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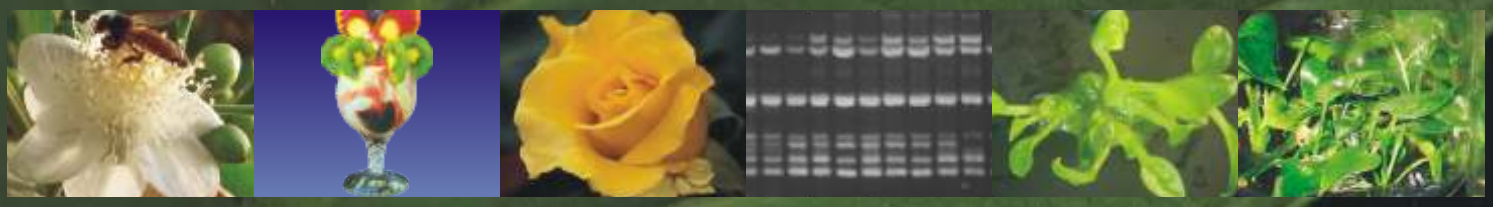

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Reduced ethylene production in transgenic carnations transformed with ACC oxidase cDNA in sense orientation

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Abstract

'Lillipot' carnation, which is usually cultivated as a potted ornamental, was transformed with a cDNA for carnation 1-aminocyclopropane-1-carboxylate (ACC) oxidase. Two lines, which harbor an *sACO* transgene, had a vase life of cut flowers more than twice longer than that of the non-transformed (NT) control. Flowers of the long vase life lines senesced with discoloring and browning in petal margins, which is typical to ethylene-independent senescence in carnation flowers. They produced negligible amount of ethylene for the first 8 day, whereas flowers of the NT control showed a climacteric ethylene production with a maximum on day 3. Transcripts for *DC-ACS1* and *DC-ACO1* were absent in petals of the long vase life flowers undergoing senescence. The present study revealed that transformation with *sACO* transgene may be useful to generate potted carnation plants with a long display time.

Key words: ACC oxidase gene (*DC-ACO1*), *Dianthus caryophyllus*, ethylene biosynthesis, flower senescence, potted carnation

Introduction

Carnations are used as ornamentals as potted plants and as cut flowers. Ethylene is a primary plant hormone involved in the senescence of cut carnation flowers (Reid and Wu, 1992). It is synthesized in a large amount, mostly from the petals, at a later stage of flower senescence (Borochov and Woodson, 1989; Abeles *et al.*, 1992; Reid and Wu, 1992; Woodson *et al.,* 1992). Increased ethylene production accelerates wilting of the petals. Inhibition of the synthesis or action of ethylene delays the onset of senescence and extends the vase life of flowers.

In senescing carnation flowers, ethylene is first produced in the gynoecium, and the ethylene evolved acts on petals and induces autocatalytic ethylene production in the petals. This results in petal wilting (Jones and Woodson, 1997; Shibuya *et al.*, 2000; ten Have and Woltering, 1997). Ethylene is synthesized through the pathway: L-methionine → *S*-adenosyl-L-methionine → ACC → ethylene. ACC synthase and ACC oxidase catalyze the last two reactions (Kende, 1993; Yang and Hoffman, 1984). So far, three genes encoding ACC synthase (*DC-ACS1*, *DC-ACS2* and *DC-ACS3*) and one gene encoding ACC oxidase (*DC-ACO1*) have been identified in carnations (Henskens *et al.*, 1994; Jones and Woodson, 1999; Park *et al.*, 1992; Wang and Woodson, 1991). Out of these genes, *DC-ACS1* and *DC-ACO1* have been shown to play a pivotal role in ethylene production in both the gynoecium and petals of senescing carnation flowers (Nukui *et al.*, 2004; Satoh and Waki, 2006).

