed Company) were sown in plastic trays, tinned in plastic bags, when the seedlings became the first true leaf, and transplanted, when they had 2 to 4 true leaves. Plants were spaced at 50 × 50 cm^2. Water was supplied whenever necessary. All samples were collected at the stage when they were being harvested for market. Samples were transferred to the laboratory as soon as possible. Only edible portions were prepared for analysis, and roots of broccoli were discarded. The data gathered were on nitrate amount of leaves, peduncles and flowers. Samples were washed successively with tap water and distilled water. Plant materials were oven dried for 24 h at 65°C and ground to pass through a 1 mm sieve. Nitrate content in plants was measured at 410 nm by a Hitachi U-2001 spectrophotometer following the nitration by salicylic acid method described by Cataldo *et al.* (1975). Yield was calculated from all harvests. All data were subjected to a one-way analysis of variance to test for Duncan's multiple range tests. All analyses were performed using SAS statistical package (Ver. 6.04, SAS, Inc., Cary, N.C.).

Result and discussion

Plants treated with 100 Mg ha^{-1} of compost had the highest yield (37.03 Mg ha^{-1}) (Table 2). Similar results were reported previously for other crops (Bazzoffi *et al.*, 1998; Mkhabela and Warman, 2005; Peyvast and Abbassi, 2006; Peyvast *et al.*, 2007). For broccoli, this amount of organic matter was needed to provide a suitable balance in soil nutrition and increase broccoli yield. Significant differences were also found on broccoli nitrate between treatments. The highest nitrate amount (0.479 %in DM) in edible part of broccoli was obtained from treatment without compost (Table 2).

Accumulation of nitrates in vegetables has been shown to be affected by the soil texture and the source of fertilizer-N (Scaife *et al.*, 1986; Gianquinto *et al.*, 1992; Gunes *et al.*, 1995), the NH_4-N-to-NO_3-N fertilizer-N ratio (McCall and Willumsen, 1998), the timing of fertilizer-N release (Tesi and Lenzi, 1998), the light intensity and duration (Behr and Wiebe, 1992; Chadjaa *et al.*, 1999; Drews *et al.*, 1995; Gaudreau *et al.*, 1995), crop season (Gianquinto *et al.*, 1992), and vegetables type and cultivar (Blom Zandstra and Eenink, 1986; Siomos, 2000., Escobar-Gutierrez *et al.*, 2002)

In view of health reasons, absorption of nitrate from vegetables to the human nutrition should be decreased. Nitrate is for all plants an important nitrogen sources and it is very mobile in plants. Phloem and xylem have the highest nitrate amounts (Krug, 1986). Reduced nitrate accumulation results have been reported for farm yard manure fertilized lettuce for one crop season only (Gianquinto *et al.*, 1992; Stopes *et al.*, 1989) and for three crops seasons (Pavlou *et al.*, 2007). Our results also showed that MSWC applications were particularly safe in terms of nitrate accumulation in broccoli (Tables 2).

Table 2. Effect of MSWC on nitrate accumulation and yield (fresh weight) in broccoli

MSWC (Mg ha^{-1})	Peduncle (% DM)	Leaves (% DM)	Flower (% DM)	Yield (Mg ha^{-1})
0	0.296^{a}	0.613^{az}	0.479^{b}	11.39^{c}
25	0.267^{a}	0.630^{a}	0.382^{b}	15.01^{bc}
50	0.235^{a}	0.640^{a}	0.197^{a}	28.29^{ab}
100	0.275^{a}	0.679^{a}	0.323^{ab}	37.03^{a}

^{z} values in a column followed by the same letter are not significantly different, $P \leq 0.01$, Duncan multiple range test.

The respective organic fertilization treatments (MSWC) did differ significantly from the control by broccoli whereas the control resulted in the highest nitrate accumulation (Table 2) so that the 25, 50 and 100 t ha^{-1} MSWC brought 9.8, 20.6 and 7.1% lower nitrate accumulation compared to control, respectively. Accumulation of nitrates results from an imbalance between the uptake and translocation of nitrates by the xylem and the reduction of these nitrates to ammonia which is subsequently rapidly incorporated into amino acids (Maynard *et al.*, 1976). However, the internal nitrate concentration in the plant seems to be controlled by a self-regulatory mechanism exerted either by negative feedback control on the net nitrate uptake rate or by passive control on nitrate efflux (Cardenas Navarro *et al.*, 1998). The viewpoint that non-structural carbohydrates and nitrates have a complementary role in maintaining cell tugor (Blom Zandstra and Lampe, 1985; Behr and Wiebe, 1988) offers a credible model for the plant nitrate regulation mechanism suggesting the accumulation of nitrates in the vacuole as an alternative osmoticum under low radiation conditions (Blom-Zandstra, 1989; Buwalda and Warmenhoven, 1999).

No significant differences were found by leaves and peduncles between the treatments. Our results showed that an accumulation of nitrates in broccoli can be affected by source of municipal solid waste compost and probably its effect on soil structure as mentioned by Scaife *et al.* (1986), Gianquinto *et al.* (1992) and Gunes *et al.* (1995). However, there was no significant tendency by tested treatments. This finding is similar to the results by Blom-Zandstra and Eenink (1986), Siomos (2000) and Escobar-Gutierrez *et al.* (2002).

From these results, it can be concluded that, in general, municipal solid waste compost didn't affect nitrate accumulation on the broccoli. Therefore, in Iran, that ranks first in soil erosion in the Middle East (Shahvali and Abedi, 2006), an application of this compost can be recommended for broccoli in every 2-3 years to reduce this important problem. On the other hand, by reducing

Table 1. Physical and chemical characteristics of MSWC and soil used in the experiment

	pH	EC (dS m^{-1})	Total Porosity (% by vol.)	Total N (%)	Total C (%)	C/N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})
MSWC	7.1	5.2	55.5	2.10	33.8	13.0	10.2	6.8	53.2	3.3
Soil	6.8	0.08	_	2.70	1.3	0.48	68	110	12	_

application of chemical fertilizer in farm lands, the saline and sodium problems which are sometimes the result of excessive chemical fertilization and irrigation can be solved.

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Hydroponic cultivation of carrots using modified rockwool blocks

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Abstract

Three varieties of carrot (*Daucus carota* L.), 'Tokinashigosun', 'Hitokuchi' and 'Kurodagosun' were cultured hydroponically with rockwool blocks (56×8×30 cm high) in a greenhouse for 90 days. Two types of rockwool block, with holes and without holes were used in the study. Rockwool blocks with holes had seven rooting holes (10 cm in depth and 2.5 cm in diameter) 8 cm apart, which were filled with vermiculite for promoting root development. Rockwool blocks without holes were used as the control for comparison. Two rockwool blocks were placed side by side in a plastic box (58×18×18 cm high) and 14 plants were grown in each plastic box. The rockwool blocks were automatically sub-irrigated with a nutrient solution containing 35 ppm total N, 14 ppm P, 59 ppm K, 23 ppm Ca, 10 ppm Mg, 0.62 ppm Fe, 0.12 ppm Mn, 0.06 ppb B, 0.02 ppm Cu, 0.04 ppm Zn and 0.01 ppm Mo. The solution was added to the plastic boxes twice a day to keep the depth of the solution at 15 cm. The fresh and dry weights of the storage roots were 2 to 3 times greater in the rockwool blocks with holes than those without holes in each variety. The storage roots produced in the rockwool blocks with holes were 2 times longer than without holes in all the varieties. The diameter of storage roots was also greater in rockwool blocks with holes than without holes. Greater weights of the whole-plant and percent harvest index were obtained in the rockwool blocks with holes than in the without holes in all the varieties. Carbon dioxide concentration inside the rockwool blocks at a depth of 8 cm from the top surface and 1 cm beside the storage roots were lower in the rockwool blocks with holes (0.08%) than in the without holes (0.11%). Carbon dioxide gas diffusion coefficient in the rockwool media was greater in the rockwool blocks with holes than in the without holes. The hardness of the growing media was lower in the rockwool blocks with holes containing vermiculite than in the without holes. Therefore, better aerating conditions inside the rockwool blocks with holes containing vermiculite and lower hardness of the media would partly account for the better growth of storage roots in the rockwool blocks with holes than in the without holes in all the tested varieties.

Key words: CO_2 concentration, CO_2 gas diffusion coefficient, *Daucus carota,* growth, harvest index, storage roots, yield

Introduction

The part of the Asia extending from the west coast of India to Japan covers about 15% of the world land surfaces, but contains more than 50% of the world's population (Herald *et al.,* 1993). The population density is roughly six times that of the rest of the world. Furthermore, most of the increase in the world's population in the next decades is expected to be in this region (Herald *et al.,* 1993). Recently, shortages of food and energy have become serious problems in these countries due to the rapid increase of the population and subsequent decrease in arable lands. The challenge facing global agriculture is to more than double food production. Recently, consumption of vegetables has been increasing in the developing countries of the world due to the rapidly growing population. Now it is necessary to develop some suitable methods and techniques for increasing vegetable production in limited space with increasing cropping intensity to meet an acute shortage of vegetable crops for the hungry millions to solve severe malnutrition problem of the densely populated countries of the world.

Carrot is an important vegetable crop especially in tropical and subtropical areas. It is rich in carotene, a precursor of vitamin A, and contains appreciable quantities of thiamine and riboflavin. Deficiency of vitamin A is the cause of xerophthalmia, which can cause blindness in children, and was formerly common in India, Africa and Southeast Asia (Phillip and Rix, 1993). Therefore, the extension of carrot cultivation can be achieved with diverse growing methods under different environmental conditions increasing the cropping intensity, yield and production in a limited space.

Carrot yields and root length are strongly influenced by the water table and the water content of the growing media during the vegetative period (Henkel, 1970; Millette, 1983; White, 1992 and Islam *et al.*, 1998). The storage roots of carrots generally show poor growth under water-saturated conditions due to inadequate air permeability of the rooting medium, resulting in short and forked storage roots (White and Strandberg, 1979). So far the authors knowledge, there are no reports available on the successful cultivation of carrots using hydroponic or soil-less method of cultivation.

The goal of the present study was to develop a method to produce carrots following a simple method of hydroponics. Two types of rockwool blocks were examined in the study to provide the aerial spaces in the storage roots growing zone in order to obtain better growth and development of storage roots of carrots in hydroponics. In the present study, the growth characteristics and yield of three carrot varieties were investigated to examine the

possibility of producing carrots using suitable method of use of rockwool blocks in the hydroponic cultivation.

Materials and methods

Cultural conditions and treatments: The experiment was conducted in a greenhouse of Osaka Prefecture University (34°33′ north latitude and 135°31′ east longitude with 30 m elevation on the sea level), Japan during the period from mid May to mid August. The average temperature during the growing period inside the greenhouse was maintained at 27±4°C. Two types of rockwool block, with holes and without holes were used in the study. Rockwool blocks with holes had seven rooting holes (10 cm in depth and 2.5 cm in diameter) 8 cm apart, which were filled with vermiculite for promoting root development (Fig. 1). Rockwool blocks without holes were used as the control for comparison. Two rockwool blocks (56×8×30 cm high) were placed side by side in a plastic box (58×18×18 cm high) and 14 plants were grown in each plastic box. The rockwool blocks were sub-irrigated with a nutrient solution containing 35 ppm total N, 14 ppm P and 59 ppm K, 23 ppm Ca, 10 ppm Mg, 0.62 ppm Fe, 0.12 ppm Mn, 0.06 ppb B, 0.02 ppm Cu, 0.04 ppm Zn and 0.01 ppm Mo. The solution was automatically added to the plastic boxes twice a day to keep the depth of the solution at 15 cm.

Three cultivars of carrot, 'Tokinashigosun', 'Hitokuchi' and 'Kurodagosun' were cultured hydroponically. Seeds were placed on seven points 8 cm apart on each rockwool block either having holes or without holes. The seeds were germinated one week later. Insecticides were sprayed three times to control the infestations of insects during the growing period.

Harvesting and data recording: Twenty plants from four boxes in the rockwool blocks with holes containing vermiculite and rockwool blocks without holes in each variety excluding the border plants were randomly sampled and harvested after 90 days of seed sowing for data recording. Fresh weight of above-ground parts and the storage roots were determined just after harvesting. Their dry weights were determined after drying for 72 hours at 80°C in an oven. The number of leaves, the lengths of the laminas and petioles, and the mean lengths and diameters of the storage roots were also recorded just after harvesting. The percentage of the storage roots that were split or forked was recorded according to the presence of radial longitudinal cracking and branching, respectively. A SPAD-502 (Minolta Co., Ltd. Japan) chlorophyll meter was used for measuring the chlorophyll content.

Shoot-root ratio (S/R) was calculated as S/R = (above-ground dry weight / storage root dry weight). Percent harvest index (HI) was calculated as HI = (storage root dry weight / whole-plant dry weight) ×100. Carbon dioxide concentration was determined by using a gas chromatograph (Model 633, Hitachi Co. Ltd., Japan). Carbon dioxide and oxygen gas samples were collected inside the rockwool blocks at a depth of 8 cm from the top surface and 1 cm beside the storage roots using a syringe and needle. Carbon dioxide gas diffusion coefficient was estimated based on the water content inside the materials following the relationships between water content and gas diffusion coefficient for rockwool and vermiculite (Yabuki and Kitaya, 1984; Kitaya, 1995). The hardness of the rockwool media was determined using a cone penetrometer (Rheo meter, Fudoh Co. Ltd., Tokyo, Japan).

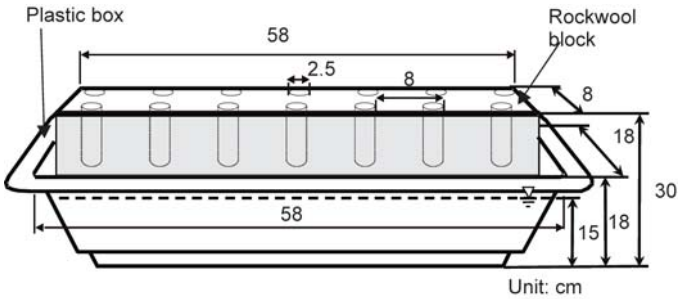

Fig.1. Schematic diagram of the culture box with rockwool blocks in hydroponic cultivation.

Experimental design and data analysis: The experiment was laid out in a randomized block design with four replications. Fisher's Least Significance Difference (LSD) method was used for comparing the growth characteristics and yield of three varieties of carrots and two types of rockwool after an ANOVA test. Mean differences of the parameters in the treatments were compared using Fisher's LSD Test at 5% level of significance.

Results

The differences in growth and morphological characteristics of storage roots and above-ground parts between the rockwool blocks with holes and the rockwool blocks without holes had almost the similar trend in all the varieties. Storage roots of carrots showed better growth in the rockwool blocks with holes than in the without holes in all the varieties (Fig. 2). The fresh and dry weights of storage roots were 2 to 3 times greater in the rockwool blocks with holes than in the without holes in all the varieties (Fig. 3). In the rockwool blocks with holes, the storage roots of the varieties Tokinashigosun and Kurodagosun had higher fresh and dry weights than the variety Hitokuchi.

Storage roots had greater length and diameter in the rockwool blocks with holes than in without holes in all the varieties (Table 1). The length of the storage roots of each variety in the rockwool blocks with holes were almost double of those in the without holes. Cracking of the storage roots was observed only in the variety Tokinashigosun. In this variety, the percent of cracking was higher in the rockwool blocks without holes than in the with holes. The percent of storage roots that were forked was higher in the rockwool blocks without holes than in the with holes in all the varieties.

The growth of the above-ground plant parts was also greater in the rockwool blocks with holes than in the rockwool blocks without holes in each variety. The varieties Kuradagosun and Tokinashigosun showed 2.2 and 1.7 times greater fresh and dry weights of above-ground parts, respectively in the rockwool blocks with holes than without holes. The fresh and dry weights of the total phytomass per plant were 1.7 to 2.4 times greater in the rockwool blocks with holes than in the rockwool blocks without holes (Table 2).

Number of leaves per plant was greater in the rockwool blocks with holes than in the rockwool blocks without holes in the varieties, Tokinashigosun and Kurodagosun. The length of laminas of the Kurodagosun was higher in the rockwool blocks with holes than in the rockwool blocks without holes. Petiole lengths in the rockwool blocks with holes and rockwool blocks without holes were not significantly different in any of the

varieties. Significantly greater chlorophyll content was recorded in the rockwool blocks with holes than without holes only in the variety, Tokinashigosun (Table 3).

In each variety, the shoot-root ratio was significantly (1.6 to 2.9 times) greater in the rockwool blocks without holes than with holes (Table 4). Percent harvest index was significantly (1.1 to 1.7 times) greater in the rockwool blocks with holes than without holes in the varieties, Toknashigosun and Hitokuchi (Table 4).

Carbon dioxide concentrations inside the rockwool blocks were lower in the rockwool blocks with holes than without holes (Fig. 4). Carbon dioxide gas diffusion coefficient was greater in the rockwool blocks with holes than in the rockwool blocks without

Fig. 2. Photograph of carrots grown on rockwool blocks with holes (right) and without holes (left) at harvest.

Table 1. Morphological characteristics of storage roots of carrots grown on rockwool blocks with rooting holes containing vermiculite or without rooting holes

Varieties	Rockwool block	Length (cm)	Diameter (cm)	Cracking (%)	Forking (%)
Tokinashigosun	With holes	12.1a	2.7a	10	0
	Without holes	6.1bc	2.0b	20	50
Hitokuchi	With holes	12.4a	2.2a	0	10
	Without holes	7.6b	1.6b	0	60
Kurodagosun	With holes	11.0a	2.7a	0	0
	Without holes	5.3c	2.2a	0	20
Varieties (V)		**	**	-	-
Rockwool types (R)		**	*	-	-
V×R		**	*	-	-

Figures with same letter in a same column do not differ significantly at 5% level by Fisher's LSD test.

Analysis of variance was applied for 6 treatments; 3 varieties and 2 rockwool types.

* and ** indicate significant differences at the P=0.05 and P=0.01, respectively.

Table 2 Weight of total phytomass and above-ground parts of carrots grown on rockwool blocks with rooting holes containing vermiculite or without rooting holes

Varieties holes	Rockwool block	Whole-plant weight (g plant^{-1})		Above-ground parts weight (g plant^{-1})	
		Fresh	Dry	Fresh	Dry
Tokinashigosun	With holes	79.0b	11.5b	36.7b	6.1b
	Without holes	35.4d	5.5d	20.5d	3.5c
Hitokuchi	With holes	54.9c	7.8c	25.6c	4.1c
	Without holes	32.4d	5.3d	21.8d	3.8c
Kurodagosun	With holes	118.3a	18.9a	79.5a	13.6a
	Without holes	48.6c	7.4c	35.6b	5.7b
Varieties (V)		**	**	**	**
Rockwool types (R)		**	**	**	**
V×R		**	**	**	**

Figures with a same letter in each column do not differ significantly at 5% level by Fisher's LSD test.

Analysis of variance was applied for 6 treatments; 3 varieties and 2 rockwool types.

** indicates significant differences at P=0.01

Table 3. Characteristics of leaves of carrots grown on rockwool blocks with rooting holes containing vermiculite or without rooting holes.

Varieties	Rockwool block	Number of leaves plant	Lamina length (cm)	Petiole length (cm)	Chlorophyll content
Tokinashigosun	With holes	12.8a	20.8c	26.3a	52.7a
	Without holes	9.5b	19.6c	27.0a	46.2c
Hitokuchi	With holes	9.2b	21.0c	23.9b	50.1ab
	Without holes	9.4b	21.3c	24.5b	48.2bc
Kurodagosun	With holes	13.9a	32.1a	27.1a	54.2a
	Without holes	10.1b	26.0b	26.5a	50.1ab
Varieties (V)		**	*	NS	NS
Rockwool types (R)		**	*	NS	*
V×R		**	*	NS	NS

Figures with same letter in a same column do not differ significantly at 5% level by Fisher's LSD test.
Analysis of variance was applied for 6 treatments; 3 varieties and 2 rockwool types.
* and ** indicate significant differences at the P=0.05 and P=0.01, respectively. NS indicates non significant.

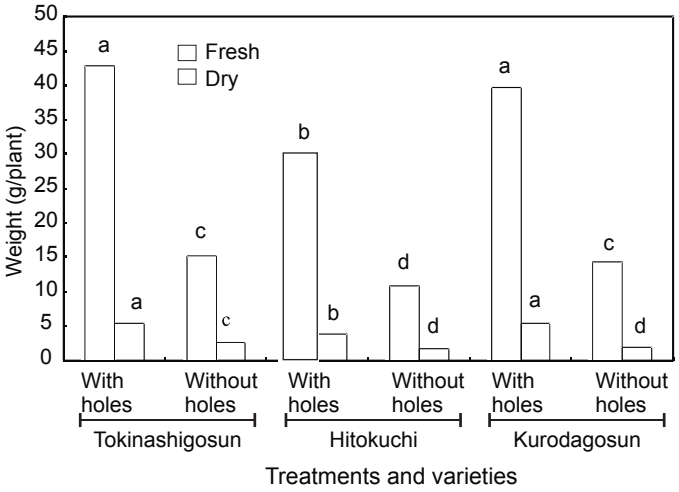

Fig. 3. Weight of storage roots of carrots grown in hydroponic method using rockwool blocks.