Currently, prevention of senescence of carnation flower is being attained by treatment of the flower with chemical preservatives which inhibit the synthesis or action of ethylene (Veen, 1979; Midoh *et al.*, 1996). Another option for preventing senescence is the generation of transgenic flowers with suppressed production or action of ethylene. So far, the lines transformed with cDNAs for carnation 1-aminocyclopropane-1-carboxylate (ACC) oxidase (*DC-ACO1*) and ACC synthase (*DC-ACS1*) in sense or antisense orientation (Savin *et al.*, 1995; Kosugi *et al.*, 2000, 2002; Iwazaki *et al.*, 2004) and a line harboring an *Arabidopsis thaliana etr1-1* allele capable of rendering ethylene insensitivity (Bovy *et al.*, 1999) have been generated. Cut flowers of the transgenic lines have a prolonged vase life compared with those of non-transgenic plants.

The preservatives described above are considered to be not applicable to potted plants, since they are usually administered to cut flowers through vascular transport by immersing the cut stem end in solutions containing the preservative. Therefore, the generation of transgenic plants is a promising way to retard senescence of flowers of potted carnations, *i.e.*, to lengthen their display time. Kinouchi *et al.* (2006) recently generated potted carnation plants transformed with cDNAs for carnation ACC synthase (*DC-ACS1*, *s/aACS* transgenes) or ACC oxidase (*DC-ACO1*, *s/aACO* transgenes) in sense or antisense orientation or mutated carnation ethylene receptor cDNA (*DC-ERS2'*) by *Agrobacterium*-mediated gene transfer. They partly characterized the transformants by investigating the conversion of exogenously-applied ACC to ethylene in leaflet segments. A performance test of the transformants as potted plants remains to be carried out. However, we should first know the synthesis and action of ethylene in flowers in each transformant to select the best line for the large scale performance test. Therefore, in this study, we cultivated several lines of the transformants on soil until flowering, and characterized their senescence, ethylene production and gene expression in cut flowers.

Materials and methods

Plant materials: Plantlets of the transgenic lines of carnation (*Dianthus caryophyllus* L. cv. Lillipot), generated previously (Kinouchi *et al.*, 2006), and the NT control were grown *in vitro* to about 5 cm in height, were transplanted into a commercial horticulture soil in a plastic container, under conditions described previously (Iwazaki *et al.*, 2004) in a containment green house

at Tohoku University. Out of 39 transgenic carnation lines generated previously (Kinouchi *et al.*, 2006), 6 transgenic and the non-transformed (NT) control lines were used since these lines flowered one year after transplanting to soil. Three transgenic lines studied were pMLH-sACO-2, -3 and -12, which were transformed with carnation *DC-ACO1* cDNA in sense orientation (*sACO* transgene) in a pMLH2113 vector. Two other transgenic lines, pIG-sACS-1 and pIG-sACO-1, were transformed with carnation *DC-ACS1* and *DC-ACO1*, respectively, in sense orientation (*sACS* and *sACO* transgenes) using the pIG121 vector. Finally, pIG-DC-ERS2'-2 was transformed with a mutated carnation ethylene receptor cDNA (*DC-ERS2'*) in the pIG121 vector. Flowering started around one year after transplanting. Flowers were harvested during the following 5-6 months. Only the first and second flowers opening on each stem were used.

Analysis of vase life of cut flowers: Three to ten flowers, depending on the line, of each of the transgenic and NT control lines were harvested at the full opening stage (day 0; their outermost petals were at right angles to the stem of flower). Stems were trimmed to 0.5 cm in length, and placed with their cut end in distilled water in 5-ml plastic vials. The flowers were left at 23°C under a 16-h photoperiod using white fluorescent light (20-30 $\mu mol\ m^{-2}\ sec^{-1}$). The water was replaced daily. Senescing flowers were observed and photographed daily to record in-rolling and subsequent wilting of petals, the desiccation, and discoloration of the petal margins. Vase life in days is expressed as the mean ± SE of given numbers of flowers.

Assay of ethylene production: Ethylene production from carnation flowers was monitored daily by enclosing individual flowers in plastic vials in 140-mL glass containers (1 flower per container) for 1 h at 23°C. A 1-mL gas sample was taken with a hypodermic syringe from inside the container through a rubber septum of a sampling port on the container and injected into a gas chromatograph (Shimadzu GC-14A, Kyoto, Japan), equipped with an alumina column and a flame ionization detector to determine ethylene content.