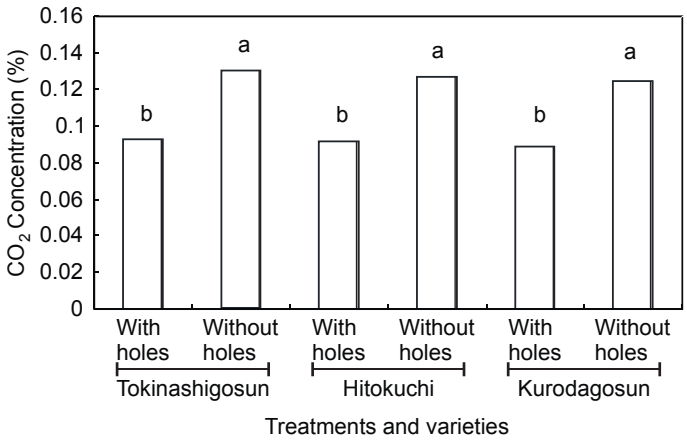

Fig. 4. CO_2 concentration inside the rockwool blocks with holes and without holes at a depth of 8 cm from the top surface and 1 cm beside the storage roots of carrots.

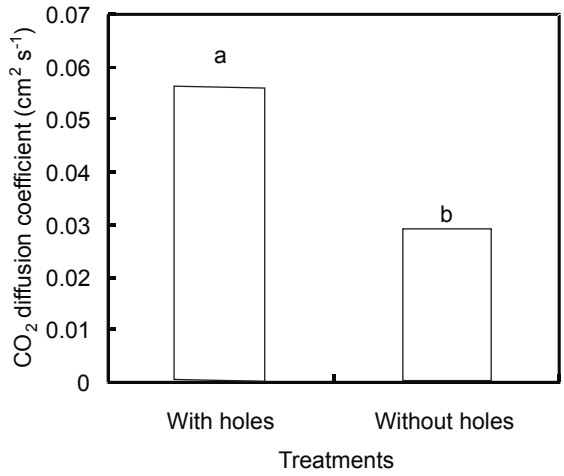

Fig. 5. CO_2 gas diffusion coefficients of vermiculite in the holes of rockwool blocks and rockwool blocks without holes.

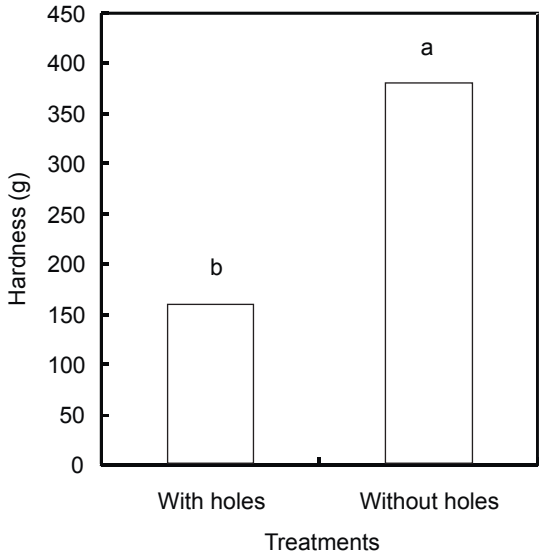

Fig. 6. Hardness of the rockwool media used as with holes and without holes.

holes (Fig. 5). The hardness of vermiculite in the rockwool blocks with holes was lower than in the rockwool blocks without holes (Fig. 6).

Discussion

The whole-plant, above-ground parts and storage roots showed better growth and development in the rockwool blocks with holes than without holes regardless of the variety. The fresh and dry weights, and length and diameter of storage roots showed greater growth and development in the rockwool blocks with holes containing vermiculite than in the rockwool blocks without holes. The short and forked storage roots of carrots were also observed by White and Strandberg (1979) when grown at water-saturated conditions due to inadequate air-permeability of the rooting medium. In a previous study, Islam *et al.* (1998) found that the length of storage roots of carrots was shorter when grown

Table 4. Shoot-root ratio and harvest index of carrots grown on rockwool blocks with rooting holes containing vermiculite or without rooting holes

Varieties	Rockwool block	Shoot-root (S/R) ratio	Harvest index (%)
Tokinashigosun	With holes	1.2c	46.5a
	Without holes	2.2b	33.6b
Hitokuchi	With holes	1.3c	47.2a
	Without holes	3.8a	28.3bc
Kurodagosun	With holes	2.7b	28.8bc
	Without holes	4.3a	25.6c
Varieties (V)	**	**	
Rockwool types (R)	**	**	
V×R	**	**	

Figures with same letter in a same column, do not differ significantly at 5% level by Fisher's LSD test.
†Analysis of variance was applied for 6 treatments; 3 varieties and 2 rockwool types.
** Significant differences at P=0.01

with higher water table levels. The forking of the storage roots was prominent in the rockwool blocks without holes regardless of the varieties. The better aeration and less hardness of the vermiculite would partly account for the better growth of carrot in the rockwool blocks with holes containing vermiculite.

Insufficient aeration in the root zone is a great problem for plant growth and thus has received a great deal of attention by the researchers. Poor aeration inhibits water and nutrient absorption of plants and thus inhibits growth (Lowton, 1945; Russell, 1977). This inhibition has been partly attributed to suppression of root respiration caused by low oxygen concentration (Glinski and Stepniewski, 1985) and high carbon dioxide concentration (Yabuki and Kitaya, 1984; Kitaya *et al.*, 1992). Generally, root damage in excess moisture environments has been attributed to the lack of oxygen (Williamson and Kriz, 1970). White and Strandberg (1979) reported that carrot root growth can be depressed by water-saturated conditions during early growth but growth continues if the root tips are not damaged. The carbon dioxide concentration was shown to increase considerably with increased water content (Yabuki and Kitaya, 1984; Kitaya *et al.*, 1984 and 1987 and Islam *et al.*, 1998). In the present study, the lower carbon dioxide concentration and higher carbon dioxide gas diffusion coefficient in the rockwool blocks with holes containing vermiculite were helpful in promoting better growth and development of storage root of carrots. Eavis (1972) reported that under compacted conditions, the effect of mechanical impedances and aeration deficiency on root growth was more apparent. In the rockwool blocks without holes, it would be expected that the storage roots of carrots would have had to exert a high pressure to penetrate the medium for growth as indicated by the greater hardness (Fig. 6). Under these conditions, carrot growth was restricted presumably because of the high mechanical impedance resulting in shorter, forked and poor storage root growth.

Therefore, better aerating conditions and looseness of the growing media around the roots would partly account for the better growth and development of storage roots of carrot in the rockwool blocks with holes containing vermiculite than in the rockwool blocks without holes.

The hydroponic method is applicable for carrot cultivation using loose growing media capable of providing sufficient aeration in the root zone. The method developed in the present study can be applicable to other vegetable crops as well.

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Screening for genetic divergence in tomato genotypes against tomato leaf curl virus

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Abstract

During summer 2005 out of 50 genotypes screened for tomato leaf curl virus under field conditions, none of the lines tested were resistant, however, six genotypes showed mild infection and nine genotypes showed moderate infection. In the second season, *i.e.*, 2006 only Nandi and Vybhav showed moderate resistant reaction, along with the new commercial hybrids Hy-558, Hy-530, NS-563 and NS-719. The variety Vybhav was found superior over other varieties against the disease. The presence of virus in the symptomatic hosts was confirmed by ELISA and PCR. The plant height of the genotypes contributes to maximum extent (52.21 %) to the divergence followed by yield per plant and per cent disease incidence (10.86 % each), but the vector population contributed least (0.97 %). As a result of D^2 clustering, the commercial hybrids possessing lot of diversity fall in to four different clusters, cluster II had got six entries, cluster III 3 entries, cluster IV 6 entries and cluster V only one entry wheras cluster I had 50 entries.

Key words: Tomato leaf curl, ELISA, PCR and screening

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is an important and most widely grown vegetable crop in India and ranks second in importance among vegetables. It is grown for its edible fruit, which can be consumed, either raw or cooked or in the form of various processed products.

The tomato leaf curl virus disease (ToLCVD) is caused by a range of circular single ssDNA virus (more than 20 species) species in the genus Begomovirus (Geminiviridae: subgroup – III) (Polston and Anderson, 1997; Faquet and Stanely, 2003) which are transmitted by the whitefly *Bemisia tabaci* Genn. and is the most important and destructive viral pathogen in many parts of India (Vasudeva and Sam Raj, 1948: Sastry and Singh, 1973; Saikia and Muniyappa, 1989; Harrison *et al.*, 1991). The symptoms of ToLCVD includes leaf curling, leaf chlorosis, vein clearing and stunting. If the infection occurs at the early stage it may lead to sterility.

The incidence of ToLCV in tomato growing areas of Karnataka ranged from 17-100 per cent in different seasons. The per cent yield loss observed ranged from 50-70 per cent in tomato cv. Pusa Ruby grown in February-May (Saikia and Muniyappa, 1989). Diversity in tomato leaf curl begomoviruses (TLCBs) in southern India has been apparent since the early 1980s when Reddy *et al.* (1981) reported that in a single tomato variety, TLCB isolates gave rise to five distinct symptom types. Variability was subsequently also found in the epitope profiles of TLCBs collected from Karnataka (Muniyappa *et al.,* 1991b), with groupings suggesting that the tomato crop and some neighboring weed species were hosts to the same TLCB strains/species.

The severity and rate of spread of ToLCV has become a major limiting factor for cultivation and challenging to farmers and scientific community. The existence of variability among the virus isolates and vector is the main reason for the break down of resistance in the leading varieties. Therefore, the screening and identification of resistance source is an important practice in the management of the disease.

Materials and methods

Tomato varieties, cultivars, commercial hybrids and breeding lines, were sown in nursery beds and 24-25 days old seedlings were transplanted in the main field. Each variety/ line, was planted in two rows of 6 meter length during summer of 2005 and the lines which showed mild and moderate infection were screened during second season (summer 2006) along with some commercial hybrids. An artificial inoculation was carried out for lines showed mild and moderate rection in summer 2005, along with commercial hybrids using standard procedures (Muniyappa, 2000).

The presence of virus in the symptomatic plants was confirmed by ELISA and PCR using coat protein specific primers (Deng, 1994). Observations were recorded on appearance of first symptom and incidence of both diseased plants and vector population upto 12 weeks after planting.

The following scale was employed for scoring the disease reaction, suggested by Muniyappa *et al.* (1991a).

Resistant (R)	: No symptom.
Mild infection (M)	: Light yellowing along the margins but no curling and very few plants are infected.
Moderate infection (Mo)	: Slight yellowing along the margins, slight curling, puckering and stunting.
Susceptible	: Very severe curling, puckering, stunting, reduction in leaf size, and reduced fruit formation.

The time taken for first symptom appearance after transplanting was recorded in all the variety/ lines/genotypes.as following:

Days taken for first appearance of symptoms after transplanting	Characteristics
10-12	Very Early (VE)
21-30	Early (E)
31-40	Moderately Late (ML)
41-50	Late (L)
51 and above	Very Late (VL)

Genetic divergence: The Mahalonobis's D^2 analysis was used for assessing the genetic divergence among the tomato genotypes. The different characteristics like PDI, vector population, plant height, number of branches and number of fruits/plant, fruit weight, yield plant^{-1}, were taken in to consideration. The square of the Mahalonobis's generalized distance between any two populations is given by the formula (Mahalonobis, 1936):

$D^2 = \Sigma \Sigma\ i\ j\ \delta_i\ \delta_j$

Where, D^2 = Square of the generalized distance

i j =Reciprocal of the common dispersal matrix.

$\delta_i = (\mu_{i1} - \mu_{i2})$

$\delta_j = (\mu_{j1} - \mu_{j2})$

μ= Vector of mean values for all the characters.

Clustering of the D^2 values: All the n (n-1)/2 D^2 values were clustered using Toucher's method (Rao, 1952). The following method of clustering D^2 values was used. The two genotypes having the lowest D^2 value between them were selected and a third genotype which had on an average the smallest D^2 values from the first two was added.

Similarly, fourth was chosen which showed, smallest average D^2 from the first three. If at any stage increase in average D^2 values due to addition of a new genotype exceeded the average of those already included, then that genotype was taken out. The genotypes that were already included in that group were considered as the first cluster. The procedure was repeated for other genotypes omitting those that are already included in the former cluster.

The average distance of all the genotypes within the cluster and between any two clusters (inter cluster distance) were calculated.

Results

The results of the study revealed that out of 50 breeding lines (Table 1) screened, none of them was resistant. However, six lines *viz*., Alcobasa, Vybhav, L-32, S-21, Sankranti and Nandi showed mild infection. The per cent infection on these lines was 5, 5, 5, 2, 2 and 8 per cent, respectively and symptom expression was delayed. Twelve lines namely, Alcobasa-V, L-10, L-15, L-17, L-24, L-25, L-26, L-30, L-34, D-4, PKM-1 and V-1 showed moderate infection, while all other lines showed susceptible reaction to ToLCV (Table 2). The time taken for expression of symptoms varied from genotype to genotype. Majority of them showed early (21-30 DAT) to very early (10-20 DAT) infection symptoms.

In the second season, 15 breeding lines which showed mild and moderate infection of ToLCV during summer 2005 were used along with seventeen commercial hybrids. Results of the study showed that none of the lines or hybrids tested were

Table 1. Response of tomato genotypes against Tomato leaf curl virus under field conditions during summer 2005

Sl. No.	Genotype	Symptom Expression (DAT)	Disease incidence (%)	Whitefly population (Number/ plant)	Yield/ plant (g)	Disease reaction
1	L-01	VE	40	2.8	1125	S
2	L-02	E	40	3.2	1100	S
3	L-03	VE	60	4.0	400	S
4	L-04	VE	85	2.0	552	S
5	L-05	VE	30	2.4	870	S
6	L-06	ML	80	3.6	900	S
7	L-07	E	60	2.0	720	S
8	L-08	VE	55	3.0	375	S
9	L-09	E	30	2.0	500	S
10	L-10	E	20	1.0	240	MR
11	L-11	VE	45	3.0	1160	S
12	L-13	VE	35	2.0	572	S
13	L-14	VE	40	2.6	400	S
14	L-15	VE	20	2.4	750	MR
15	L-16	VE	40	2.0	37.5	S
16	L-17	VE	15	1.0	385	MR
17	L-19	VE	35	2.8	350	S
18	L-21	VE	20	2.2	360	S
19	L-23	VE	15	0.4	700	MR
20	L-24	E	20	2.0	216	MR
21	L-26	VE	15	0.2	520	MR
22	L-30	VE	15	1.0	225	MR
23	L-31	VE	25	2.2	150	S
24	L-32	E	5	0.4	1800	M
25	L-33	VE	20	1.8	360	S
26	L-34	E	20	0.4	150	MR
27	L-35	VE	55	2.0	200	S
28	L-35-1	VE	40	2.0	1705	S
29	L-36	VE	35	3.2	240	S
30	L-38	VE	30	2.2	300	S
31	L-39	VE	35	3.2	780	S
32	L-43	VE	40	3.0	350	S
33	L-44	VE	50	2.8	72	S
34	L-49	VE	30	2.0	336	S
35	L-58	E	45	2.2	450	S
36	L-86	VE	45	3.6	230	S
37	Alcobasa- B	VL	5	0.2	1350	M
38	Alkobasa-V	E	10	1.2	560	MR
39	Arka Vikas	VE	55	1.0	1350	S
40	CA-1	VE	30	2.0	660	S
41	D-4	VE	10	0.4	840	MR
42	D-25	VE	40	2.2	1500	S
43	Sankranti	VL	2.0	1.0	656	M
44	Nandi	VL	8	1.2	620	M
45	Vybhav	VL	5	0.2	1116	M
46	PKM-1	VL	10	0.2	765	MR
47	S-21	VL	2.0	0.4	720	M
48	UC-204B	VE	40	2.0	600	S
49	V-1	VE	20	2.0	1100	MR
50	Megha	VE	100	2.0	780	S

Reaction types: R- Resistant, M-Mild Reaction, MR-Moderate Reaction, S-Susceptible.

Table 2. Grouping of genotypes screened during summer 2005 in to different reaction types against tomato leaf curl virus disease

Sl. No	Reaction types	Genotypes
1	Resistant	-
2	Mild Reaction	Alcobasa-B, Vybhav, L-32, S-21, Sankranti and Nandi.
3	Moderate Reaction	Alcobasa-V, L-10,L-15, L-17, L-23, L-24, L-26, L-30, L-34, D-4, PKM-1 and V-1.
4	Susceptible	L-1, L-2, L-3, L-4, L-5, L-6, L-7, L-8, L-9, L-11, L-13, L-14, L-16, L-19, L-21, L-31, L-33, L-35, L-35-1, L-36, L-38, L-39, L-43, L-44, L-49, L-58, L-86, UC 204 B, D-25, Arka Vikas, CA-1 and Megha

found resistant, but few lines such as Nandi, Vybhav, Hy 558, HY 530, NS-503 and NS-719 showed mild reaction of ToLCV and Sankranti, PKM-1 and Utsav showed moderate reaction. All other lines were found susceptible. It was interesting to note that majority of the lines, which showed mild, and moderate reactions during summer 2005 became susceptible during, 2006. The cultivars like Nandi (8.33 PDI), Vybhav (6.89 PDI) were found better performed both from the point of view of resistance and yield. Among the hybrids, HY 558, HY 530 (Sungro hybrids) NS-719 and NS-563 (Namdhari hybrids) showed good response both for yield and for disease resistance (Table 3 and 4).

The results of the ELISA showed that lines which showed mild and moderate reactions had less virus titer compared to susceptible lines. The results of artificially inoculated plants showed that under high disease pressure Nandi and PKM-1 were also susceptible.

Genetic divergence: Plant height of the genotypes contributed maximum extent (52.21 %) to the divergence followed by yield per plant and per cent disease incidence (10.86 % each) but the vector population contributed least (0.97 %) to the divergence (Table 5).

Based on the extent of divergence in the genotypes they were grouped into five clusters (Table 6). The results indicated that majority of them were under cluster 1, (50 genotypes) having less divergence. Commercial hybrids possessed lot of diversity fall in

Table 3. Response of tomato genotypes against Tomato leaf curl virus under field conditions during summer 2006

Sl. No	Natural					Artificially inoculated			
	Genotype/ variety	Symptom expression (DAT)	Disease incidence (%)	Whitefly population (Number/ plant)	Yield plant^{-1} (g)	Disease reaction	Disease incidence (%)	ELISA absorbance values	PCR reaction
1	L-5	E	100.00	2.0	880	S	100.00	1.50	+
2	L-10	E	100.00	0.6	260	S	100.00	1.28	+
3	L-15	E	90.90	2.8	620	S	90.00	1.29	+
4	L-17	E	100.00	0.8	320	S	100.00	1.32	+
5	L-23	E	81.81	0.6	660	S	90.00	1.41	+
6	L-26	E	100.00	2.2	520	S	100.00	1.63	+
7	L-30	E	100.00	3.8	220	S	100.00	1.54	+
8	L-32	E	94.44	2.4	1500	S	90.00	1.46	+
9	Sankranti	ML	13.04	3.6	620	MR	10.00	1.10	+
10	Nandi	ML	08.33	4.2	580	M	20.00	1.04	+
11	Vybhav	ML	06.89	2.2	1260	M	0.00	1.06	+
12	V-1	E	100.00	2.2	400	S	100.00	1.80	+
13	PKM-1	ML	36.36	0.4	650	MR	40.00	1.09	+
14	Alkabasa-V	E	100.00	3.2	350	S	100.00	1.60	+
15	Arka Vikas	E	100.00	0.2	800	S	100.00	1.50	+
16	NS-53	E	100.00	1.2	460	S	100.00	1.40	+
17	NS-563	E	08.69	2.2	1529	M	10.00	1.02	+
18	NS-564	ML	100.00	0.4	272	S	100.00	1.64	+
19	NS-585	L	33.33	0.2	1495	MR	30.00	1.20	+
20	NS-658	E	76.66	0.8	840	S	80.00	1.66	+
21	NS-719	L	06.66	0.4	650	M	10.00	1.06	+
22	NS-816	E	100.00	1.4	256	S	100.00	1.56	+
23	NS-812	E	65.21	1.2	720	S	70.00	1.64	+
24	NS-2530	E	100.00	2.6	258	S	100.00	1.70	+
25	NS-2535	E	86.66	1.2	300	S	80.00	1.81	+
26	Utsav	E	36.00	2.2	1230	M	40.00	1.00	+
27	Indira	ML	77.77	1.4	1254	S	70.00	1.54	+
28	Malini	E	50.00	0.8	864	S	70.00	1.70	+
29	Sonam	L	64.28	0.8	1240	S	80.00	1.60	+
30	HY-530	L	07.60	0.8	1385	M	10.00	1.00	+
31	HY-558	L	09.09	0.6	2448	M	10.00	1.20	+
32	Megha	E	100.00	0.4	780	S	100.00	1.32	+

Table 4. Grouping of genotypes and commercial hybrids screened during summer –2006 in to different categories against tomato leaf curl virus disease

Sl. No	Reaction types	Genotypes
1	Resistant	-
2	Mild Reaction	Nandi, Vybhav, Hy-530, Hy-558, NS-563 and NS-719
3	Moderate Reaction	Sankranti, PKM-1 and Utsav.
4	Susceptible	L-5, L-10, L-15, L-17, L-23, L-26, L-30, L-32, Alkobasa-V, Arkavikas, NS-564, NS-53, NS-658, NS-812, NS-816, NS-2530, NS-2535, V-1, Malini, Sonam, Indira and Megha

Table 5. The per cent contribution of each character towards divergence in tomato

Sl. No.	Character	Contribution
1	Per cent disease incidence	10.86
2	Vector population	00.97
3	Plant height (cm)	52.21
4	Number of branches	08.99
5	Number of fruits/plant	10.06
6	Fruit weight (g)	06.01
7	Yield /plant (g)	10.86
Total		100.00

to four different clusters indicating considerable genetic distance among them. The cluster II had six entries, III had 3 entries, cluster IV had 6 entries and cluster V had only one entry.