Treatment with exogenous ethylene of flowers of the transgenic lines: Two to five cut flowers each of the respective transgenic lines and the NT control were enclosed in a 60 L glass chamber and exposed to ethylene at 10 $\mu l\ L^{-1}$ for 16 h at 23°C under white fluorescent light (50 $\mu mol\ m^{-2}\ s^{-1}$). After the treatment, the flowers were held in open air for 1 h to let exogenous ethylene diffuse. They were subsequently encapsulated and their ethylene production was determined by gas-chromatography. The petals and gynoecia were immediately excised and prepared for total RNA extraction.

Northern blot analysis: Total RNA was isolated by the SDS-phenol method (Palmiter, 1974) from the petals and with RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) from gynoecium of cut flowers of the respective transgenic lines and the NT control line at given time after the full opening of flowers (day 0). Pistils and petals were detached form one to three flowers, depending on lines, at the given time and combined to make one sample each. Also, total RNA was isolated from the gynoecium and petals of the flowers treated with ethylene as described above. Ten µg of total RNA was denatured, separated on a 1.0% agarose gel, transferred onto nylon membranes (Hybond N^+, Amersham Pharmacia Biotech, Tokyo, Japan) and hybridized with the DNA probes for *DC-ACS1* and *DC-ACO1* transcripts. The DNA probe for *DC-ACS1* transcript was 560 bp which corresponded to the position 1 bp to 560 bp of the coding region of *DC-ACS1* cDNA (GenBank Accession No. M66619), and that for *DC-ACO1* transcript 560 bp corresponded to the position 261 bp to 820 bp of the coding region of *DC-ACO1* cDNA (GenBank Accession No. M62380). The DNA probes were labeled with HRP and hybridized with the blot by using ECL Direct™ (Amersham Pharmacia Biotech) according to the manufacturer's instruction. Hybridization signals were detected by exposure to X-ray film (RX-U, Fuji Photo Film, Tokyo, Japan).

Results

Vase-life and senescence profile of the transgenic flowers: Cut flowers of each of the transgenic lines had vase-life of various length varying from 3.0 ± 0.4 to 7.6 ± 0.4 days, whereas that of the NT control line was 2.8 ± 0.2 days (Table 1). Vase lives of the pMLH-sACO-2 and -12 lines were 7.6 ± 0.4 and 6.3 ± 0.5 days, respectively (significantly different from the NT control, at P=0.001 by t test). Other transgenic lines had vase lives that were not significantly different from that of the NT control, except for the pIG-DC-ERS2'-2 line. We did not investigate further the pIG-DC-ERS2'-2 line.

Flowers of the NT control remained turgid until day 3, showed in-rolling of petals on day 4, and completely wilted thereafter. On contrast, flowers of the pMLH-sACO-2 and -12 lines remained turgid without petal in-rolling until day 6 or more, but eventually began to show desiccation and discoloration in the rim of petals. Petal in-rolling at the onset of wilting is a well-known characteristic of ethylene-dependent senescence of carnation flowers. Desiccation, discoloration, and browning of the rim of petals are characteristics of ethylene-independent senescence of carnation flowers. These findings suggested little or no function of ethylene during the senescence of petals of the pMLH-sACO-2 and -12 flowers. In the following experiments, we characterized ethylene production, expression of genes for ethylene biosythesis and response to exogenous ethylene of flowers of the pMLH-sACO-2 and -12 lines by comparison with those of the NT control.