The intra cluster distance varied from 11.23 in cluster III to 16.05 in IV. This indicates the presence of divergent genotypes within different clusters. The inter cluster D^2 values also ranged widely with minimum value of 19.49 between cluster I and IV to maximum of 31.55 between cluster II and IV indicating only some diversity among the genotypes (Tables 7 and Table 8).

Discussion

In general, majority of the lines tested were found susceptible and only few lines showed moderate reaction. The variety Vybhav, which was found superior over other varieties was on par with some of the better hybrids used in the study. Similar to this, screening of genotypes for managing the disease have been reported by Som and Choudhary (1976), Hassan *et al.* (1984), Banerjee and Kalloo (1987a), Pilowsky and Cohen (1990) and Muniyappa *et al.* (1991a). The cultivars such as Vybhav, HY-530 and HY-558, which were found tolerant could be used in the areas of high disease pressure (Table 3 and 4).

Success in locating resistance to ToLCV breeding is directly related to the availability of diversity in germplasm for resistance either to ToLCV or its vector. The genes for resistance to ToLCV have been reported in wild species like *L. hirsutum*, *L. peruvianum*, *L. pimpenellifolium* (Banerjee and Kalloo, 1987, and Pilowsky and Cohen, 1990). But transfer of these genes to cultivated species was possible in very stray cases. In view of that an attempt was made to screen 65 genotypes/hybrids. The reaction of genotypes to ToLCV under epiphytotic conditions during the summer seasons was assessed. It was found that majority of the genotypes were susceptible to ToLCV under field conditions. However, few were found resistant and they had

Table 6. Clustering pattern of 65 tomato varieties/ genotypes/hybrid following D^2 analysis

Cluster No.	Number of entries	Genotypes
I	50	B-Alcobasa-V, Vybhav, L-32, S-21, Sankranti, Nandi, Alcobasa-V, L-34, L-23, D-4, L-24, L-26, L-30, PKM-1, V-1, L-17, L-15, L-10, L-43, L-39, L-49, L-38, L-36, L-35-1, L-21, L-33, L-31, CA-1, L-58, L-44, UC 204 B, L-86, L-35, D-25, Arkavikas, L-1, L-11, L-16, L-19, L-14, L-13, L-6, L-9, L-8, L-7, L-4, L-2, L-5, L-3 and Megha,
II	6	NS-564, NS-2635, NS-816, NS-812, NS-53 and NS-2530.
III	3	Utsav, Sonam and Indira.
IV	6	Malini, Hy-530, Hy-558, NS-585, NS-719 and NS-563.
V	1	NS-658.

Table 7. The average inter and intra cluster distances for 65 tomato genotypes

Cluster	I	II	III	IV	V
I	15.14	26.94	20.27	19.49	25.16
II		13.53	18.28	31.55	22.56
III			11.23	19.79	18.51
IV				16.05	25.23
V					0.00

Diagonal values indicate intra cluster distance. Above diagonal values indicate intercluster distance.

Table 8. Cluster mean of seven characters in tomato

Cluster	Character						
	1	2	3	4	5	6	7
I	31.65	1.54	654.77	31.20	20.25	8.49	50.12
II	89.26	1.25	422.05	47.72	9.05	3.72	41.16
III	59.78	1.13	1225.77	67.33	19.00	4.44	48.22
IV	18.45	0.64	1305.72	65.22	20.16	5.27	57.00
V	73.55	0.86	860.00	70.00	13.66	4.66	83.66

good fruit set and growth. There was lot of diversity among the genotypes towards ToLCV with the per cent disease incidence varying from 6.89-100 per cent. This was confirmed from the data on D^2 values that distributed genotypes into five clusters. The resistant/tolerant groups of genotypes were congregated in to cluster I and cluster IV. Further it was found that plant height contributed the highest to divergence (52.21 %) followed by per cent disease incidence and fruit yield per plant. It is clear from the results that hybrids have more divergence than the genotypes or advanced breeding lines.

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Occurrence of *Pseudomonas syringae* pv. *syringae* the causal agent of bacterial canker of stone fruits in Guilan province of Iran

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Abstract

Pseudomonas syringae pv. *syringae* causes canker, leafspots and necrosis of the bark of cherry, plum, and peach fruit trees. Symptoms caused by this pathogen on leaves, blossoms, and fruit, reported as common else where, are rare in Guilan cherry orchards. In this research, during survey from cherry, plum, and peach orchards in different areas of Guilan province (Talesh, Hashtpar, Astaneh-Ashrafieh and Lahijan), samples were taken from infected tissues of disease trees. For isolation of bacteria causing disease, infected tissue were crushed in bacteriological saline (0.85% w/v NaCl) and 100µL of juice was cultured on nutrient agar (NA) and King's B medium. Strains of bacteria rod-shaped, gram negative and aerobic bacterium were isolated. The strains produced Levan on media including sucrose. All strains made Hypersensitive Reaction (HR) on tobacco and geranium leaves. All of the isolated bacteria were oxidase, nitrate, tween 80 hydrolysis, indole and starch hydrolysis negative and could not rot potato tuber slices, produced H_2S, and grew at 36°C. The isolates could use citrate and urease. The isolates produced acid from sorbitol, galactose, myo-inositol, manitol, xylose, maltose and sucrose. Their gelatin test were positive. Based on morphological, physiological, biochemical, pathogenicity properties and total cellular protein profiles (SDS-PAGE), the predominate pathogenic type was identified as *P. s.* pv. *syringae.* This is the first report of the existence of *P. s.* pv. *syringae* on stone fruit trees in Iran.

Key words: Stone fruit trees, *Pseudomonas syringae* pv. *syringae,* canker, Iran

Introduction

Bacterial canker disease is caused by *Pseudomonas syringae pv. syringae*, a bacterium. Although most serious on sweet cherries, bacterial canker also affects peaches, prunes, plums, apricots and almonds. Trees already weakened by frost, wounds, early pruning, water stress and poor nutrition are vulnerable to cankers (Anonymous, 2004; Ogawa *et al.,* 1995). Disease outbreaks are sporadic and more frequent on sweet cherry than on sour cherry. *P. s.* pv. *syringae* is found on peach (Jones and Sutton, 2004). Bacterial canker is an important disease affecting stone fruits in all production areas (Roberts and Smith, 2002). The disease attacks most parts of the tree. Leaves on the terminal portions of cankered limbs and branches may wilt and die in summer or early autumn if girdled by a canker. Occasionally, large scaffold limbs are killed. Leaf and fruit infection occur sporadically, but they can be of economic significance in years with prolonged wet, cold weather during or shortly after bloom (Jones and Sutton, 2004). The pathogen produces a potent phytotoxin called syringomycin that is estimated to nearly double its virulence (Gross *et al.*, 1997). The bacteria can survive from one season to the next in bark tissue at canker margins, in apparently healthy buds and systemically in the vascular system (Jones and Sutton, 2004). Cankers can continue to develop in lateral branches and the trunk. This disease has been difficult to study and control, as the pathogen is very widespread, lives as an epiphyte on the host and weeds, invades host tissues without inducing symptoms, and causes disease that has symptoms similar to those caused by other pathogens (Roberts and Smith, 2002). Cherry trees are commonly hosts of the causal organism, but disease does not occur unless the climate is conducive and the host is predisposed (Timothy and Kupferman, 2003). Young cherry trees are the most seriously affected, as trunks are often girdled or severely damaged. Trees affected during the first three seasons after planting are often killed outright or grow so poorly that they must be removed (Jones and Sutton, 2004). Molecular techniques (AFLP) are used for distinguishing between both pathovars, as well as for the assessment of the genetic diversity of the pathogen. In view of relating this diversity to differences in pathogenicity, a method for artificial infection was developed (King *et al.,* 1954). We studied the presence of *P. s.* pv. *syringae* on stone fruit trees such as cherry, plum, and peach orchards in the Guilan province of Iran.

Materials and methods

Bacterial isolations: Samples were collected from orchards in Talesh, Hashtpar, Astaneh-Ashrafieh and Lahijan during 2002–2003. Small tissue pieces from stem lesion margins, surfaces of cankers and leaf tissue showing necrotic lesions and blight symptoms were removed aseptically, ground in bacteriological saline (0.85% w/v NaCl), and left at room temperature (20°C) for 10 min. The suspensions were streaked onto Nutrient Agar (NA) and King's medium B (KB) and incubated at 26°C. Bacterial colonies growing from the suspensions were re-streaked onto KB to obtain single colonies. Isolates were routinely grown on KB at 26°C and stored at 4°C for up to 2 weeks. For long-term storage bacterial strains were stored in freezing medium at -80°C.

Biochemical and physiological tests: Strains were characterized based on the following tests: Gram test in 3% KOH (Sulsow

et al., 1982), oxidative/fermentative test (Hugh and Leifson, 1953) production of fluorescent pigment on KB, hypersensitive reaction (HR) in tobacco and geranium leaves (Lelliot *et al.,* 1987), oxidase test, levan formation, catalase, urease, gelatin liquefaction, litmus milk, salt tolerance (5%) and gas formation from glucose. In addition, tests for arginine dehydrolase, hydrogen sulfide production from peptone, reducing substance from sucrose, tyrosinase casein hydrolase, nitrate reduction, indole production, 2-keto gluconate oxidation lecitinase, starch hydrolysis, phenylalanine deaminase, esculin and Tween 80 hydrolysis and optimal growth temperature were performed as per Schaad *et al.* (2001). The presence of DNase was tested on DNA agar (Diagonistic Pasteur, France). Carbohydrate utilization using Ayer basal medium was carried out and the results were recorded daily upto 8 days (Hildebrand, 1988).

Pathogenicity test on leaves, fruit and branches of cherry: Characteristic colonies of *P. s.* pv. *syringae* that grew on NA and KB were subcultured. Pathogenicity tests were carried out on leaves, immature sweet cherry fruit and branches of young shoots of cherry. Leaves were removed from young shoots and sterilized with 70% ethanol. In toward of midrib was injured T shape and then 50 µL droplet of bacterial suspension (3×10^8 CFU mL^{-1}) was placed on them. The leaves were maintained under humid (95%) conditions at 27°C for 10 days. Immature sweet cherry fruit assay was carried out essentially as described by Jones (1971) and Latorre and Jones (1979). Immature sweet cherry fruits were collected during September. The fruits were surface sterilized with 70% ethanol and 100 µL of bacterial suspension (3×10^8 CFU mL^{-1}) was injected on them.

Branches of young cherry shoots were placed in an Erlenmeyer flask with water. Shoots, 45 cm long, were tip-inoculated by injection of 100 µL of bacterial suspension $2x10^8$ CFU mL^{-1} with a hypodermic needle; they were maintained at 26°C for 6 weeks. For each strain tested, five branches were used. Control were treated with sterile distilled water.

SDS-PAGE: Electrophoresis of soluble proteins was carried out in a discontinuous SDS polyacrylamide gel according to the method of Laemmli (1970), with some modifications as described by Rahimian (Rahimian, 1995). Strains were grown on NA medium at 24 °C for two days. A bacterial suspension in distilled water was prepared in Eppendorf tube and centrifuged at 13,000 x g for 12 min. The pellet was washed and resuspended in sterile .distille water. The cell suspension optical density was adjusted to 1.0 at 630 nm. Each sample was mixed with 5x sample buffer [sample buffer: 63 ml, Tris-HCl (pH 6.8), 10 % (v/v) glycerol, 2.0% (w/v) SDS and 0.25% (w/v) bromophenol blue] and heated at 95°C for 5 min. To each 950 µL sample buffer, 50 µL of 2-mercaptoethanol, as a reducing agent, was added just before boiling. Samples were centrifuged at 15,000 x g for 10 min. Fifty µL of soluble proteins was loaded in each well in a 13 x 17 cm polyacrylamide slab with 0.75 mm thickness. Proteins were fractionated in 10% resolving gel at a constant current of 20 mAmps for four hrs. The gel was stained in methanol, water and acetic acid (5: 5: 1) containing 0.5% coomassie brilliant blue G250 overnight and destained in the same solution without dye. The gel was kept in 7% acetic acid.

Antibiotic sensitivity test: *P. s.* pv. *syringae* strains were tested for antibiotic sensitivity using antibiogram disks. A bacterial suspension was spread on nutrient agar medium. Two disks for each antibiotic were placed on medium. After 48 hrs of incubation at 28°C, inhibition zone was measured.

Results and discussion

Biochemical and physiological tests: All strains were gram, oxidase, catalase negative, and unable to utilize glucose under anaerobic conditions (Table 1). None of the strains were able to produce reducing compounds from sucrose or show lecithinase, arginine dihydrolase activity or produce gas from glucose. All strains were esculin positive and capable of hydrolyzing gelatin, None of the strains were able to hydrolyze Tween 80, produce indole, reduce nitrate and oxidize 2-keto-gluconate. All strains of *P. s.* pv. *syringae* were able to produce syringomycin and showed ice nucleation activity. All strains were able to utilize citrate, L-Lysine and produce acid from manitol, xylose, D (+) galactose, inositol, maltose, sorbitol, manose and sucrose. None of the strains were capable of utilizing L-arabinose, trihalose and L-tartrate. The presence of DNase was tested on DNA agar (Diagonistic Pasteur, France).

Pathogenicity test: All strains caused dark brownish spots with depressions on the surface of immature cherry fruits 7-10 days after inoculation, whereas a slight discoloration was observed in the control. Gumming occured at the margins of the cankers and often was heavy**.** The strains also caused water-soaked spots and necrosis in the injured region on cherry leaves after a week. These symptoms did not occur in the control. Further, inoculation of young shoots on cherry trees with individual strains caused chlorotic spots that eventually became necrotic and dried. Cankers typically ooze amber-colored gum and often become entry sites for borers (Richard, 2002). These symptoms did not occur in the control.

Serious infections have occurred on young trees that were wetted by rain or irrigation within a few days of planting or after suckers were removed from trunks. Frost, especially when closely followed by rain or heavy dew, leads to bacterial blast of blossoms. In areas of the world that have cool, wet winters, infection of pruning wounds during the fall or winter is common (Roberts and Smith, 2002; Beers *et al.,* 1993)**.**

Protein profile: Total protein pattern of isolates were compared to standard strain. Protein bands of strains were nearly similar to protein bands of standard strain of *P. s.* pv. *syringae* (Fig. 1). Analysis of the ERIC fingerprints from *P. s.* pv. *syringae* strains showed that the strains isolated from stone fruits formed a distinct cluster separate from most of the strains isolated from other hosts. These results provide evidence of host specialization within the diverse pathovar *P. s.* pv. *syringae* (Little *et al*., 1998).

Antibiogram test: *P. s.* pv. *syringae* strains were tested in duplicates for sensitivity toward 16 different antibiotics (Table 2). They were classified into three groups: resistant (without an inhibition zone), relatively sensitive (inhibition zone with a diameter less than 10 mm) and sensitive (inhibition zone greater than 10 mm in diameter).

Control of bacterial canker is often difficult and sometimes impossible. As with most plant problems, keep trees as healthy as possible so that they are less vulnerable to attack**.** Most chemical

Table 1. Phenotypic characteristics of *P. syringae* pv. *syringae* strains tested

Characteristics	Isolates of *P. s.* pv. *syringae*
Gram reaction	-
Oxidative/Fermentative	-
Fluorescent pigment	+
HR on tobacco	+
Ice nucleation	+
Growth at 39°C	-
Syringomycin production	+
Leaf blight on pear	+
Pectinase	-
Acetoin	-
Argenine dihydrolase	-
Levan formation	+
Nitrate reduction	-
Catalase	-
Tween 80 hydrolysis	-
Oxidase	-
Starch hydrolysis	-
Gelatin hydrolysis	+
Esculin hydrolysis	+
DNase activity	+
Indole formation	-
H_2S from cysteine	-
Casein hydrolysis	-
Urease	+
MR	-
Utilization of	
L-lysine	+
Citrate	+
lecithinase	-
Growth in 5% NaCl	-
Acid from	
L-Arabinose	-
Myo-Inositol	+
Manitol	+
Xylose	+
Trihalose	-
Maltose	+
L-tartrate	-
D-Galactose	+
D-Sorbitol	+
Sucrose	+
D-Rafinose	-
D-Manose	+
D-Glucose	+
Cellobiose	-
Inolin	-
Froctose	+
Lactose	-
Ribose	-
D-Adnitol	-
Glycerol	+

control of this disease is based on fungicides containing copper (Roberts and Smith, 2002). The strategy of planting clonal cherry material, supposedly resistant to bacterial canker, can become very risky because of the high phenotypic and genetic variation of *P. syringae* isolated from wild cherry trees, which was demonstrated by the diagnostic techniques (De Cuyper and Steenackers, 2003).

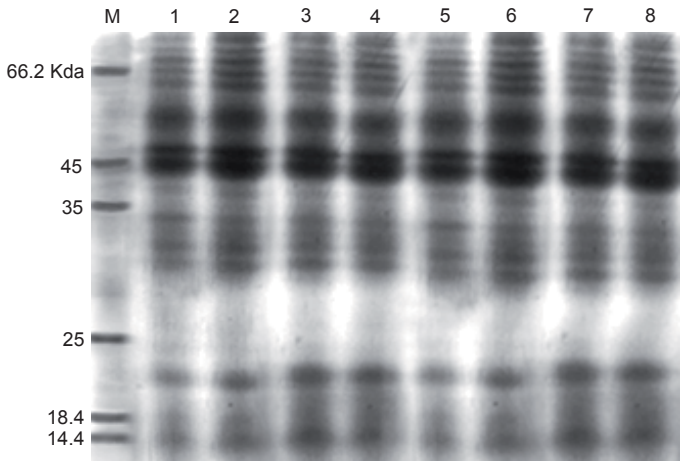

Fig. 1. SDS-PAGE analysis of total soluble proteins of bacterial isolates from stone fruit trees, M, Marquer, lane 1, *Pseudomonas syringae* pv. *syringae* CFBP 2212 (standard strain), lanes 2 to 8 isolates of *P. s.* pv. *syringae* in polyacrylamide gel

Table 2. Antibiotic sensivity (25 µg/disk) of *P. syringae* pv. *syringae* strains used in this study

Antibiotic	Sensitive (%)	Relatively Sensitivity (%)	Resistant (%)	Range of inhibition zone (mm)
Oleardoomycin	6	6	88	11.5
Phosphomycin	0	0	100	0
Spectinomycin	0	26	74	4
Streptomycin	6	33	61	4.75
Doxycyline	13	40	47	4.1
Cephalexin	13	27	60	6.6
Novobiocine	13	27	60	5.3
Ampicillin	0	0	100	0
Rifamycin	46	64	0	10.3
Kanamycin	6	60	34	10.6
Oxytetracycline	73	27	0	4.3
Chloramphenicol	100	0	0	4

The bacteria overwinter inside the host plants, usually along the edge of cankers that grew the previous season, or in infected but symptomless buds or other host tissue (Roberts and Smith, 2002). Bacteria spread from overwintering sites to grow epiphytically on tree, leaf, flower and weed leaf surfaces. Moist, cool weather favors the spread and growth of bacterial colonies. Rain and wind serve as the primary means of local dispersal. Temperatures below 80 °F, dry weather, and low relative humidity cause a rapid decline in epiphytic populations of the bacteria, which survive the summer inside of host tissues. Temperatures over 95°F may greatly reduce the numbers of bacteria surviving inside the plant tissues (Roberts and Smith, 2002).

Isolates of. *P. s.* pv. *syringae* were used in this study, came from various locations whitin Guilan province and this is first report of *P. s.* pv. *syringae* revealing high incidence on cherry, plum, and peach fruit trees from Iran. The study indicates the importance of investigation on a large population of isolates in different regions of Iran.

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Fungicide soil application efficiency for the control of black scurf (*Rhizoctonia solani*) on three potato cultivars

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Abstract

In the 2004 and 2005 seasons, experiments were carried out at INTA Balcarce, Argentina on potato cultivars Kennebec, Innovator and Shepody, with different susceptibility to *Rhizoctonia solani* Kuehn. A randomized block design with four replications was created. Visual observations during the crop cycle were carried out in order to record the number of healthy and infected plants, with symptomatology of stem canker produced by *R. solani.* After harvest, the potatoes in each block were washed and weighted, the total yield recorded and the marketable and "seed" potatoes were classified into healthy, cracked or malformed tubers. The best results on the reduction of infected plants were obtained with the highest dose of fluazinam (as Frowncide 50SC) and pencycuron (as Monceren 25SC). Commercial and "seed" tuber yields in the Spunta cultivar showed no significant differences among treatments, but with the Shepody cultivar, when the severity of the disease was high (2004) both fungicide treatments surpassed the untreated check. When the severity of the disease was low (2005), all chemical treatments registered higher commercial and "seed" tubers yields than the untreated check. In Kennebec and Spunta cultivars, all the treatments – except the lowest dose of fluazinam – accomplished a higher quantity of healthy tubers and surpassed the untreated check; although only the highest doses showed the highest yield of healthy tubers, showing a positive relationship with respect to the applied dose. Only the highest doses of fluazinam considerably reduced diseased tuber incidence in comparison to the untreated check.