Ethylene production of the transgenic flowers: Flowers of the NT control showed a climacteric rise in ethylene production, attaining a maximal rate on day 3 (Fig. 1). Flowers of the pMLH-sACO-2 and -12 lines produced very small amounts of ethylene during senescence period of 8 days. Their maximum ethylene production rates were around 10% that of the NT control. The

Table 1. Senescence of flowers of the NT control and transgenic carnations

Lines	Number of flowers tested	Vase life [a] (days)	Senescence pattern[b]
NT control	6	2.8±0.2	W
pMLH-sACO-2	7	7.6±0.4	D
pMLH-sACO-3	6	3.2±0.4	W
pMLH-sACO-12	4	6.3±0.5	D
pIG-aACS-1	10	3.0±0.4	W
pIG-aACO-1	3	3.3±0.9	W
pIG-DC-ERS2'-2	4	4.5±1.0	W

[a] Each value is the mean ± SE.
[b] W, in-rolling and wilting of the petals; D, desiccation, discoloration and necrosis of the petals.

lack of petal in-rolling and prolonged vase-life in flowers of the pMLH-sACO-2 and -12 lines coincided with a marked reduction in ethylene production.

Transcript levels for *DC-ACS1* and *DC-ACO1* in the gynoecium and petals of the transgenic flowers: As described in introduction, *DC-ACS1* and *DC-ACO1* play a pivotal role in ethylene production in both the gynoecium and petals of senescing carnation flowers (Nukui *et al.*, 2004; Satoh and Waki, 2006). We examined the transcript levels for *DC-ACS1* and *DC-ACO1* in the gynoecium and petals of the transgenic flowers undergoing senescence.

On day 0 (at the time of full opening of flowers), *DC-ACS1* and *DC-ACO1* transcripts were absent in both the gynoecium and petals of carnation flowers (Fig. 2). With the NT control flowers, *DC-ACS1* and *DC-ACO1* transcripts accumulated abundantly in the gynoecium on day 2 (the day before petal in-rolling), and significantly in the petals on day 3 when a maximum ethylene production from flowers occurred (Fig. 1). In the pMLH-sACO-2 and -12 lines, tissue sampling was conducted on days 0, 3 and 6. In gynoecia of the pMLH-sACO-2 line, *DC-ACS1* and *DC-ACO1* transcripts accumulated, although to small amounts in the latter, on day 3. These transcripts diminished on day 6. Both transcripts were absent in the petals on days 3 and 6. Similarly, with pMLH-sACO-12 line, *DC-ACS1* and *DC-ACO1* transcripts accumulated abundantly in the gynoecium on day 3 and diminished on day 6, but they were absent in the petals on both days. Absence of *DC-ACS1* and *DC-ACO1* transcripts in the petals of pMLH-sACO-2 and -12 lines coincided with the negligible amount of ethylene production from flowers of these lines (Fig. 1).

Responses to exogenous ethylene of flowers of the transgenic lines: In carnation flowers, the expression of *DC-ACS1* and *DC-ACO1* genes in petals can be induced by exogenously applied ethylene and by ethylene produced endogenously from the gynoecia (Shibuya *et al.*, 2000). The response of the transgenic lines and the NT control to exogenously applied ethylene was investigated after treatment with ethylene at 10 µl L^{-1} for 16 h. Ethylene evolution was determined from flowers at the beginning

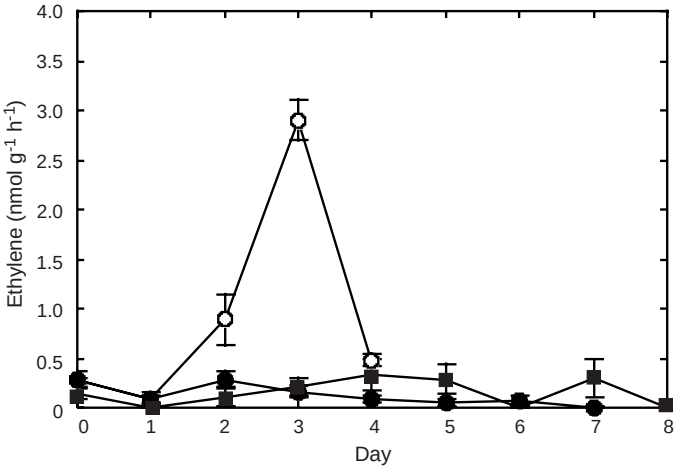

Fig. 1. Ethylene production from cut carnation flowers during the senescence period in the NT control and the transgenic lines. Flowers (numbers shown in Table 1) of three lines were harvested at full opening stage (day 0) and their ethylene production was monitored daily. Data are shown by the mean ± SE.
o, NT control; ●, pMLH-sACO-2; ■, pMLH-sACO-12.

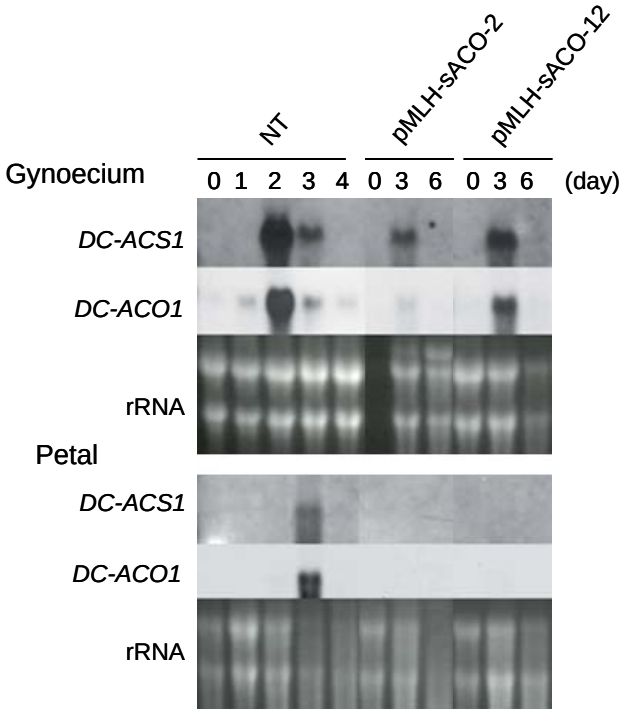

Fig. 2. RNA gel blot analysis of *DC-ACO1* and *DC-ACS1* transcripts in the gynoecium and petals of the NT control and in two transgenic lines during natural senescence. Gynoecium and petals were isolated from cut flowers at given days after full opening of flowers; days 0, 1, 2, 3, and 4 for the NT control, but days 0, 3 and 6 for pMLH-sACO-2 and -12 transgenic lines which did not show wilting. Ten µg of total RNAs isolated from respective flower tissues were separated on an agarose gel and hybridized to DIG-labeled *DC-ACS1* and *DC-ACO1* probes. Equal loading of total RNAs was checked by ribosomal RNAs visualized by ethidium bromide staining of the agarose gel. No data for the gynoecium of pMLH-sACO-2 on day 0.