Key words: Potato, black scurf, control, Argentina, fluazinam, pencycuron

Introduction

Rhizoctonia solani can manifest itself during various stages of the potato crop. The young shoots of planted tubers can become infected with the pathogen, which causes their death and thus diminishes the emergence plant stand. Once the plant has emerged, the roots, stolons and stalks can be infected, producing cankers and higher severity can cause death (Hide, 1981; Jeger and Velvis, 1994; Jeger *et al*., 1996). In stalks, the cankers modify normal physiological functions of the plant, inducing lower growth, rolling, and red coloring in apical leaves. If the canker develops to the base of the stalk, the most aggressive symptom of the disease can be observed: inhibition of the movement of nutrients to the tubers, which impedes tuber development and leads to the formation of aerial tubers. The affected plants that complete their cycle produce smaller and malformed tubers due to cracking. In the surface of the tubers, the pathogen develops mycelium, which will later form sclerotes (Scholte, 1992; Schöber and Turkensteen, 1992). These are strongly fastened to the skin and cannot be removed by washing without causing damage. The pathogen also produces intense cracking of variable depth, where high concentration of sclerotes can be observed. If the cracks are deep and numerous, they cause commercial quality loss of the tuber (Simons and Gilligan, 1997).

Usage of seed free from *R. solani* is highly recommended to avoid the introduction of the pathogen as a source of infection in the crop (Carmona *et al*., 2003). Deep plantation in cold and excessively humid or easily flooded soils should be avoided as rapid emergence of the cultivar helps prevent infection. Chemical control is an advisable practice that reduces the effects caused by the disease (Eyherabide and Mantecón, 1981; Mantecón and Eyherabide, 1992). The fungicides can be applied to the seed tuber before planting (highly recommended when a seed with presence of the pathogen is used) and/or to the ground at plantation time (advisable when a seed free from the pathogen is used). Chemical treatment can reduce the disease symptoms by 70% in plants and 55% in tubers (Scholte, 1992). The use of integrated control is necessary to attenuate the incidence of the black scurf of the potato. Infected seed plantation in cold and wet soils, severely reduces the effectiveness of chemical control. The objective of this work was to evaluate the efficacy of fungicides applied on the planting row of cultivars with different behaviour in the face of the disease.

Material and methods

In the 2004 and 2005 seasons, experiments were carried out at INTA Balcarce, Argentina. Kennebec, Spunta and Shepody cultivars with different susceptibility to *R. solani*, were used for the experiment. The cut potato seeds used during test trials were healthy and without any symptoms of the disease. Planting was carried out on the 11/01/04 and 11/30/05 by means of an experimental four-row planting machine and was hand harvested on the 04/21/05 and 06/19/06, respectively. A randomized block design with four repetitions was created. The plots consisted of four 4-m wide rows every 0.8 m. The distance between plants along the rows was 0.2 m. Fungicide was applied, to the ground of the row furrows, after mechanical planting of the cut potato seed. In order to inoculate with the pathogen each planted cut seed and rebuild the row furrows by means of a weeding machine and artificial inoculation was carried out through the superficial distribution of milled oat grains, which were previously sterilized

and colonized by a severely pathogenic strain of *R. solani* (AG3) which causes stem canker and black scurf of potato plants.

During the crop cycle, the trial was sprayed weekly with difenoconazole (as Bogard 25EC) with a dose of 0.25 L ha^{-1} and fluazinam (as Frownside 50SC) with a dose of 0.5 L ha^{-1} to control early and late blight, *Alternaria solani* Jones & Grout and *Phytophthora infestans* De Bary, respectively. Visual observations during the crop cycle were carried out in order to register the number of healthy and infected plants, with symptomatology of stem canker produced by *R. solani.* After harvest, the yield of each block was washed and weighed, recording the total yield, in addition to the marketable and "seed" yield which were later classified into healthy, cracked or malformed tubers. In each case, the two external rows of each block were not taken into account. Data were statistically analyzed and significance between means was tested by LSD (P=0.05).

Results and discussion

During the two years of evaluations, climatic conditions were favorable for the development of both the crop and the disease. Artificially inoculated seeds, cultivar management and frequent watering allowed a significant development of the disease during test trial. In the Kennebec and Shepody cultivars, all chemicals showed a lower percentage of infected plant than untreated check; while with Spunta cultivar, the lowest dose of fluazinam did not reduce the percentage of disease plants (Table 1, 2, 3). The best results on the reduction of infected plants were obtained with the highest dose of fluazinam and the chemical check pencycuron. These treatments surpassed the untreated check, which, during the two years, showed disease average 62.5% with Kennebec; 42.5% with Spunta and 29.5% with Shepody plants at full tuberization. A clear and direct relationship between the dose of usage and reduction of the percentage of diseased plants could be observed. The lowest levels of diseased plants (%) were obtained with highest dose of fluazinam and with pencycuron. When the marketable commercial tubers yields were evaluated (Tables 1, 2, 3) it can be seen that fluazinam surpassed the untreated check with doses from 1000 to 1500 mL ha^{-1} with the Kennebec cultivar. The lower dose of 750 mL ha^{-1} showed similar yield in commercial tubers to the untreated check. Differences between treatments was observed when the yield of "seed" tubers was analyzed. The largest quantity of diseased tubers was in untreated check, which was expected due to stress caused by the disease resulting into a smaller size of tubers.

With regards to commercial and "seed" tuber yield in the Spunta cultivar, there was no significant differences among treatments. However, with the Shepody cultivar, when the severity of the disease was high (2004), pencycuron and fluazinam had higher seed tuber yield than the untreated check at the highest, but not at the lowest evaluated dose. There was no noticeable difference in tuber "seed" yield. When the severity of the disease was low (2005), all chemical treatments registered higher commercial and "seed" tuber yield than the untreated check.

In the Kennebec and Spunta cultivars, all the treatments (except the lowest dose of fluazinam) accomplished higher quantity of healthy tubers than the untreated check and a positive relationship could be observed with respect to the applied dose. Only the highest doses of fluazinam considerably reduced the quantity of diseased tubers in comparison to the untreated check. With the Shepody cultivar, when the severity of the disease was high (2004), only pencycuron and the highest doses of fluazinam had better results than the untreated check, registering a larger number of healthy tubers. However, when the severity of the disease was low (2005), all chemical treatments registered higher commercial and "seed" tubers yields than the untreated check.

Efficacy to control the disease by pencycuron and fluazinam with the highest doses are good because they reduced its incidence and notably increased commercial and "seed" tuber yield than the untreated checks. With excellent climatic conditions for the development of the disease, high disease levels were registered and only the highest doses of fluazinam notably reduced it. When the incidence of the disease was low, generally all chemical treatments reduced it significantly. Chemical treatment efficacy in the control of the disease did not increase due to the use of a cultivar like Shepody, which presented the lowest susceptibility to the disease.

Table 1. Diseased plant percentage, marketable, diseased and healthy tubers registered in Kennebec cultivar

Treatments and dose (mL ha^{-1})	Diseased plants (%)		Yield (kg/plot)				
	45 days	70 days	Healthy tubers	Healthy tubers (%)	Diseased tubers	Marketable tubers	Seed tubers
Season 2004							
Check	32 a*	60 a	12.2 c	62.0 d	10.7 a	16.3 b	6.6 a
Fluazinam 500	20 b	28 b	17.1 b	68.7 cd	7.8 ab	20.4 ab	4.5 ab
Fluazinam 750	15 bc	22 b	19.0 ab	74.5 bc	6.5 bc	22.5 a	3.0 b
Fluazinam 1000	10 cd	10 c	21.6 a	87.1a	3.5 c	21.2 a	3.6 ab
Fluazinam 1500	8 d	8 c	21.5 a	84.2ab	4.4 bc	23.7 a	3.2 b
Pencycuron 2000	6 d	5 c	20.8 a	83.0ab	3.9 c	21.8 a	2.9 b
Season 2005							
Check	25.0 a	65.0 a	14.3 c	62.4 c	8.6 a	15.9 c	7.0 a
Fluazinam 500	12.0 b	32.0 b	17.5 bc	73.2 bc	6.4 ab	19.5 bc	4.4 b
Fluazinam 750	6.0 cd	8.5 cd	21.8ab	82.0 ab	4.8 b	22.0ab	4.6 b
Fluazinam 1000	4.0 cd	4.0 d	25.4a	82.2 a	5.5 b	24.5a	6.4ab
Fluazinam 1500	2.5 d	3.0 d	24.6a	83.1 a	5.0 b	25.0a	4.6 b
Pencycuron 2000	5.0 cd	8.0 cd	23.9a	83.6 a	4.7 b	23.2ab	5.4ab

Within a column, means followed by the same letter are not significantly different (P=0.05).

Table 2. Diseased plant percentage, marketable, diseased and healthy tubers registered in Spunta cultivar

Treatments and dose (mL ha^{-1})	Diseased plants (%)		Yield (kg/plot)				
	45 days	70 days	Healthy tubers	Healthy tubers (%)	Diseased tubers	Marketable tubers	Seed tubers
Season 2004							
Check	29 a*	45 a	14.5 c	65.9 c	7.5 a	17.5 a	3.5 a
Fluazinam 500	25 a	40 a	16.1abc	73.5 bc	5.8 ab	18.1 a	3.7 a
Fluazinam 750	5 b	12 b	19.9ab	81.6 ab	4.5 ab	20.6 a	3.8 a
Fluazinam 1000	4 b	8 bc	20.2a	84.2 ab	3.9 b	21.0 a	3.1 a
Fluazinam 1500	5 b	8 bc	19.9ab	85.8 a	3.4 b	19.5 a	3.8 a
Pencycuron 2000	6 b	6 c	21.0a	87.5 a	3.0 b	20.9 a	3.1 a
Season 2005							
Check	22.0 a	40.0 a	11.5 c	59.0 c	8.0a	14.1 b	5.4 a
Fluazinam 500	5.5 b	9.5 bc	19.1ab	74.4 bc	6.5ab	21.4a	4.2 a
Fluazinam 750	0.0 c	5.5 c	22.2a	82.1 a	4.8 bc	22.6a	4.4 a
Fluazinam 1000	0.0 c	4.0 c	23.1a	87.3 a	3.4 c	21.5a	5.0 a
Fluazinam 1500	0.5 c	8.5 c	21.4a	79.9 ab	5.4 bc	22.5a	4.3 a
Pencycuron 2000	9.0 b	15.5 b	16.0 b	78.5 ab	5.4 bc	19.2a	4.2 a

* Within a column, means followed by the same letter are not significantly different (P=0.05).

Table 3. Diseased plant percentage, marketable, diseased and healthy tubers registered in Shepody cultivar

Treatments and dose (mL ha^{-1})	Diseased plants (%)		Yield (kg/plot)				
	45 days	70 days	Healthy tubers	Healthy tubers (%)	Diseased tubers	Marketable tubers	Seed tubers
Season 2004							
Check	32 a*	35 a	10.8 c	55.7 c	8.6 a	14.0 b	5.4 a
Fluazinam 500	22 b	28 ab	13.5 bc	62.8 bc	8.0 a	15.4 ab	6.1 a
Fluazinam 750	22 b	25 b	14.0 b	65.1 bc	7.5 ab	16.0 ab	5.5 a
Fluazinam 1000	12 c	14 c	17.1ab	79.5 ab	4.4 b	15.7 ab	5.8 a
Fluazinam 1500	10 c	10 c	19.1a	82.0 a	4.2 b	18.2 a	5.1 a
Pencycuron 2000	10 c	12 c	18.9a	82.5 a	4.0 b	18.0 a	4.9 a
Season 2005							
Check	10.5 a	24.0 a	14.4 b	70.4 b	8.6 a	13.0 b	7.5 a
Fluazinam 500	2.5 b	11.0 b	19.4 a	85.0 a	3.4 b	18.6a	4.2 b
Fluazinam 750	0.0 b	2.5 c	21.1 a	89.5 a	2.5 b	18.4a	3.6 b
Fluazinam 1000	0.0 b	3.5 c	20.0 a	91.0 a	2.0 b	18.6a	3.4 b
Fluazinam 1500	0.0 b	1.5 c	21.6 a	90.5 a	2.3 b	19.9a	4.0 b
Pencycuron 2000	1.5 b	1.5 c	21.3 a	92.0 a	1.9 b	18.8a	4.4 b

* Within a column, means followed by the same letter are not significantly different (P=0.05).

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Influence of ethanol on the longevity and delayed senescence of bougainvillea flower

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Abstract

The study was carried out to investigate the effect of ethanol (ET) at different concentrations on longevity and senescence delay in bougainvillea flowers. The treatments were water (control), 2, 4, 8, 10, 20, 30, 40, 50 and 70% ET. Positive response was found in case of 4, 8 and 10% of ET after a certain period of treatment application. Dry weight was higher in lower concentrations of ethanol and lower in higher concentrations. Flower longevity was 2 days longer in 4, 8 and 10% ET than in water control and other concentrations of ethanol. Petal wilting and abscission occurred 2 days later in 4, 8 and 10% ET than in control. Perianth abscission also appeared 2 days later in 4, 8 and 10% ET than in control. However, petal discoloration (color change) was later in control, 2, 4, 8 and 10% than in 20, 30, 40, 50 and 70% ET. The results showed that flower vase life was significantly affected by ethanol concentrations as well as longevity was longer in 4, 8 and 10% ET than in water control and other concentrations.

Key words: Bougainvillea flower, vase life, senescence, ethanol

Introduction

Bougainvilleas are popular ornamental plants and are used as decorative flowers in most areas with warm climates, including Australia, India, Malaysia, the Mediterranean region, Mexico, South Africa, Taiwan, and Arizona, California, Florida, Hawaii, and southern Texas in the United States. Bougainvilleas are used to decorate fences and arbors with explosion of color in the house corridor, office and play ground. A bougainvillea tree can make guarding the entry or framing a window. Bougainvillea is a great vine for large containers to decorate hot patios and plazas. Bougainvillea is also used to create beautiful flowering bonsai specimens. Bougainvillea flower are dropped having a short vase life. Cameron (1981) reported that bougainvillea bracteoles were attractive at the end of a 6-day observations period but 32.2% dropped in treated with STS (0.5 oz/Gallon) whilst, 100% dropped in the control.

Pun *et al.* (1999) reported that vase life of carnation flowers increased with treatment of 4 and 6% ethanol and cultivars showed variable response to ethanol treatment with regards to vase life increment, and delay in bud opening. They also mentioned that treatment with 4% ethanol inhibited ethylene production as well as sensitivity to ethylene.The effectiveness of ethanol in extending vase life correlated closely with the longevity, ethylene production and sensitivity.

Longevity of vase life is an important factor in consumer preference and considerable research has been carried out on the causes of carnation senescence (Menguc and Usta, 1994; Reid *et al.,* 1980, 1983). Senescence of cut flowers is induced by several factors, *e.g.*, water stress (Sankat and Mujaffar, 1994), carbohydrate depletion (Ketsa, 1989), micro-organisms (Witte and Van Doom, 1991), and ethylene effects (Wu *et al.,* 1991). Climacteric senescence can be prevented, and hence longevity of the flowers increased, with the use of various chemicals (Staby *et al.,* 1993) as a pre-treatment or in the vase solution (Reid *et al.,* 1980), resulting in inhibition of either ethylene biosynthesis or ethylene action or both. Of the available chemicals, silver thiosulphate (STS) is the most effective and widely-used commercial postharvest treatment for carnation cut flowers (Reid *et al.,* 1980). Ethanol has been found to be effective in increasing the vase life of carnation flowers by inhibiting ethylene biosynthesis (Heins and Blakely, 1980; Wu *et al.,* 1992) as well as its action (Wu *et al.,* 1992). The concentration of ethanol effective in increasing vase life of carnation flowers ranges from 2% for an unknown cultivar (Heins and Blakely, 1980) to 8% (Wu *et al.,* 1992).

Podd and Staden (2004) stated that acetaldehyde and ethanol, when applied at low concentration in holding solutions both extended the vase life of cut carnation flowers by inhibiting the action of ACC synthase. Treatment of cut carnation flowers with low concentrations of ethanol increases their vase life significantly (Heins, 1980; Podd and Staden, 1998; Wu *et al.,* 1992). The aims of this project are to develop develop techniques for retaining bougainvillea flower quality (color development, longevity, expansions and delay senescence) by applying different ethanol concentrations.

Materials and methods

Plant material: Three-year-old bougainvillea plants were used in this experiment for collecting flower sample. Bougainvillea flowers (purple) were collected from nursery, University of Malaya campus. The plant was 0.75 m of height and canopy length was 1.0 m. The plant consisted of 4 branches. Flowers were harvested from each branch randomly.

Flower harvesting and measurement: The flowers were harvested on January 18, 2007. Flowers were weighed immediately after harvest and used for setting treatments.

Treatment setting: Treatments were set following Complete Randomized Block design. Each treatment was replicated 4 times. Total 40 flowers of 4 branches were collected for 10 treatments. The treatments were water (control), 2, 4, 8, 10, 20, 30, 40, 50 and 70% ethanol (ET). Flower stems (petiole) were placed individually in distilled water immediately after cutting and sprayed to the petal and perianth with different concentrations of ethanol solutions. The samples were placed at 28 °C of room temperature.

Response characters determination: Response characters were observed. Positive (+) indicates freshness of flower just before wilting. Negative (-) was considered as wilting of flower.

Vase life, petal wilting, scar (color changed) and senescence evaluation: Vase life was observed by counting day. Flower status was observed everyday. Percent petal wilting was calculated by taking the total petal area divided by wilted petal area multiplying by 100. Color changing (petal scar) was determined by visual observation. After wilting phase, petal senescence was evaluated by observing petal abscised position.

Fresh and dry weight measurements: Fresh weight was measured immediately after harvest on 18th January, 2007. Dry weight was measured after all flowers were abscised.

SPAD measurement: SPAD value was measured by SPAD-502, Minolta Co. Japan. The petal was inserted into the meter and SPAD value measured 5 times from different parts of a single petal.

Petal abscission measurement: Flowers were forced with air using a fan. The flowers were kept in front of a table fan for 5 min. Petal abscission was calculated by counting the percentage of petal drop.

Statistical analysis: Mean seperations were done by Duncan`s multiple range test (DMRT).

Results

Response to ethanol was positive (before wilting) from 12 h-6 days after treatment (DAT) and afterwards negative in case of all treatments (Table 1). The highest positive response (6d) was found at 8 and 10% ET treated flower and the lowest (1st day) was found at 70% ET treated flower. In case of water (control), wilting occurrence was observed on 5th day, while it occured at 7 DAT for 8 and 10% ET treated flower (Fig. 1). The wilting occurred from 1 DAT for 50 and 70% ET, 2 DAT for 30 and 40 % ET, 3 DAT for 20 % ET, 6 DAT for 4% ET, 5 DAT for 2 % ET and water (control), 7 DAT for 8 and 10 % ET treated trees (Fig. 1). In case of water control 100% wilting was observed on 7 DAT, while it was found in 9 DAT for 8 and 10% ET treated flower. Percent petal abscission was earlier for water control, 2% ET than 4, 8 and 10% ET (Fig. 2). The petal abscission range was 3-12 days in different concentrations of ethanol.

The abscission order was 70< 50 < 30 < 20 < control and 2 <4, <8 and 10% ET. The similar trend was found for percent perianth abscission (Fig, 3). But the perianth abscission was 1 day later than petal abscission. The 100% perianth abscission was found for 8 and 10% ET on 12th day while, on 11th day for water control, 2, 4 and 20% ET treated flower. Percent petal discoloration was earlier in water, 2, 4 20, 30, 40, 50 and 70% ET than 4, 8 and 10% ET (Fig, 4). The similar increasing (day) trend was found in case of all ET treated flowers. Petal discoloration started 4 DAT for water contol and completed (100%) 11 DAT, whereas, it started 5 DAT and completed (100%) 11 DAT for 8 and 10% ET treated flowers.

Vase life was extended 1 day with 4% ET and 2 days with 8 and 10% ET than control and 2% ET treated flowers (Table 2). The vase life gradually decreased as 6, 5, 4, 2, 1 and 0.5 day following the order of 8 and 10> 4>water control and 2 > 20 > 30 and 40> 50 and 70% ET. Fresh weight (before wilting) was measured and there was no significant difference among all treatments (Table1). Dry weight was measured after abscission. Dry weight significantly reduced in case of all treatment but more significantly reduced at 40, 50 and 70% ET treated flower. Fresh and dry weight ratio was lower at 2, 4, 8 and 10% ET than control, 20, 30, 40, 50 and 70% ET (Table 1). Thirty three per cent petals shed at 4, 8 and 10% ET, 66.6% at water control, 2 and 20% and 100% at 30, 40, 50 and 70% ET.

Initially SPAD value was almost same in different treatments. However, finally it was higher in 8 and 10% ET than all other treatments. Fig. 5 shows the different flower structures and color changes after treatment application at different stages.