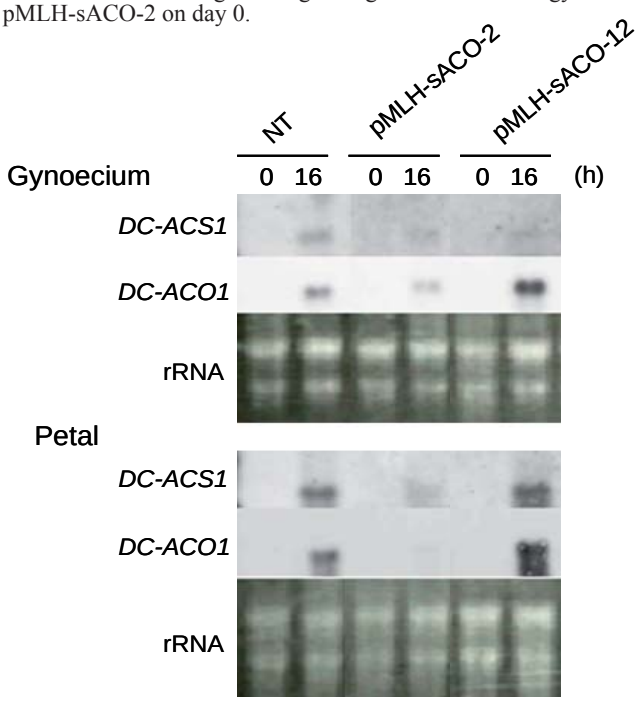

Fig. 3. RNA gel blot analysis of *DC-ACS1* and *DC-ACO1* transcripts in gynoecium and petals of the NT control and two transgenic flowers before and after ethylene treatment. Cut carnation flowers of respective lines were treated with 10 µl L^{-1} ethylene for 16 h. Ethylene production from flowers was measured before and after the ethylene treatment by enclosing them for 1 h and measuring ethylene produced. Numbers of flowers used were 2, 5 and 3 for the NT, pMLH-sACO-2 and -12 in this order. After ethylene assay, the flowers were subjected to analysis of amounts of *DC-ACS1* and *DC-ACO1* transcripts as described in the legend to Fig. 2.

and end of ethylene treatment. We also determined transcript levels for *DC-ACS1* and *DC-ACO1* in the gynoecia and petals. Treatment with exogenous ethylene for 16 h caused petals of the transgenic lines and the NT control to wilt, indicating that flowers of the transgenic lines were responsive to ethylene.

At the beginning of experiment, ethylene production from flowers was not detected or negligible (< 0.2 nmol $g^{-1} h^{-1}$). In flowers of the NT control treated with ethylene for 16 h, ethylene production was 9.38 ± 0.27 nmol $g^{-1} h^{-1}$. Ethylene evolution from the transgenic lines was less than that of the NT control; 1.96 ± 0.54 and 4.46 ± 1.34 nmol $g^{-1} h^{-1}$ for the pMLH-sACO-2 and -12 lines, respectively. Exogenous ethylene treatment caused an accumulation of *DC-ACS1* and *DC-ACO1* transcripts in the gynoecium and petals of the NT control and the transgenic lines, although the level of *DC-ACS1* and *DC-ACO1* transcripts in pMLH-sACO-2 line was lower than that in the NT control (Fig. 3).

Discussion

In this study we used six transgenic lines, which flowered a year after transplanting and cultivation, in soil, out of 39 transgenic lines generated previously (Kinouchi *et al.*, 2006). In the six transgenic lines, two lines transformed with pMLH2113-Hm/*sACO* construct, pMLH-sACO-2 and -12 lines, had a vase life more than twice longer than that of the NT control. The *sACO* transgene in the pMLH2113 vector efficiently suppressed ethylene production, which resulted in longer-lasting flowers.

Flowers of the pMLH-sACO-2 and -12 lines did not show petal in-rolling and wilting which are typical for ethylene-dependent senescence in carnation petals. Instead, the flowers showed browning and drying in the rim of petals, which spread out to all the portion of the petals, and eventually the flowers faded out at the late stage of vase life, which was about twice that of the NT control flowers. These are typical of ethylene-independent senescence of carnation flowers.

DC-ACS1 and *DC-ACO1* transcripts were absent in the petals of both pMLH-sACO-2 and -12 flowers undergoing natural senescence (Fig. 2). This explained the reduced ethylene production in flowers of the two lines (Fig. 1). Previously, Kosugi *et al.* (2002) suggested that the *sACO* transgene integrated into carnation inhibited ethylene production in the flowers by cosuppression of expression of endogenous *DC-ACO1* gene in flower tissues. This seems also true in the present *sACO* transgenes with a long vase life. Kosugi *et al.* (2002) showed that the integrated *sACO* transgene might act first in the gynoecium, inhibiting the expression of *DC-ACO1* and suppressing ethylene production in the gynoecium and, subsequently, the expression of *DC-ACO1* and *DC-ACS1* in the petals. This was also found as an explanation for the absence of *DC-ACS1* transcript in the petals of both pMLH-sACO-2 and -12 flowers undergoing natural senescence.