Discussion

The results showed that ethanol was effective as ethylene inhibiting component in bougainvillea flower. It was observed that the most effective ethanol concentrations were 8 and 10% ethanol. Results indicated that sensitivity to ethylene developed several

Table 1. Response of bougainvillea flower as affected by different concentrations of ethanol

Treatment	Response										
	12h	1d	2d	3d	4d	5d	6d	7d	8d	9d	10d
Water	+	+	+	+	+	-(W)	-(W)	-(W)	-(A)	-(A)	-(A)
2 % Ethanol	+	+	+	+	+	-(W)	-(W)	-(W)	-(A)	-(A)	-(A)
4 % Ethanol	+	+	+	+	+	+	-(W)	-(W)	-(W)	-(A)	-(A)
8 % Ethanol	+	+	+	+	+	+	+	-(W)	-(W)	-(W)	-(A)
10 % Ethanol	+	+	+	+	+	+	+	-(W)	-(W)	-(W)	-(A)
20 % Ethanol	+	+	+	-(W)	-(W)	-(W)	-(W)	-(A)	-(A)	-(A)	-(A)
30 % Ethanol	+	+	-(W)	-(W)	-(W)	-(W)	-(A)	-(A)	-(A)	-(A)	-(A)
40 % Ethanol	+	+	-(W)	-(W)	-(W)	-(A)	-(A)	-(A)	-(A)	-(A)	-(A)
50 % Ethanol	+	-(W)	-(W)	-(A)	-(A)	-(A)	-(A)	-(A)	-(A)	-(A)	-(A)
70 % Ethanol	+	-(W)	-(A)	-(A)	-(A)	-(A)	-(A)	-(A)	-(A)	-(A)	-(A)

+ : positive (before wilting symptom), -: negative (wilting symptom just appeared), W: wilting, A: abscission, d: day

Table 2. Fresh and dry weight of bougainvillea flower as affected by different concentrations of ethanol

Treatments	Initial fresh weight (g)	Dry weight after senescence (g)	Ratio (FW/DW)	Vase life (Day)	Petal drop (%)	SPAD value)	
						Initial	Final
Water	0.57±0.10a	0.301±0.05b	1.89±0.20a	4.5±0.32c	66.3±5.8b	3.8±0.5a	0.3±0.02b
2 % Ethanol	0.62±0.12a	0.345±0.06c	1.79±0.11a	4.5±0.34c	66.3± 5.6b	3.6±0.4a	0.3±0.02b
4 % Ethanol	0.48±0.09a	0.261±0.05b	1.83±0.13a	5.5±0.33d	33.3±3.8a	3.7±0.3a	0.2±0.01a
8 % Ethanol	0.66±0.08a	0.395±0.05c	1.67±0.12a	6.5±0.45d	33.3±3.3a	3.7±0.3a	0.5±0.02c
10 % Ethanol	0.62±0.11a	0.393±0.05c	1.57±0.15a	6.5±0.38d	33.3±3.3a	8.0±0.3a	0.6±0.02c
20 % Ethanol	0.51±0.06a	0.275±0.04b	1.85±0.16a	2.5±0.27b	66.3± 00b	3.6±0.4a	0.2 ±0.01a
30 % Ethanol	0.60±0.11a	0.310±0.04b	1.93±0.23a	1.5±0.22a	100± 00c	3.8±0.3a	0.2±0.01a
40 % Ethanol	0.67±0.10a	0.181±0.04a	3.70±0.28b	1.5±0.12a	100±00c	3.6±0.4a	0.2±0.01a
50 % Ethanol	0.55±0.08a	0.132±0.03a	4.16±0.37c	0.5±0.04a	100±00c	3.7±0.5a	0.1±0. 005a
70 % Ethanol	0.64±0.09a	0.097±0.02a	6.60±0.51d	0.5±0.04a	100±00c	3.8±0.5a	0.05±0.005a

Mean±SE (n =4). FW: Fresh weight, DW: Dry weight, Means followed by the same letters in column are not significantly different at the 5% level by Duncan`s multiple range test(DMRT).

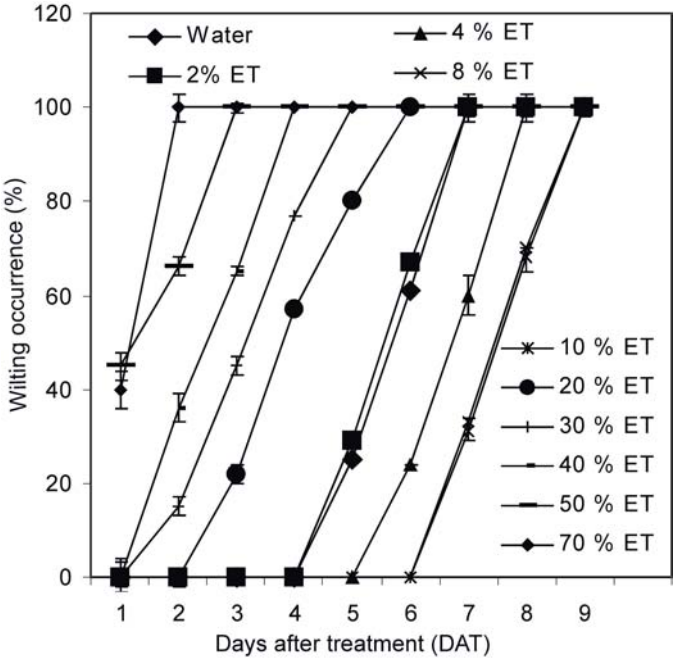

Fig.1. Wilting occurrence at different days after treatment at different ethanol concentrations. Bars represent SE.

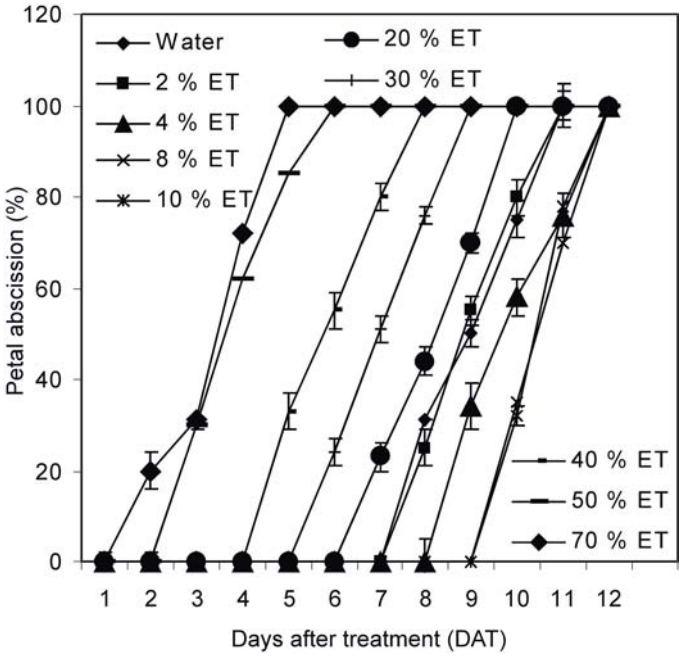

Fig. 2. Petal abscission at different days after treatment at different ethanol concentrations. Bars represent SE.

days after flower opening that ethanol only had a limited ability to delay vase life as well as petal abscission. It was reported that ethylene was the major coordinator of senescence in many flowers (Nickols, 1968). Podd and Staden (2004) stated that ethanol, when applied at low concentration in holding solutions, extended the vase life of cut carnation flowers. They also mentioned that low concentration of ethanol apparently decreased the formation of ethylene by inhibiting the action of ACC synthase. Ethanol has been found to be effective in increasing the vase life of carnation flowers by inhibiting ethylene biosynthesis (Heins and Blakely, 1980) as well as its action (Wu *et al.,* 1992).

In our results we found ethanol most effective in case of 8 and 10%. The concentration of ethanol effective in increasing vase life of carnation flowers ranged from 2% (Heins and Blakely, 1980) to 8% (Wu *et al.,* 1992) for the different cultivars. This variation in response could be due to differences in cultivar sensitivity to ethylene (Mayak and Triosh, 1993; Serrano *et al.,* 1991).

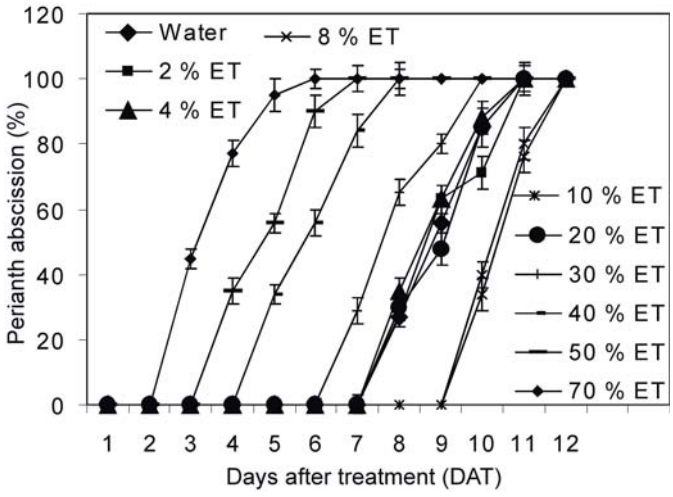

Fig. 3. Perianth abscission at different days after treatment at different ethanol concentrations. Bars represent SE.

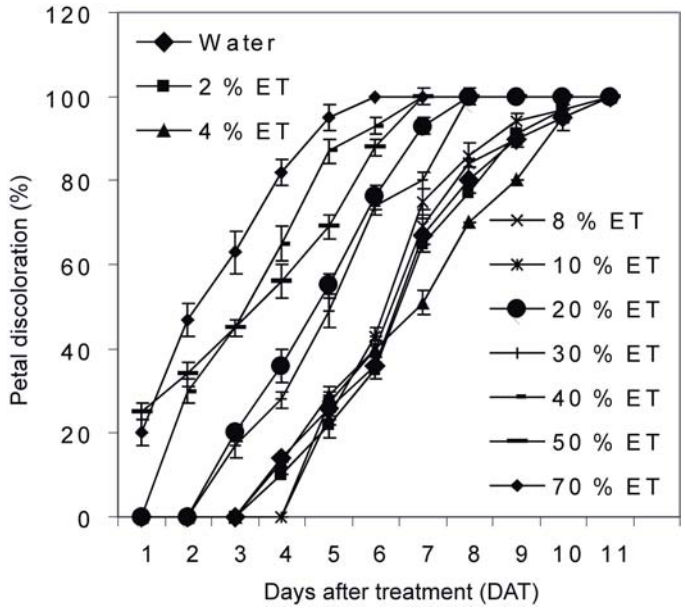

Fig. 4. Petal discoloration followed by days after treatment at different ethanol concentrations. Bars represent SE.

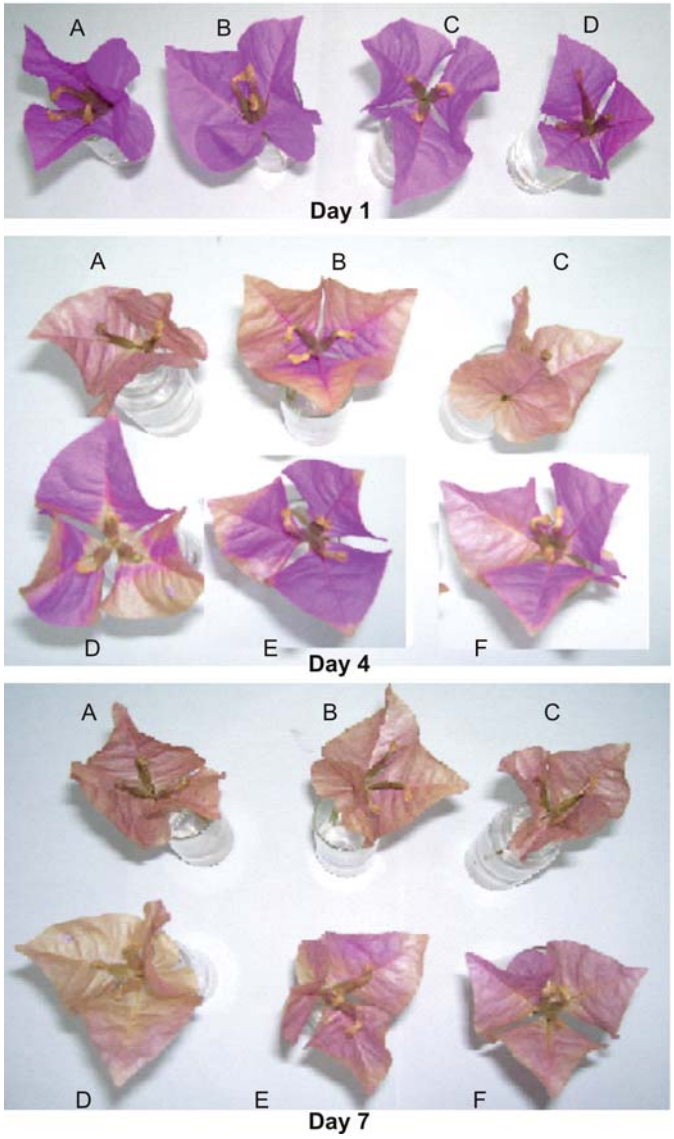

Fig. 5. Flower shape and color after treatment. A: Control, B: 2%ethanol, C: 4% ethanol, D: 8% ethanol, E: 10% ethanol, F: 70% ethanol

Treatment of cut carnation flowers with low concentrations of ethanol increased their vase life significantly (Heins, 1980; Podd and Staden, 1998; Wu *et al.,* 1992). In normally senescing cut carnation flowers, irreversible wilting of the petals and a concurrent swelling and "greening" of the ovaries is well documented (Cook and Staden, 1983, 1986; Nichols, 1968). Concentrations of ethanol (2-8%) has been found effective in extending vase life of cut carnation flowers by several wrokers and in this experiment our result highlighted the similar effect on bougainvillea.

Our results show that it was possible to extend vase life of bougainvillea using 4, 8 and 10% ethanol by causing senescence delay. Lower concentrations of ethanol decreased the formation of ethylene by inhibiting the action of ACC synthase as a result over all flowers (wilting, abscission, scar and color changes) were affected.

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Effect of exogenous putrescine on postharvest life of sweet cherry (*Prunus avium*) fruit, cultivar "Surati-e Hamedan"

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Abstract

The purpose of this study was to investigate the effect of exogenous putrescine on postharvest life and quality of sweet cherry fruit, cultivar "Surati-e Hamedan" at 2˚C. Fruits were treated with 0.5, 1, 2, 3 and 4 mM putrescine as well as distilled water (Control) for 10 minutes, then transferred into the fridge (2˚C). The rate of ethylene production, weight loss, tissue firmness, soluble solids content, titratable acidity and pH of fruits were determined 5, 10, 15, 20 and 25 d after the beginning of storage. Parameters associated with ripening processes, including softening and loss of titratable acidity, significantly decreased by application of putrescine. Soluble solids content of cherries also increased by the putrescine treatment. In addition, cherries treated with higher concentrations of putrescine showed lower rate of ethylene production. Weight loss of the fruits was affected by putrescine in a concentration dependent manner, while putrescine did not affect pH of fruit juice.

Key words: Sweet cherry, *P. avium,* Surati-e-Hamedan, putrescine, postharvest life

Introduction

Sweet cherries (*Prunus avium*) are very perishable commodity. Optimum extension of the postharvest life of fleshy fruits is critically dependent on the reduction and/or retardation in the physiological process of maturation and senescence. An important approach to minimize or eliminate this problem is to apply fruit ripening retardants (*i.e.* polyamines).

Polyamines (PAs) are low molecular weight polycationic organic compounds existing in all living organisms (Liu *et al.*, 2006). The major forms of PAs including diamine putrescine, triamine spermidine and tetraamine spermine are found in every plant cell. Less common PAs (*i.e.* 1,3-diaminopropane and homospermidine) differ from the major forms of these compounds in terms of the number of methylenic moieties between their amine groups. The association of the uncommon PAs with the capacity of some biological systems to grow or function under extreme conditions has provided opportunities for new investigations into their potential functions (Pandey *et al.*, 2000). Polyamines are implicated in various plant growth and developmental processes. These include stimulation of cell division, DNA and protein synthesis, dormancy breaking of tubers and germination of seeds, response to environmental stresses and regulation of rhizogenesis, embryogenesis, senescence, floral development, and fruit ripening (Kakkar and Sawhney, 2002; Tassoni *et al.*, 2003). Polyamines may mediate the action of hormones as a part of their signal response, and are thus suggested as hormonal second-messengers (Kakkar and Sawhney, 2002). Polyamines also serve as precursors for secondary metabolites such as nicotine, and can be conjugated with phenolic acids to produce plant defense-related compounds (Martin-Tanguy, 1997). The biological activity of PAs is attributed to the cationic nature of these molecules. Polyamines occur in plants in free form, bound electrostatically to negatively charged molecules, and conjugated to small molecules (*i.e.* phenolic acids) and proteins (Walters, 2000). Interactions of PAs with phosphate groups of DNA and RNA, anionic components of phospholipids and cell wall components (such as pectic polysaccharides) have been reported (Kakkar and Sawhney, 2002). PAs also bind to the negatively charged phospholipids or other anionic sites on membranes, resulting in altering the stability characteristics of such membranes. PA binding is also known to affect membrane fluidity. Therefore, PAs may indirectly modulate the activities of membrane-associated enzymes (Slocum *et al.*, 1984). Binding of PAs to proteins in *Petunia* protoplast (Mizrahi *et al.*, 1989) also suggests the direct interaction between the PAs and the membranes. PAs protect the damage of DNA by neutralizing charge and/or conformational changes of DNA (Kakkar and Sawhney, 2002).

Polyamines are reported to be effective anti-senescence agents and found to retard chlorophyll loss, membrane deterioration and to increase in RNAse and protease activities, all of which help to slow down the senescence process (Evans and Malmberg, 1989). Exogenously applied polyamines are potent inhibitors of senescence in oat leaf protoplasts and leaves and storage tissues of several plants (Kaur-Sawhney *et al.*, 1982).

Polyamines, especially spermidine and spermine compete with ethylene for a common substrate, S-adenosylmethionine (SAM) and make it plausible to modulate postharvest fruit development. Enormous works have demonstrated that exogenously applied polyamines affect fruit quality, through some change in fruit firmness, weight loss, ethylene evolution, soluble solutions and titratable acids. Spermidine and spermine treatment retards softening of apple (Kramer *et al.*, 1991) and strawberry fruits (Ponappa *et al.*, 1993). Exogenously applied putrescine results in the reduction of mechanical damage and increasing firmness of lemon (Martinez-Romero *et al.*, 1999), apricot (Martinez-Romero *et al.*, 2002) and plum (Perez-Vicente *et al.*, 2002). Application of polyamines has also reduced or delayed browning, peroxide level and ethylene production, coupled with elevated levels of

polyamines in litchi fruits stored at 5°C (Jiang and Chen, 1995). Plum fruits treated with 1 mM putrescine have shown a delay and/or reduction in ethylene production, together with an increase in fruit firmness and a decrease in soluble solutions, titratable acids and weight loss and also a delay in color change, leading to extended storage life (Valero *et al.*, 2002). Apricot fruits treated with putrescine have significantly shown lower weight loss compared with the untreated fruits (Martinez-Romero *et al.*, 2002). In kiwifruit, putrescine treatment reduced or slowed color change, ethylene emission and respiration (Petkou *et al.*, 2004). Similarly, application of putrescine by vacuum infiltration has notably increased lemon fruit firmness and delayed the color change compared with the control (Valero *et al.*, 1998). It is known that, fruit softening is primarily due to the breakdown of the cell wall. The above-mentioned effects of polyamines on fruit texture (firmness and softening) could be ascribed as a part of their properties such as inhibiting enzymes degrading pectic acids (Martinez-Romero *et al.*, 2002) and/or their ability to bind the cell wall and membrane (Ponappa *et al.*, 1993), leading to rigidification of cell wall and stabilization of membrane. Inhibited ethylene production might also account for the enhanced firmness and delayed softening. The reason why polyamines modify soluble solutions and titratable acids has remained unclear. The objective of this study was to investigate the effect of exogenous putrescine treatment on improvement of the characterstics related to the postharvest life of sweet cherry cv. "Surati-e Hamedan".

Materials and methods

Sample preparation: Thirty kg of flawless cherries, cv. "Surati-e Hamedan" were harvested by hand at the commercial maturity stage (as soon as their shine colour turned into pink) from a local orchard in Hamedan, put in the wooden boxes and transported immediately to the laboratory for the experiments. Experiment was conducted in a completely randomized design including 6 treatments (0.5, 1, 2, 3 and 4 mM putrescine and distilled water "control") and three replications. Two kg of fruits was used for each replicate. Putrescine (98%) was purchased from Merck Chemical Co. Fruits were immerged in putrescine solutions (5 L, 20°C) as well as distilled water (control) and left for 10 min, transferred into the baskets for 20 min to dry. Putrescine treated fruits together with controls were then transferred into 2 L plastic containers and kept into the fridge (2°C). Observations were carried out 5, 10, 15, 20 and 25 d after the beginning of storage.

Ethylene determinations: Ethylene production was measured by placing five fruits in a 1 L glass jar tightly fitted with a rubber cap for 1 h. One mL of the holder atmosphere was withdrawn using a gas syringe, and the ethylene was quantified by a Gas Chromatograph (Shimadzu, C-R 4A, Japan) apparatus. Results were expressed in nL of ethylene released per g of fruit tissue per h (nL kg^{-1} h^{-1}). Tissue firmness determination was carried out using a penetrometer (Wagner, Model FDK 32, Italy) apparatus through measurement of the force required for a 3 mm probe to penetrate fruit tissue. Titratable acidity was determined by titration with 0.1 N NaOH up to pH 8.1 and expressed as g of malic acid per 100 g fresh weight. Soluble solids content was determined using a portable refractometer (Atago N1, Japan) at 20°C, and expressed as °Brix. pH of fruit juice was measured using a Jenway 3320 pH meter calibrated by pH 4 and 6.4 buffer solutions. To determine the weight loss, ten fruits for each treatment were weighed at the beginning of the experiment and during storage. Results were expressed as percentage of weight loss toward the initial value.

Statistical analysis: The data was analyzed using MSTATC statistical software and the means were compared by Duncan's Multiple Range Test.

Results and discussion

Fruit tissue firmness: Putrescine application had a significant effect on fruit firmness. Tissue firmness was higher in putrescine-treated cherries as compared with controls. There was also a significant difference between the various concentrations of putrescine in terms of their effects on tissue firmness ($P<0.05$). The highest tissue firmness was observed when 4 mM putrescine applied at all determination times (5, 10, 15, 20 and 25 days after the beginning of storage), while the lowest rate of firmness was related to distilled water treatment (Fig. 1). The inhibitory role of exogenous putrescine on fruit softening has been reported in apples (Kramer *et al.*, 1991), strawberry (Ponappa *et al.*, 1993), tomato (Law *et al.*, 1991), lemon (Valero *et al.*, 1998), peach (Bregoli *et al.*, 2002) and plum (Serrano *et al.*, 2003). The effect of putrescine on increasing fruit firmness and reducing softening could be attributed to cross-linking properties of this compound to pectic substances which result in cell wall rigidification. This binding also blocks the access of degradative enzymes to the cell wall, which reduces the rate of tissue softening during storage (Valero *et al.*, 1998). Putrescine can also inhibit the activity of pectin degrading enzymes such as polygalacturonase through binding to pectic acid (Kramer *et al.*, 1991).

Titratable acidity: Titratable acidity of cherries stored at 2°C decreased during storage time. It was observed that the acidity increased with increasing putrescine concentrations. As seen in Fig. 2, the titratable acidity of cherries in all treatments was significantly different at $P<0.05$. However, titratable acidity was not significantly different at 5, 10 and 25 days of storage for cherries treated with 3 and 4 mM putrescine. Since organic acids are substrates for the enzymatic reactions of respiration, a reduction in the acidity and an increase in pH values are expected. No marked changes in pH value were observed during fruit storage in the present study. Putrescine application reduces the respiration rate (Perez-Vicente *et al.*, 2002) which causes a delay in utilization of organic acids.

Soluble solids content: Soluble solids content of cherries slightly decreased during storage time. However, these changes were significantly lower in putrescine-treated ($P<0.05$) than the control (Fig. 3). At all determination times, the soluble solids content was significantly higher in putrescine-treated cherries than those found in controls, but the difference between cherries treated with various concentrations of putrescine was significant only at 10th and 15th days. Moreover, the higher putrescine concentration significantly increased the soluble solids content of cherries at these two storage times. It is difficult to explain how in the putrescine-treated cherries soluble solids increase but it seems to be due to a reduction in the respiration of the fruits.

Weight loss: Fruit weight loss increased throughout storage period, although increase was significantly higher in control (Fig. 4). The weight loss was lower in putrescine-treated cherries as

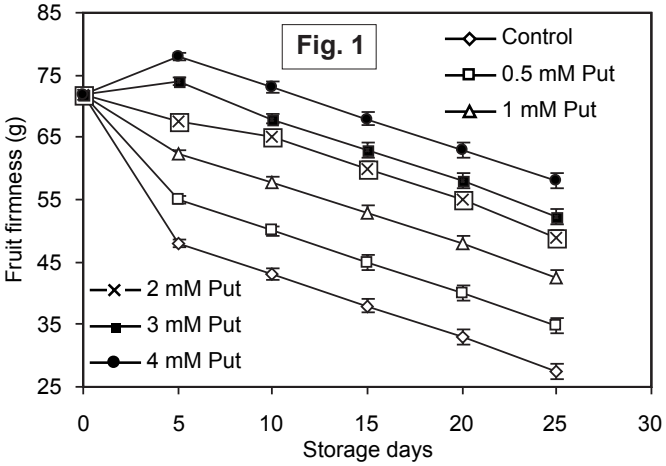

Fig. 1. Changes in fruit firmness of sweet cherry, cv. Surati-e Hamedan during storage at 2˚C.

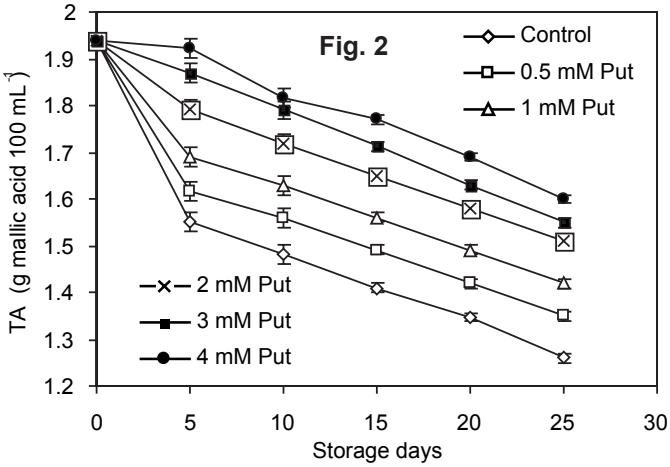

Fig. 2. Changes in titratable acidity of sweet cherry, cv. Surati-e Hamedan during storage at 2˚C.

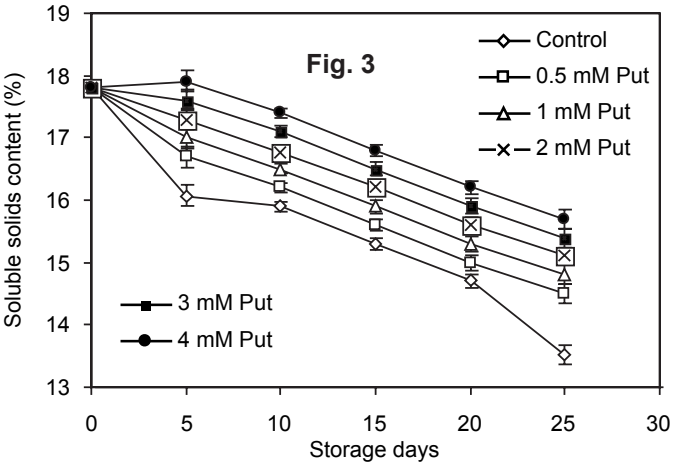

Fig. 3. Changes in soluble solids content of sweet cherry, cv. Surati-e Hamedan during storage at 2˚C.

compared with the controls at all determination times, but the difference between cherries treated with various concentrations of putrescine was significant only at 10th and 15th day. The loss of weight during storage of fruit is caused by water exchange between the internal and external atmosphere, the transpiration rate being accelerated by cellular breakdown. Putrescine treatment might have maintained membrane integrity and delayed the removal of epicuticular waxes which play an important role in water exchange through the skin, as has been reported in mandarin

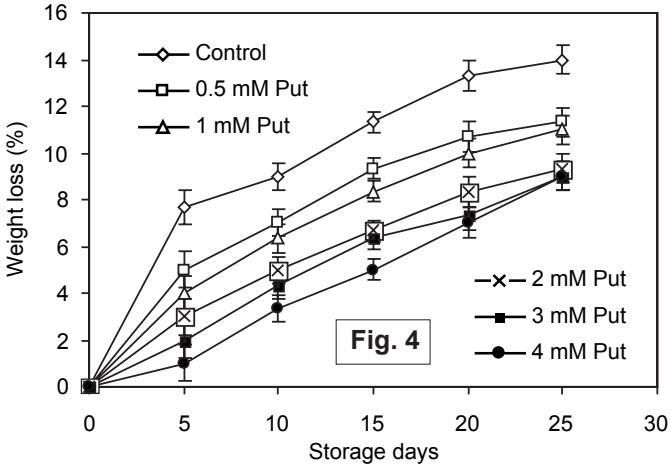

Fig. 4. Changes in weight loss of sweet cherry, cv. Surati-e Hamedan during storage at 2˚C.

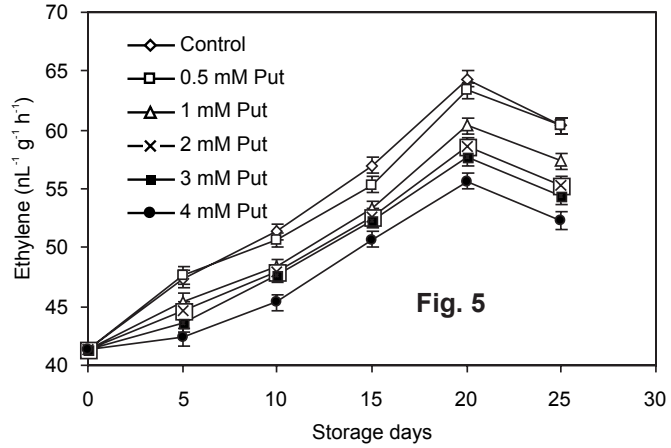

Fig. 5. Changes in ethylene production of sweet cherry, cv. Surati-e Hamedan during storage at 2˚C.

(Schirra and D'Hallewin, 1997), apricot (Martinez-Romero *et al.*, 2002) and plum (Serrano *et al.*, 2003).

Ethylene production: There were no significant difference between 0.5 mM putrescine-treated and untreated (Control) cherries for ethylene production, but the difference between controls and cherries treated with higher concentrations were significant ($P<0.05$). The highest and lowest rates of ethylene production occurred in control and 4 mM putrescine treatment, respectively at all sampling dates (Fig. 5), but there was no difference between various concentrations of putrescine in terms of ethylene production.

pH: The pH of the sweet cherry juice was not significantly altered by various concentrations of putrescine during storage time (data not shown).

By taking into account the parameters related to fruit quality including firmness, soluble solids content, titratable acidity, ethylene production and weight loss as well as the visual appearance of the fruits, the estimated postharvest life of the control cherries was 17 days. The postharvest life increased up to 19, 21, 23, 27 and 28 days for cherries treated with 0.5, 1, 2, 3 and 4 mM putrescine, respectively.

In conclusion, the exogenous application of putrescine is an effective method to prolong storability and increase shelf life of sweet cherry during storage at 2˚C.

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Relationship of arbuscular mycorrhizal fungi and *Azotobacter* with plant growth, fruit yield, soil and leaf nutrient status of mango orchards in north-western Himalayan region of India

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Abstract

The present investigation was undertaken with the objective to find out the nutritional status of mango orchards cv. Dashehari located in north- western Himalayan region of India and to establish the relationship of soil microflora especially, arbuscular mycorrhizal (AM) fungi and *Azotobacter* with growth, fruit yield, and soil and leaf nutrient contents. The study revealed that the correlation between AM spore population and shoot extension growth, leaf area, fruit yield, available Cu and Zn content and leaf N, P, Cu, Zn and Mn contents was found to be positive and significant, whereas, the relationship with soil as well as leaf K content was negative but significant. *Azotobacter* count was positively and significantly correlated with fruit yield, soil organic carbon (OC) and leaf Fe content, while, it was negative and significant with leaf K content. The relationship of per cent root colonization with soil OC and available N content of orchard soil was found to be positive and significant, and with shoot extension growth, leaf area, fruit yield, electrical conductivity, available P, K, Cu, Zn and Mn content and P, K and Cu contents of leaf, it was negative but non-significant.

Key words: AM fungi, *Azotobacter,* root colonization, correlation, mango.

Introduction

Arbuscular mycorrhizal (AM) fungi form mutualistic symbiosis in roots of several horticultural crops (Nemec and Vu, 1990). AM fungi promote growth of host plants by enhancing minerals, mainly P and water uptake (Marschner and Dell, 1994). Mycorrhizal fungi can absorb, accumulate and transport a large quantity of phosphates within their hyphae and release it to cells of the root tissues. It has been shown that mycorrhizal plants can absorb and accumulate several times more phosphate from the soil solution than non-mycorrhizal plants (Smith and Dowd, 1981). These plants also accumulate P, K, Ca, Cu and Mn in the leaf in higher concentration than non-mycorrhizal plants (Nopamornbodi *et al.*, 1987). Besides this, AM fungi also improve growth of horticultural plants in part, by enhancing the acquisition of mineral nutrients especially, P, Cu and Zn (Morin *et al.*, 1994). AM inoculation has been reported to improve growth, dry matter production and concentrations of Ca and Fe in the leaves (Skinner *et al.*, 1988). The increase in uptake of nutrients by mycorrhizal plants attributed to solubilization of the elements by increased root surface resulted by hyphal strands in soil regions inaccessible by the root hairs.

Azotobacter, a non-symbiotic, free-living, aerobic nitrogen fixing diazotroph, is known to add nitrogen to the soil through biological nitrogen fixation. It also results in production of plant growth regulators *viz.*, indole acetic acid and gibberellins, enhances the uptake of NO_3, NH_4, H_2PO_4 and Fe and improves nitrate reductase enzyme activity (Wani, 1990). Dual inoculation of AM fungi and *Azotobacter* is of great significance to fruit crops and is well documented in the literature. Therefore, the objective of the present study was to establish the relationship of AM fungi and *Azotobacter* with plant growth, fruit yield and nutritional status of mango orchards of north- western Himalayan region of India particularly, Himachal Pradesh.

Materials and methods

Ten full bearing mango orchards of cultivar Dashehari at each of the nineteen locations namely. Dadd, Gehrwin, Kalol, Jukhala, Jarol, Kotlu, Berthin, Bari, Nihari, Dashlera, Railli, Talai and Dadhol of Bilaspur district, Bijjar, Railli- Jajjri of Hamirpur district and Nagrota, Nagni, Utreh and Jachh of Kangra district of Himachal Pradesh were selected for the studies during 2002-2004. A comprehensive soil sampling for AM spore population and *Azotobacter* count in the rhizosphere was conducted. Ten samples from each orchard were collected. Soil throughout the top 25 cm was taken especially from basin area of the bearing trees. AM spores were isolated from the soil samples by wet sieving and decanting method as suggested by Gerdmann and Nicolson (1963). AM fungal spores were counted with the most probable number (MPN) method, used to enumerate the AM spore count using by 10-fold series of soil dilution (Powell, 1980).

Ten different AM species of the genera *viz.*, *Glomus*, *Gigaspora*, *Acaulospora*, *Scutellospora*, *Entrophospora* and *Sclerocystis* were isolated and characterized. Taxonomic identification of the AM species was done in accordance with the synoptic keys (Schenck and Perez, 1988). Trinocular biological microscope model LEICA DMLB was used to count the spore population and their morphological identification was done with the help of image analysis software system. To determine the extent of root colonization, roots from randomly selected trees were taken. The samples were cleaned and stained fine roots were prepared (Phillips and Hayman, 1970). These samples were assayed for AM fungal colonization using the Gridline Intersect method

(Giovannetti and Mosse, 1980). The serial dilution technique was employed for the isolation of viable *Azotobacter* count on Jenson's medium. Taxonomic identification of *Azotobacter* isolates was done according to Bergey's Manual of Systematic Bacteriology (Tchan, 1984).

Twenty uniform and healthy shoots all over the tree canopy in all directions were randomly selected. The length of each shoot was measured at the beginning and ends of growing season between the points of initiation of new growth to the extremity of the shoot tip and was expressed in centimeters. For measuring leaf area, 50 leaves were randomly sampled from all over the tree canopy and their accumulative area was recorded with the help of Leaf Area Meter model-3100 and was expressed in square centimeters. Fruit yield in kilogram per tree was recorded at the time of harvest.

Soil samples collected from different mango orchards were analyzed for pH, electrical conductivity (EC), organic carbon (OC) and available nutrient contents by using standard methods of estimation.

Leaf samples were also analyzed for total N by Nitrogen Autoanalyzer- Kjeltech Foss Tecator model-2300, for P by Phosphovanadomolybdate method (Jackson, 1973) and for K and micronutrients (Cu, Zn, Fe and Mn) by Atomic Absorption Spectrophotometer.

A correlation analysis was performed for AM spore population, per cent root colonization and *Azotobacter* count with growth parameters, fruit yield and nutritional status of mango orchards according to Snedecor and Cochran (1980).

Results and discussion

Relationship of AM Fungi and* Azotobacter *with growth and nutrients content

Growth and fruit yield: Under different mango orchards surveyed, AM spore population, per cent root colonization and *Azotobacter* count ranged between 1200 and 3850 kg^{-1} soil, 3.2 and 13.9% and 2.4 x 10^6 and 6.3 x 10^6 CFU, respectively (Table 1). The data on the growth parameters and fruit yield indicated that the shoot extension growth, leaf area and fruit yield varied from 7.0-34.0 cm, 39.5-85.4 cm^2 and 135-410 kg $tree^{-1}$, respectively. The highest average shoot extension growth (26.5cm), leaf area (63.7 cm^2) and fruit yield (340 kg $tree^{-1}$) was exhibited by orchards located in Nihari, whereas, it was minimum in Daad with corresponding values of 9.5 cm, 42.8 cm^2 and 160 kg $tree^{-1}$ (Table 1). Linear correlation analysis revealed that AM spore population exhibited positive and significant correlation with leaf area, shoot extension growth and fruit yield with respective r- values of 0.691, 0.825 and 0.779, respectively. Correlation of *Azotobacter* count with yield was positive and significant (r= 0.303), whereas, with leaf area and shoot extension growth, it was positive but non-significant. However, per cent root colonization was negatively but non-significantly correlated with leaf area, shoot extension growth and yield (Table 4).

The positive relationship of AM spores and *Azotobacter* with growth and yield could be attributed to more dry matter and plant biomass production and enhanced nutrient uptake by roots from the soil. Furthermore, the enhancement in shoot extension growth is also attributed to an increased nutrient uptake particularly, phosphorus apart from an increased uptake of micronutrients (Mathews *et al.,* 2003). This resulted in the improvement of photosynthetic rate and changed microbial plant biomass induced in the host. The positive influence of AM fungi however, might be due to growth promotory effect of AM fungi that had increased phosphorus availability and thereby causing higher protein synthesis resulted in more morphological growth (Singh and Singh, 2004). The results of the present studies were also in accordance with those of Rana and Srivastava (1984), who also reported positive and significant relationship of AM spore

Table 1. AM spore population, per cent root colonization, *Azotobacter* count, growth parameters and fruit yield of mango orchards

Locations	AM Spore number (kg^{-1} soil)	Root colonization (%)	*Azotobacter* count (x 10^6 CFU*)	Shoot extension growth (cm)	Leaf area (cm^2)	Fruit yield (kg $tree^{-1}$)
Dadd	1200	7.6	4.7	7-12 (9.5)	41.3-44.3 (42.8)	135-185 (160.0)
Gehrwin	2300	8.4	3.3	14-19 (16.5)	39.5-54.4 (46.9)	165-210 (187.5)
Kalol	3300	3.2	3.9	18-23 (20.5)	53.5-62.3 (57.9)	290-360 (325.0)
Jukhala	3800	8.9	5.0	25-28 (26.5)	51.6-75.8 (63.7)	310-370 (340.0)
Jarol	2500	6.7	4.7	16-21 (18.5)	40.5-51.8 (46.2)	250-340 (295.0)
Kotlu	2300	12.6	3.8	12-18 (15.0)	40.6-48.4 (44.5)	170-270 (220.0)
Berthin	3600	11.7	4.9	19-25 (22.0)	52.1-66.9 (59.5)	300-350 (325.0)
Bari	3050	13.9	6.3	17-22 (19.5)	40.8-64.4 (52.6)	240-310 (275.0)
Nihari	3850	10.6	4.2	20-34 (27.0)	63.3-85.4 (74.4)	350-410 (380.0)
Bijjar	2100	12.4	3.2	9-16 (12.5)	39.8-50.5 (45.2)	150-180 (165.0)
Railli-Jajjri	2200	11.2	2.9	12-15 (13.5)	40.6-53.4 (47.0)	150-200 (175.0)
Talai	3150	9.3	5.2	16-24 (20.0)	45.7-57.9 (51.8)	280-320 (300.0)
Dashlehra	2800	6.8	3.2	13-20 (16.5)	40.1-59.7 (49.9)	180-240 (210.0)
Railli	2500	4.9	2.4	16-19 (17.5)	43.7-62.1 (52.9)	220-310 (265.0)
Dadhol	3200	5.6	2.7	18-23 (20.5)	47.1-69.9 (58.5)	240-360 (300.0)
Nagrota	2700	11.3	2.9	9-16 (12.5)	43.2-58.5 (50.9)	185-290 (237.5)
Nagni	2900	13.8	5.6	20-25 (22.5)	45.3-67.6 (56.5)	165-250 (207.5)
Utreh	3300	10.8	5.2	17-22 (19.5)	46.9-59.9 (53.4)	145-220 (182.5)
Jachh	3000	8.7	4.9	12-20 (16.0)	45.1-52.6 (48.9)	175-250 (212.5)
Mean	2828.9	9.4	4.2	18.1	50.3	250.7

* Colony forming units, Figures in parentheses are the average values

Table 2. Soil chemical characteristics of mango orchards

Locations	pH	EC (dSm^{-1})	OC ($g\ kg^{-1}$)	Macronutrients (kg ha^{-1})			Micronutrients (ppm)			
				N	P	K	Cu	Zn	Fe	Mn
Dadd	6.1-6.8 (6.45)	0.33-0.36 (0.33)	4-6 (5.00)	120-149 (134.5)	17-19 (18.0)	170-189 (179.5)	1.5-2.1 (1.80)	2.5-5.8 (4.15)	19-31 (25.0)	11-19 (15.0)
Gehrwin	5.9-6.2 (6.05)	0.32-0.34 (0.33)	2-3 (2.50)	147-166 (156.5)	18-21 (19.5)	165-180 (172.5)	1.9-2.4 (2.15)	3.1-3.8 (3.45)	23-29 (26.0)	13-18 (15.5)
Kalol	6.2-6.8 (6.50)	0.32-0.34 (0.33)	4-5 (4.50)	152-175 (163.5)	11-13 (12.0)	131-146 (138.5)	4.1-5.3 (4.70)	4.9-5.8 (5.35)	26-39 (32.5)	15-23 (19.0)
Jukhala	6.1-6.7 (6.40)	0.30-0.33 (0.31)	7-8 (7.50)	125-134 (129.5)	9-10 (9.5)	126-156 (141.0)	4.1-5.6 (4.85)	5.8-6.6 (6.20)	42-47 (44.5)	19-28 (23.5)
Jarol	6.0-6.5 (6.25)	0.39-0.41 (0.40)	6-8 (7.00)	139-145 (142.0)	13-16 (14.5)	161-174 (167.0)	2.6-3.2 (2.90)	3.5-4.2 (3.85)	31-48 (39.5)	21-29 (25.0)
Kotlu	6.0-6.5 (6.25)	0.31-0.34 (0.32)	4-6 (5.00)	152-180 (166.0)	12-14 (13.0)	158-178 (168.0)	1.8-2.8 (2.30)	3.3-4.5 (3.90)	35-48 (41.5)	18-31 (24.5)
Berthin	6.1-6.8 (6.45)	0.33-0.37 (0.35)	3.5-5 (4.30)	138-160 (149.0)	8-12 (10.0.)	130-146 (138.0)	3.9-5.5 (4.70)	5.5-6.3 (5.90)	23-34 (28.5)	11-19 (15.0)
Bari	6.1-6.5 (6.30)	0.38-0.43 (0.40)	8-10 (9.00)	169-195 (182.0)	13-16 (14.5)	136-155 (145.5)	2.9-4.1 (3.50)	4.3-4.8 (4.55)	29-41 (35.0)	15-26 (20.5)
Nihari	5.5-6.5 (6.00)	0.34-0.38 (0.36)	5-6 (5.50)	146-149 (147.5)	16-18 (17.0)	116-124 (120.0)	4.3-5.8 (5.05)	6.4-6.8 (6.60)	33-52 (42.5)	21-32 (26.5)
Bijjar	6.2-6.7 (6.45)	0.32-0.34 (0.33)	4-5 (4.50)	143-159 (151.0)	12-15 (18.5)	168-192 (180.0)	1.5-2.3 (1.90)	2.5-5.9 (4.20)	36-46 (41.0)	26-35 (30.5)
Raili-Jajjri	6.1-6.8 (6.45)	0.31-0.33 (0.32)	5-6 (5.50)	152-171 (161.5)	9-13 (11.0)	161-186 (173.5)	1.7-2.6 (2.15)	2.8-3.4 (3.10)	42-58 (50.0)	20-28 (24.0)
Talai	6.4-6.8 (6.60)	0.30-0.34 (0.32)	6-7 (6.50)	143-156 (149.5)	10-14 (12.0)	140-155 (147.5)	2.8-3.3 (3.05)	4.9-5.4 (5.15)	46-62 (54.0)	32-42 (37.0)
Dashlehra	5.2-6.0 (5.60)	0.36-0. 38 (0.37)	3-5 (4.00)	152-166 (159.0)	8-10 (9.0)	146-170 (158.0)	2.9-3.8 (3.35)	4.1-5.9 (5.00)	40-52 (46.0)	25-31 (28.0)
Railli	6.3-6.7 (6.50)	0.39-0.41 (0.40)	3-6 (4.50)	139-149 (144.0)	15-19 (17.0)	152-173 (162.5)	2.8-3.9 (3.35)	4.2-5.1 (4.65)	32-43 (37.5)	23-34 (23.5)
Dadhol	5.9-6.2 (6.05)	0.37-0.41 (0.39)	4-6 (5.00)	134-136 (135.0)	20-23 (21.5)	135-144 (139.5)	3.4-4.5 (3.95)	4.7-6.9 (5.80)	28-39 (33.5)	21-30 (25.5)
Nagrota	5.3-5.8 (5.55)	0.34-0.38 (0.36)	3-6 (4.50)	146-165 (155.5)	12-18 (15.0)	128-139 (133.5)	3.1-4.2 (3.65)	4.1-5.2 (5.55)	20-30 (25.5)	35-48 (41.5)
Nagni	5.9-6.5 (6.20)	0.38-0.42 (0.40)	5-7 (6.00)	121-154 (137.5)	16-21 (13.5)	131-138 (134.5)	2.7-3.9 (3.30)	3.7-4.4 (4.05)	26-35 (30.5)	23-31 (27.0)
Utreh	5.7-6.3 (6.00)	0.36-0.40 (0.38)	5-7 (6.00)	135-160 (147.5)	18-23 (20.5)	123-146 (134.5)	2.9-4.2 (3.55)	3.1-4.6 (3.85)	44-56 (50.0)	28-36 (32.0)
Jachh	6.2-6.8 (6.50)	0.39-0.43 (0.41)	6-9 (7.50)	130-155 (142.5)	14-26 (20.0)	132-141 (136.5)	2.7-3.8 (3.25)	3.7-4.5 (4.10)	48-65 (56.5)	17-26 (21.5)
Mean	6.23	0.36	5.49	150.2	15.1	151.1	4.99	4.70	38.9	25.1

Figures in parentheses are the average values

number with growth and yield of litchi orchards. The positive correlation was observed between mycorrhizal spore population in the rhizosphere soil with leaf area and crop yield (Sharma *et al.,* 2005). *Azotobacter* produced growth regulators like IAA and GA besides nitrogen fixation, favoured the availability of N in the soil and its uptake by the crop reflecting on higher fruit yield (Venkateswarlu and Rao, 1983), and hence positively influenced plant growth and fruit yield (Rao and Das, 1989).

Soil nutrient status: Soil analysis (Table 2) showed that pH in different mango orchards ranged from 5.55 (Nagrota) to 6.60 (Talai). The highest average EC (0.41dSm^{-1}) was recorded in soil samples collected from Jachh, whereas, it was minimum (0.31dS m^{-1}) in Jukhala area. Average soil OC content ranged between 2.5 g kg^{-1} (Gehrwin) and 9.00 g kg^{-1} (Bari). All the orchards have shown an intermediate range of soil OC content. Furthermore, all the orchards have been found in medium range with reference

to the available macro- and micronutrient contents. A perusal of the correlation data indicated that AM spore population had positive and significant correlation with soil Cu (r = 0.809), Zn (r = 0.832) and Mn (r= 0.410), but, it was negative and significant with soil K (r = -0.526). However, the spore population was positively and non-significantly correlated with OC, EC, and available N and Fe content of the orchard soils, while, negatively but non-significantly with soil pH and available P content (Table 5). Negative correlation between AM spores and soil K contents might be due to negative effect of K fertilizers on the development and function of AM fungi.

Correlation between *Azotobacter* count and soil OC content was positive and significant (r = 0.572), whereas, with soil pH, EC, available N, P, K, Cu, Zn, Fe and Mn content of orchard soils was non-significant. This relationship is in agreement with those of

Table 3. Leaf nutrient status of mango orchards

Locations	Macronutrients (%)			Micronutrients (ppm)			
	N	P	K	Cu	Zn	Fe	Mn
Dadd	1.03-1.11 (1.07)	0.07-0.09 (0.08)	1.15-1.29 (1.22)	7-9 (8.0)	13-21 (17.0)	33-44 (38.5)	22-34 (28.0)
Gehrwin	1.08-1.12 (1.10)	0.06-0.10 (0.08)	1.09-1.20 (1.15)	10-14 (12.0)	13-22 (17.5)	38-54 (46.0)	26-35 (30.5)
Kalol	1.20-1.31 (1.26)	0.11-0.15 (0.13)	0.92-1.01 (0.97)	15-20 (17.5)	27-31 (29.0)	45-56 (50.5)	35-44 (39.5)
Jukhala	1.33-1.46 (1.40)	0.13-0.19 (0.16)	0.75-0.88 (0.82)	18-23 (20.5)	25-31 (28.0)	52-63 (57.5)	29-36 (32.5)
Jarol	1.09-1.18 (1.14)	0.09-0.13 (0.11)	1.03-1.13 (1.08)	11-15 (13.0)	18-26 (22.0)	58-74 (66.0)	33-42 (22.5)
Kotlu	1.13-1.38 (1.26)	0.07-0.11 (0.09)	0.99-1.07 (1.03)	10-13 (11.5)	33-40 (36.5)	60-79 (69.5)	31-45 (38.0)
Berthin	1.24-1.40 (1.32)	0.11-0.18 (0.15)	0.88-0.95 (0.92)	17-22 (19.5)	24-29 (26.5)	52-66 (59.0)	28-38 (33.0)
Bari	1.31-1.44 (1.38)	0.10-0.14 (0.12)	1.01-1.12 (1.07)	15-17 (16.0)	21-29 (25.0)	59-74 (66.5)	29-37 (33.0)
Nihari	1.31-1.49 (1.40)	0.14-0.21 (0.17)	0.56-0.65 (0.61)	19-25 (22.0)	29-35 (32.0)	62-82 (72.0)	37-46 (41.5)
Bijjar	1.02-1.19 (1.11)	0.06-0.10 (0.08)	1.09-1.23 (1.16)	8-11 (9.5)	10-17 (13.5)	42-58 (50.0)	39-48 (43.5)
Railli-Jajjri	1.11-1.19 (1.15)	0.08-0.11 (0.10)	1.09-1.20 (1.15)	10-14 (12.0)	15-23 (19.0)	38-56 (47.0)	32-41 (36.5)
Talai	1.18-1.33 (1.26)	0.08-0.14 (0.11)	0.90-1.10 (1.00)	14-17 (15.5)	25-29 (27.0)	44-62 (53.0)	42-54 (48.0)
Dashlehra	1.21-1.36 (1.29)	0.09-0.13 (0.11)	1.03-1.14 (1.09)	12-16 (14.0)	19-27 (23.0)	40-52 (46.0)	34-43 (38.5)
Railli	1.29-1.40 (1.35)	0.11-0.15 (0.13)	1.02-1.18 (1.10)	12-15 (13.5)	20-25 (22.5)	46-59 (52.5)	31-40 (35.5)
Dadhol	1.18-1.31 (1.25)	0.09-0.13 (0.11)	0.90-0.99 (0.95)	16-19 (17.5)	22-25 (23.5)	35-48 (41.5)	33-38 (35.5)
Nagrota	1.14-1.18 (1.16)	0.07-0.13 (0.10)	0.95-1.04 (1.00)	14-18 (16.0)	18-23 (20.5)	31-45 (38.0)	42-56 (49.0)
Nagni	1.19-1.22 (1.21)	0.12-0.15 (0.14)	0.99-1.12 (1.06)	15-21 (18.0)	19-26 (22.5)	38-46 (42.0)	38-46 (42.0)
Utreh	1.12-1.21 (1.17)	0.09-0.13 (0.11)	0.88-0.97 (0.93)	16-19 (17.5)	20-24 (22.0)	45-56 (50.5)	36-47 (41.5)
Jachh	1.16-1.24 (1.20)	0.10-0.14 (0.12)	0.94-1.08 (1.02)	15-20 (17.5)	19-23 (21.0)	49-55 (52.0)	29-38 (33.5)
Mean	1.24	0.12	1.02	15.3	23.6	52.5	39.9

Figures in parentheses are the average values

Table 4. Correlation (r- values) of AM spore number, per cent root colonization and *Azotobacter* count with growth parameters of mango orchards

Parameters	Shoot extension growth	Leaf area	Fruit yield
AM spore number	0.825**	0.691**	0.779**
Root colonization	-0.102	-0.021	-0.137
Azotobacter count	0.222	0.162	0.303*

*, ** Significant at *P*=0.05 and *P*=0.01, respectively

Tiwary *et al.* (1999), who reported positive relationship between *Azotobacter* population and soil N, Cu and Zn content. Per cent root colonization showed positive and significant relationship with OC (r = 0.306), and available N (r = 0.344), however, this relationship was non-significant with EC, available P, K, Cu, Zn and Mn content of orchard soils. Most of the biological species/strains of mycorrhizal fungi and *Azotobacter* are soil and agro-climatic specific. This limits their widespread and foolproof use with expected performance, which in turn favoured the establishment of arbuscular- mycorrhizae and *Azotobacter* symbiosis.

Leaf nutrient status: The leaf N, P, K, Cu, Zn, Fe and Mn content of mango orchards of Himachal Pradesh varied between 1.07-1.40%, 0.08- 0.17%, 0.61- 1.22%, 8.0- 22.0 ppm, 13.5- 36.5 ppm, 38.5- 72.0 ppm, 22.5- 48.0 ppm per cent, respectively (Table 3). The relationship with soil microflora revealed that the AM spore population had a positive and significant correlation with leaf N, P, Cu and Zn contents with respective r- values of 0.635, 0.651, 0.802 and 0.571, respectively, but negative and significant with leaf K content (r = -0.310) (Table 6). Mycorrhizae can absorb several times more phosphates from soil than the non-infected roots (Gianinazzi *et al.,* 1981). This greater phosphates absorption by AM fungi could be because of its superior efficiency of uptake from liable forms of soil phosphate.

The positive relationship between AM fungi and Cu as well as Zn content of leaf attributed to increased root colonization, which increased the surface area for nutrient absorption. AM fungi enhanced the uptake of slowly immobile nutrients from the soil especially, Cu, Zn, Fe and Mn contents. The application of AM fungi to rhizosphere converted slowly immobile nutrients to the available forms so that these become easily available to plants (Sharma and Bhutani, 2000).

Azotobacter count was positively and significantly correlated with leaf Fe (r= 0.371) and non-significantly related with N, P, Cu and Zn content of leaf, while, negatively but significantly correlated with leaf K (r = -0.314). The positive influence with nitrogen might be due to its enhanced availability in the rhizosphere resulting in better uptake. These results are also in accordance with Rao and Das (1989), who attributed high leaf N to more dry matter production by *Azotobacter chroococcum* in ber and pomegranate. Higher status of N and organic carbon has shown positive relationship with microbial population and root colonization (Sharma *et al.*, 2005). Furthermore, the secretion of IAA and GAs by *Azotobacter* in rhizosphere might have lowered pH of soil and thereby enhanced uptake of nutrients. The relationship between root colonization and N, Zn, Fe, Mn P, K and Cu content of leaf was non-significant.

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Table 5. Correlation (r- values) of AM spore number, per cent root colonization and *Azotobacter* count with soil chemical characteristics of mango orchards

Parameters	pH	OC	EC	N	P	K	Cu	Zn	Fe	Mn
AM spore number	-0.188	0.206	0.037	0.116	-0.237	-0.526**	0.809**	0.832**	0.063	0.410**
Root colonization	0.092	0.306*	-0.224	0.344*	-0.216	-0.047	-0.201	-0.123	0.153	-0.294
Azotobacter count	0.172	0.572**	0.141	0.045	-0.227	-0.136	0.227	0.232	0.027	0.174

*, ** Significant at *P*=0.05 and *P*=0.01, respectively

Table 6. Correlation (r- values) of AM spore number, per cent root colonization and *Azotobacter* count with leaf nutrient contents of mango orchards

Parameters	N	P	K	Cu	Zn	Fe	Mn
AM spore number	0.635**	0.651**	-0.310*	0.802**	0.571**	-0.222	0.099
Root colonization	0.058	-0.041	-0.010	-0.057	0.063	0.291	0.276
Azotobacter count	0.215	0.218	-0.314*	0.194	0.256	0.371*	-0.221

*, ** Significant at *P*=0.05 and *P*=0.01, respectively

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In vitro and *ex vitro* seed-based propagation methods of *Echinops kebericho* Mesfin: A threatened medicinal plant

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Abstract

Effects of seed sterilization, storage time, and temperature as well as extent of seeding survival and establishment under glasshouse versus nursery conditions were studied for *E. kebericho*. Seeds sterilized for 9 and 5 minutes in 70 % ethanol and in 10% sodium hypochlorite, respectively, germinated best (95.2 ± 1.2%) on Murashige and Skoog medium, supplemented with 10 g L^{-1} phytoagar. Further increases or decreases in sterilization time decreased germination percentage and increased contamination, respectively. Unsterilized seeds (control) were completely contaminated before the emergence of radicle as a result of fungal growth. Seed germination percentage declined with increasing storage time and dropped from 94.6 ± 0.4 % to 32.2 ± 1.2% in 15 months. 25 °C was an optimal temperature for best germination (94.6 ± 2.4%) of seeds. Seeds sown in pots containing a mixture of sand, nursery soil, and animal manure in a ratio of 0.5: 2.5: 0.5 respectively, germinated significantly ($P< 0.05$) compared to other soil ratios. Increase in sand or animal manure ratios decreased germination, while increase in nursery soil increased percentage and rate of germination. High percentage (96.0 ± 0.5%) germination was obtained with the seeds sown in nursery soil–surface mixed additives compared with the control. Seedlings of nursery bed origin survived best compared to those *in vitro* or pot origin seedlings. Ultimately, seedlings growth with vigorous and orthotropic developmental pattern was obtained under nursery conditions, compared to those in the glasshouse, which showed stunted and plagiotropic developmental pattern. The study found that seeds stored for less than 5 months, and at 25 °C, were the most suitable for *in vitro* and *ex vitro* propagation of *E. kebericho*.

Key words: *Echinops kebericho*, Kebericho (globe thistle), rootstock, seed-based propagation, Asteraceae

Introduction

Echinops kebericho Mesfin, commonly known as globe thistle belongs to the family Asteraceae. The genus *Echinops* comprises 120 species, of which 12 are known to occur in Ethiopia. The 12 species that occur in Ethiopia are confined to the highlands of the country between 7° 30′ N and 38° 45′ E and at altitudes between 1700 and 2900 m.a.s. (Tadesse and Abegaz, 1990; Erko, 2006). It grows in dry and stony lateritic soils. *E. kebericho* is variable in growth habit and in dissection of the leaf blade. Thus, populations from dry, stony lateritic soils are perennial herbs whereas those growing in deep vertisols are low shrubs. However, Tadesse and Abegaz (1990) noted that the chemical compounds isolated from both populations were identical.

E. kebericho is a threatened medicinal plant with an enlarged rootsystem, used as a source of medicaments (Hymete and Afifi, 1997; Wolde and Gebre-Mariam, 2002). Ethno-botanical surveys have documented that *E. kebericho* has long been traditionally employed by the local healers to prepare medicines against migraine, mental illness, heart pain, lung TB, leprosy, kidney disease, malaria, billharzia, syphilis and amoebic dysentery (Abebe and Ahadu, 1993; Desta, 1993; Abera, 2003). Extracts such as sequiterpene lactones isolated from the rootstock have shown antitumor, antimulagenic and cytotoxic effects. The bioactive extract of *E. kebericho* has been shown to have antimicrobial effects equal to or better than penicillin, especially against *Stereptococcus beta-haemolyticus, Escherchia coli, Klebsiela aerogenes* (Debela, 2002; Desta, 1993). Alkaloid extract of the roots of *E. kebericho* has been shown to have a very strong lethal activity against earthworm (Hymete and Kidane, 1991; Hymete and Afiffi, 1997; Erko, 2006). An enlarged roots of *E. kebericho* increases soil fertility, and also reduces soil erosion, especially when growing on mountainous slopes.

Kloos *et al.* (1978) have indicated that *E. kebericho* is one of the ten medicinal plants sold in all the 19 markets surveyed, including the capital city, Addis Ababa. However, despite its high health value, few studies have been undertaken for multiplication to conserve this threatened medicinal plant.

Although *Echinops* have no hard seed coat, which restricts water uptake and gaseous exchange, these species have pubscent hairs. These pubscent hairs are the pathway for the entrance of contaminant agents into seeds and tissues, which inhibits *in vitro* germination of *E. kebericho* seeds. Furthermore, length of seed storage time and temperature affect germination (Negash, 2004). However, also to what extent seed storage time and temperature affect germination of *E. kebericho* seeds are not yet known. In addition, since the natural regeneration of *E. kebericho* is restricted to a specific microclimate the domestication of this threatened medicinal plant species is still not easy. Thus, seed-based propagation method of *E. kebericho* provides the possibility to domesticate the population for conservation and sustainable usage. *In vitro* seed culture would constitute a basis for tissue culture development and approaches to genetic improvement.

The objectives of this study were (1) to evaluate seed pretreatments, and develop *in vitro* seed-based propagation; and (2) to examine appropriate *ex vitro* germination media, and consequently seedling establishment under glasshouse or nursery conditions.

Materials and methods

Plant material: Mature *E. kebericho* fruits were collected from two population sites: (1) from naturally growing population found in Tulu Baja peasant association, Gedo district, West Shoa zone, Oromia, Southwest Ethiopia (160 km west of Addis Ababa), during October 2005; and (2) from the medicinal plant garden, located within the campus of Ethiopia Health and Nutrition Research Institute (EHNRI), Addis Ababa, during August, 2005. The fruits were removed by hand from the flower head and stored at room temperature (ca 22 ºC). *In vitro* germination experiment was conducted at the Institute of Plant Genetics and Crop Plant Research (IPK), Gateresleben, Germany, between September 2005 and February 2006. Glasshouse and nursery experiments were conducted at the Faculty of Science (Addis Ababa University) (between September 2003 and August 2005) and at Jimma Agricultural College and Veterinary Medicine (Jimma University), (March to September 2006). The effect of storage time on germination was studied 1, 3, 5, 10, and 15 months after collection whereas the effect of temperature was evaluated with seeds stored at 15, 20, 25, 30 and 35 ºC.

***In vitro* seed germination**: Seeds of *E. kebericho* were released from the thin layers of seed coat by using a scalpel under laminar flow hood. Scarification was carried out opposite to the helium in order to avoid injury of the embryo tissue of the seed as suggested by Negash (1992). Seeds were surface sterilized in 70% ethanol for 5, 7, 9, 11, 13, 15 min, then in 10% sodium hypochlorite solution for 3, 4, 5, 6, 7, 8, 9 minutes and rinsed several times using double distilled water under a laminar flow hood. The germination medium consisted of MS minerals and vitamins (Murashige and Skoog, 1962) supplemented with 6, 8, 10, 12, 14, gL^{-1} phytoagar (Sigma, St Louis MO, USA), whereas no sucrose was added. The different media were stirred; the pH adjusted to 5.75, autoclaved at 121 ºC for 20 minutes, and then cooled down in a water bath to about 50 ºC. The media (25 mL each) were then dispensed in glass baby jars (10 x 6 cm, height, width, respectively) following the method on the germination of Arabidopsis seeds (Kumlehn *et al.*, 2006). Twelve seeds per vessel were cultured with 10 replicates per treatment. Cultures were inocubated at 25 ºC under cool fluorescent (40 $\mu mol\ m^{-2}\ s^{-1}$: 16 photoperiod). Seed germination indicated by at least radicle emergence was recorded at days 5, 10, 15, 20, 25 and 30.

Pot experiments: Seeds (*i.e.* seeds with intact seed coat) were planted in plastic pots (mouth diameter 20 x depth 25 cm) filled with a mixture of sand, red soil, and animal manure in a ratio of (1.5: 1.0: 0.5, 0.5: 2.5: 0.5, 1.5: 0.5: 1.0, 1.0: 1.5: 0.5, respectively), and maintained in a glasshouse. 17 seeds were cultured in each pot with 6 replicates per treatment. Dried grass stalks were laid horizontal on top of the pots for conserving moisture, and the system was watered once a day. The grass cover was removed at the onset of germination to prevent the bending of emerging seedlings (Negash, 2003; 2004). The mean minimum and maximum temperatures of the glasshouse during the study period were 11.8 ± 1.0 ºC (nights) and 28.5 ± 2.0 ºC (days), respectively. The relative humidity (RH) ranged from 61 to 73%, and was maintained roughly throughout the experimental period by sprinkling the floor of the glasshouse with water. RH was measured using a Humidity and Temperature Sensor (Type HP- 100-A., Umweltanalytische Mess-System GmbH, Munic, Germany).

Nursery experiment: A total of 100 m^2 nursery bed was prepared and divided in parallel into 4 similar split plots (each 25 m^2). Three plots were surface mixed with three types of additives (cattle dung, horse dung, and sand). Sowing seeds on a nursery soil without additives was considered as a control. 105 decoated seeds were sown on each plot and the same numbers of intact seeds were used as control. The mean minimum and maximum temperatures of the nursery area during the study period were 10.8 ± 1.0 ºC (nights) and 24.5 ± 2.0 ºC (days), respectively and the relative humidity (RH) ranged from 64 to 75% throughout the experimental period.

Seedling survival and growth: One-month-old *in vitro*, pot (glasshouse) and nurserybed origin germinants were used to examine the survival and growth of the seedlings using five categories (I-V): (I) except nursery (which was only evaluated under nursery condition), each *in vitro* and pot origin germinants were divided into two (II-IV) and studied both under glasshouse and nursery conditions. 100-110 seedlings were used for each treatment and regularly inventoried during the growing period. Inventories were made every second day during the first month after sowing, weekly at the end of the first growing season, and monthly during subsequent growing seasons. Data on the percentage survival and measurement on the growth height were scored per month.

Statistical analysis: Data were analyzed using SPSS. ANOVA, followed by Tukey Honest Significant Difference Test, was used for detecting significant differences among means. Test for ANOVA assumptions (*i.e.*, homogeneity of variance was run using Tukeys' homogeneity test).

Results

Effects of seed sterilization and phytoagar concentration: Both seed sterilization and phytoagar concentration influenced the germination of *E. kebericho* seeds (Table 1). Unsterilized seeds were drastically affected by fungal contamination, producing a whitish mycelium on the surface of the seeds 3 days after seed incubation (control). Seeds sterilized with 70% ethanol for 5 minutes and 10% sodium hypochlorite for 3 minutes showed poor germination (27 ± 1.5%) 15 days after seed sowing. Further, the growth of germinants was limited by the expansion of fungal growth throughout the culture media, thus resulting in the death of the germinants. The best germination was obtained with seeds sterilized for 9 and 5 minutes in 70% ethanol and 10% sodium hypochlorite, respectively. Further increases or decreases in time sterilization decreased germination. The germination of *E. kebericho* seeds was also influenced by the availability of water as was adjusted by the concentration of phytogar. Concentrations of 6 and 14 $g\ L^{-1}$ resulted in poor germination. The best germination was obtained at a concentration of 10 $g\ L^{-1}$ 15 after days of seed incubation. Further increases or decreases in phytoagar concentration decreased germination. All subsequent experiments

Table 1. Effects of sterilization and phytoagar concentration on the germination of *E. Kebericho* seeds on MS medium

NaOCl (10%) (min)	70% Ethanol (min)	Phytoagar concentration	Germination (%) on different days				
			3	6	9	12	15
0	0	-	-	-	-	-	-
3	5	6	-	16 ± 0.9^{a*}	21 ± 0.6^{a*}	23 ± 0.4^{a*}	27 ± 1.2^{a*}
4	7	8	24 ± 0.9^{b*}	34 ± 0.6^{b}	46 ± 0.9^{b}	62 ± 0.6^{b}	75 ± 0.6^{c}
5	9	10	65 ± 1.2^{c}	78 ± 0.6^{d}	82 ± 0.6^{d}	88 ± 0.6^{d}	95 ± 1.2^{e}
6	11	12	43 ± 0.6^{d}	53 ± 0.6^{c}	72 ± 0.6^{c}	79 ± 0.5^{c}	84 ± 0.6^{d}
7	13	14	24 ± 1.8^{b}	33 ± 1.5^{b}	43± 2.2^{b}	47± 1.2^{b}	52 ± 0.9^{b}

* Means with standard deviation within the same column followed by different letters (a-e) are significantly different (P=0.05).

Table 2. Effects of soil ratios (sand, red soil, horse dung) on the germination of *E. kebericho* seeds sown in pots, maintained under glasshouse conditions

Seeding media**	Germination (%)			
	day 7	day 14	day 21	day 28
A	30.2 ± 0.9^{c**}	43.4 ± 1.2^{c**}	62.4 ± 0.6^{c**}	76.7 ± 1.1^{c**}
B	54.3 ± 0.6^{d}	66.5 ± 0.3^{d}	76.1 ± 1.2^{d}	93.4 ± 1.2^{d}
C	13.5 ± 0.9^{a}	23.2 ± 1.2^{a}	28.4 ± 1.5^{a}	34.6 ± 1.2^{a}
D	21.3 ± 0.9^{b}	28.5 ± 0.6^{b}	33.5 ± 1.2^{b}	43.6 ± 0.6^{b}

*Means with standard deviation within the same column followed by different letters (a-e) are significantly different (P= 0.05).
**Seeding media: sand: red soil: horse dung (A. 1.5: 1.0: 0.5, B. 0.5: 2.5: 0.5, C. 1.5: 0.5: 1.0, D. 1.0: 1.5: 0.5).

Table 3. Interactive effects of additives and nursery soil on the germination of *E. kebericho* seeds

Additives**	Germination (%)			
	Day 7	Day 14	Day 21	Day 28
Control	14.3 ± 0.6^{a**}	22.7 ± 1.2^{a**}	33.0 ± 1.5^{a**}	42.6 ± 1.2^{a**}
A	63.6 ± 1.2^{d}	73.6 ± 0.9^{d}	85.0 ± 1.1^{d}	96.0 ± 0.5^{d}
B	40.6 ± 1.2^{c}	51.6 ± 1.7^{c}	63.3 ± 1.4^{c}	73.6± 2.1^{c}
C	32.3 ± 1.2^{b}	41.3 ± 0.9 ^{b}	51.3 ± 0.9^{b}	62.0 ± 0.6^{b}

*Means with standard deviation within the same column followed by different letters (a-e) are significantly different (P=0.05).
**Additives- A. nursery soil, horse dung, B. nursery soil, cattle dong, C. nursery soil, sand. control- without additive.

were therefore performed sterilizing seeds for 9 and 5 minutes with 70% ethanol and 10% sodium hypochlorite, respectively using 10 g L^{-1} phytoagar. Seed germination indicated by at least radicle emergence was recorded at days 3, 6, 9, 12, and 15.

Effects of soil mixture ratios: The germination of *E. kebericho* seeds was tested on different mixture of soil ratio (Table 2). Seeds sown in pots containing a mixture of sand, nursery soil, horse dung soil in a ratio of (1.5: 0.5: 0.5; 1.0: 1.5: 0.5, respectively) showed poor germination. The highest percentage (93 ± 1.2%) germination was obtained in a mixture of sand, nursery soil and horse dung in a ratio of (0.5: 2.5: 0.5, respectively) 28 days after seed sowing. Decreased in germination was observed with an increment of both sand and horse dung. Increased ratio in horse dung not only reduced in germination but also steadily exposed to fungal contamination, and consequently caused the death of seedlings. No significant difference was observed between decoated and intact seeds on percentage and rate of germination of *E. kebericho* seeds.

Effects of additives: A significant (P<0.05) effect of animal manure was observed on the germination response of *E. kebericho* seeds (Table 3), which was influenced by surface-mixed animal manure and sand with a nursery soil (Table 3). Seeds cultivated on a nursery soil without additives (control) showed poor germination. The best germination (96.0 ± 0.5%) was obtained

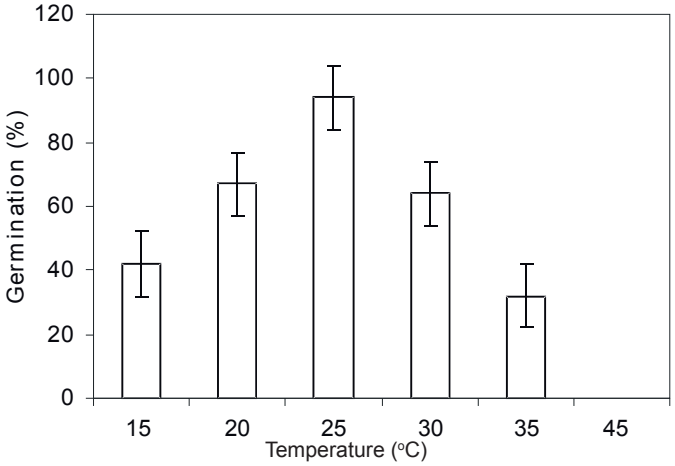

Fig. 1. Effects of temperature on *in vitro* germination of *E. kebericho* seeds. Bar represent ± S.D.

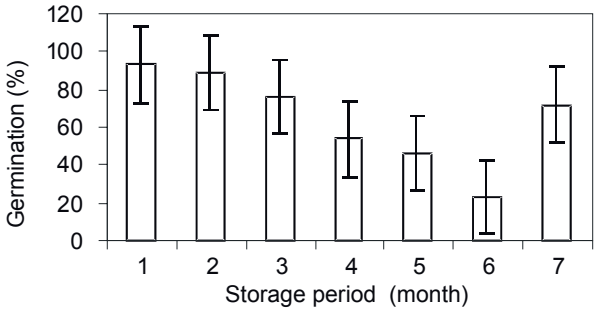

Fig. 2. Effects of storage time on in vitro germination of *E. kebericho* seeds. On the X-axis, 1, 2, 3, 4, 5 refer to the seeds stored for 1, 3, 5, 10 and 15 months while 6, and 7 indicates seeds stored at ca., 22 and 4 oC for 10 months, respectively. Bars represent ± S.D., 100-115 seeds per treatment.

from nursery soil surface-mixed horse dung followed by cattle dung (73.6 ± 2.1%). Seeds sown on a nursery soil surface-mixed sand and control also showed significant (P<0.05) with the least germination (42.6 ± 1.2%). Similar to the pot experiments, there was no significant difference between decoated and intact seeds on percentage and rate of germination of *E. kebericho* seeds.

Effects of temperature on seed germination: Seeds (*i.e.*, propagules without seed coat layers) germinated best (94.6 ± 2.4%) at 25°C, and percentage germination was significantly different (P<0.05) from those at 15, 20, 30 and 35 °C (Fig. 1). *In vitro* germination was least at 15 and 35 °C and was significantly different from all other treatments (P<0.05). There was no significant difference in germination between seeds at 15, 20 and 30 °C.

Effects of storage time: Seed viability declined with storage time (Fig. 2). The difference in seed viability stored at room temperature for 1, 3, 5 months and those stored for 10, 15, 20

Table 4. Percentage of seedling survival and height of seedlings germinated *in vitro*, in pots (glasshouse) and on a nursery soil, and maintained under glasshouse and nursery conditions for a period of five months (March-July 2006)

Seedlings**	Seedling survival (%)	Height of seedlings (cm)				
		30	60	90	120	150
A	24.6 ± 1.5^{a**}	5.0 ± 0.3^{a**}	10.4 ± 0.5^{b**}	13.2 ± 0.6^{ab**}	16.2 ± 0.8^{ab*}	19.8 ± 0^{bb*}
B	31.8 ± 1.7^{b}	4.2 ± 0.4^{a}	7.6 ± 0.2^{a}	10.8 ± 0.3^{a}	13.8 ± 0.5^{a}	15.4 ± 0.5^{a}
C	49.0 ± 2.2^{c}	7.0 ± 0.3^{b}	12.2 ± 0.3^{b}	15.8 ± 0.3^{b}	18.0 ± 0.5^{b}	21.0 ± 1.0^{b}
D	63.4 ± 1.9^{d}	13.0±1.0^{c}	17.2 ± 1.4^{c}	22.2 ± 1.3^{c}	26.2 ± 1.5^{c}	30.8 ± 1.9^{c}
E	93.9 ± 1.1^{e}	23.8 ± 0.8^{d}	34.6 ± 0.5^{d}	44.4 ± 0.8^{d}	55.0 ± 0.7^{d}	66.2 ± 0.5^{d}

**A. in vitro germinated maintained under glasshouse; B. in vitro germinated and maintained under nursery; C. germinated in pots (glasshouse) monitored in the same; D. germinated in pots (glasshouse), maintained under nursery; E. germinated on a nursery, monitored under the same conditions.
*Means within each column followed by different letters (a-d) are significantly different at 0.05% probability.

months was significant (P<0.05). A storage time of 20 months lowered germination capacity close to 0. Mean percentage germination dropped to as low as 42% after storing seeds for 10 months at room temperature. On the other hand, seeds stored for 1, 3, and 5 months (96.4 ± 0.4, 85.2 ± 0.3, 75.4 ± 1.4%, respectively) showed better germination than those stored for more than 10 months.

Seedling survival and growth: Seedlings survival and growth depended on the type of germination media and subsequent growth environment (glasshouse versus nursery) (Table 4). Only 24.6 ± 15 and 31.8 ± 1.7% of *in vitro* origin upon transfer to the glasshouse and nursery, respectively survived 150 days after sowing. A significant difference (P<0.05) in survival was observed between pot origin germinants divided and maintained under glasshouse and nursery conditions. Pot (glasshouse) origin germinants transferred to the nursery showed better survival than germinants evaluated under it's (the same) origin. *In vitro* germinated seedlings maintained under glasshouse and nursery survived less as compared to nursery germinated seedlings, continuously maintained under the same condition. The highest percentage (93.9 ± 1.1%) survival of the seedlings was obtained from nursery origin evaluated under nursery conditions. Similarly, seedlings growth with vigorous and orthotropic developmental pattern (Fig. 3) was recorded under nursery conditions with a maximum growth in height (66.2 ± 0.5 cm) compared to those in the glasshouse, which showed stunted and plagiotropic developmental pattern (Fig. 3).

Discussion

Seeds of *E. kebericho* are covered by overlapped seed coat layers. These seed coat layers contain pubescent hairs. This is generally the case that such overlapped seed coat layers facilitate the incorporation of pathogens, which caused seed contamination during *in vitro* germination. It is well known that many tropical species grow with pubescent hairs, which allows the penetration of pathogens into plant tissues that cause contamination. In this study, unsterilized seeds (control) cultured *in vitro* were completely begun to be contaminated before the emergence of radicle, three days after seed sowing. It is known that the conditions *in vitro* which favour target seed germination (plant growth), *i.e.* high levels of nutrients, humidity and warm temperatures, also favour the growth of micro-organisms which multiply and grow rapidly affecting the germination potential (Bewley and Black, 1994; Khana, 2003). Thus, contaminants affect plant growth /seed germination potential by growing on media, consequently reducing the pH below 3, metabolizing much of the nutrients (carbohydrate), and ultimately producing phytotoxic fermentation products such as ethanol and acetic acid (Bewley and Black, 1994; Khana, 2003). This action starve the carbohydrate of the plant tissue, make certain nutrients unavailable, and ultimately causes toxic effects through the production of secondary metabolites like phenol oxidates (Bewley and Black, 1994; Khana, 2003). However, sterilized seeds of *E. kebericho* with 10% sodium hypochlorite and 70% ethanol for five and nine minutes, respectively highly reduced the growth of fungi, and consequently best germination was achieved (Table 1).

Fig. 3. 8-month-old seedlings under nursery; and 8-month-old seedlings under glasshouse conditions.

Germination percentages and seedlings growth were highly variable for *E. kebericho* sown in pots containing different ratios of soil mixture maintained under glasshouse conditions (Fig. 3). This germination variability is probably due to the (1) different ratios of soil properties, including the modifications in texture and structure; (2) confinement of the root system within a limited space (in pots) which retards the developmental status of the root and shoots as plants grow older; and (3) competition for light as plants grow bigger and start to crowd one another and with other species maintained under the same condition. This is in agreement with study report of Negash (2004) on seed-based propagation of *Prunus africana.*

Uniform seed germination and orthotropic developmental pattern of the seedlings on a nurserybed under nursery condition (Fig. 1A) possibly due to the following major reasons: (1) the fertile nature of the soil of the study area (Kifle, 1997) and surface-mixed animal manure (horse dung) may enhanced the process of germination provided that in the presence of organic compounds in urea of this additive; (2) exposure of seedlings to unlimited light and other environmental conditions (humidity, temperature), may become appropriate resources; (3) with site preparation soil texture and structure may be modified such that pore size is

increased, thus becoming available to bacterial decomposition and mineralization; (4) the increased soil temperature of prepared soil can affect the availability of soil nutrients positively, as decomposition of organic matter is increased by warm soil temperatures and by mixing with mineral soil; and (5) site preparation also modifies aeration by decreasing bulk density. However, sometimes these changes in soil properties can have both beneficial and detrimental effects on seedling morphology (Sutton, 1991; 1993). For example, high nutrient and moisture availability, as well as warmer soil temperatures benefit shoot and root biomass growth (Orlander *et al.*, 1996; Sutton, 1991), whereas high soil bulk density can reduce height growth and root elongation. In addition, high nitrogen promotes branch production and modifies biomass allocation patterns in seedlings. The change of soil texture and structure with site preparation has been reported by various authors in optimizing soil nutrient contents (Hassink, 1997; Meke *et al.*, 2002), aeration (Ritari and Lahde, 1978), temperature (Kubin and Kemppainen, 1994; Fleming *et al.*, 1994), and water (Winsa, 1995) for plant growth.

Decreased germination with increasing temperature (Fig. 1) shows the effects of elevated temperature on the viability of *E. kebericho* seeds. It is reported that, in germinating seeds of other many plant species, high temperature causes greater membrane and embryo damages through lipid peroxidation (Erko, 2006). A number of studies also indicated that different temperature optima for different plant species (Negash, 1992; 2003).

E. kebericho seeds are characterized by a short viability period when stored at room temperature (Fig. 2), thus loosing their capacity for germination relatively quickly. Similar studies also confirm that seeds of other tropical plant species such as *Podocarpus falcatus, Prunus africana* stored at room temperature for a long time gradually lose their germination potential (Negash, 2003; 2004).

In summary, *in vitro* and *ex vitro* germination and plant regeneration protocol was established that allows producing multiple seedlings. The value of such basic protocol for *E. kebericho* is many-fold. Above all, it can be used as a means to preserve germplasm of this endangered species of high medical importance. Furthermore, a rapid and efficient method of plant multiplication is of particular importance in perennial species such *as E. kebericho*.

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