The accumulation of *DC-ACS1* and *DC-ACO1* transcripts in flower tissues of the transgenic carnations (pMLH-sACO-2 and -12 lines) after treatment with exogenous ethylene (10 µl L^{-1} for 16 h), indicated that the integrated *sACO* transgene did not impair their responsiveness to ethylene. The accumulation of *DC-ACO1* transcripts in the petals of the pMLH-sACO-2 and -12 flowers after exogenous ethylene treatment indicated that exogenous ethylene treatment overcame the effect of the integrated *sACO* transgene in these two lines. Kinouchi *et al.* (2006) tested *in vivo* ACC oxidase activity using leaflet segments of the transgenic plants harboring *sACO* transgene. The *in vivo* ACC oxidase activities of the pMLH-sACO-2 and -12 lines were similar to and lower than the NT control, respectively.

These results suggested that the integrated *sACO* transgene exerted its effect in the leaflet segments of pMLH-sACO-12 line, but not pMLH-sACO-2 line. In this study, however, *DC-ACO1* transcript accumulated in low amounts in the gynoecium and was absent in the petals of pMLH-sACO-2 flowers. It was present abundantly in the gynoecium but also absent in the petals of pMLH-sACO-12 flowers on day 3. These differences suggest different action (expression) of the integrated *sACO* transgene between the leaflet and flower tissues, and also a difference between the two transgenic lines.

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Effects of high temperature on floral development and flowering in spray chrysanthemum

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Abstract

Delayed flowering of chrysanthemum under high temperature conditions is a serious obstacle for all year round cut chrysanthemum flower production in southern temperate and subtropical zones. To clarify the causes of flowering delay in spray chrysanthemum, two different genotypes of spray chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitam. syn. *Chrysanthemum morifolium*) were grown under high-temperature conditions: summer-to-autumn flowering type (SA type, high temperature tolerant) and autumn flowering type (A type, high temperature sensitive). Their flower-bud initiation and development were subsequently compared. Results clarify that two independent events caused by high temperatures occur in the shoot apex of spray chrysanthemum under short-day conditions. First, high temperatures slowed floral development in inflorescence, thereby increasing the number of florets in both SA and A chrysanthemum genotypes. Secondly, high temperatures slowed the developmental speed of inflorescence after the budding stage, and the time to reach the bud break stage was prolonged, thereby delaying flowering, especially in A chrysanthemum genotypes.

Key words: Chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitam. syn. *Chrysanthemum morifolium*), floral development, high temperature.

Introduction

Chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitam. syn. *Chrysanthemum morifolium*) is one of the most globally important ornamental species. The effects of temperature, especially sub-optimal temperature, on growth and flowering of chrysanthemum have been studied intensively (van der Ploeg and Heuvelink, 2006). However delayed flowering of chrysanthemum as a result of high temperatures is still a serious problem, not only in southern temperature zones such as Japan, but also in tropical zone countries like Malaysia, Thailand and Indonesia, where production of cut chrysanthemum flowers has increased recently. Chrysanthemum is a short-day plant; consequently, its flowering can be controlled by changing the day length (Okada, 1963). Short-day treatment is essential to produce cut chrysanthemum flowers in areas where the natural day length is longer than the critical day length in summer. The greenhouse often has high temperatures when it is shaded for this purpose. It has been reported that high temperatures engendered delayed flowering in chrysanthemum (Cockshull, 1979; Cockshull and Kofranek, 1994; Nishio *et al.*, 1988; Whealy *et al.*, 1987). Nevertheless, a few studies have been done to clarify the relationship between floral development and flowering delay. It remains unclear how the delay is reflected in the development of inflorescence. Most genotypes of spray chrysanthemum grown in Europe have a critical day length of about 13.5 h. The genotypes are designated as autumn flowering (A) type. Kawata *et al.* (1987) found the absolute short-day genotypes of chrysanthemum with longer critical day length (16-19 h), for flowering in summer under natural day length in Japan. The genotypes are designated as summer-to-autumn flowering (SA) type. Heat-tolerant cultivars were bred based on the SA genotypes (Shibata and Kawata, 1987). The SA genotypes show only a little flowering delay in summer, but they often show insufficient stem elongation in winter. Because of the defect of SA genotypes, year-round production of spray chrysanthemum is established by combining SA with A genotypes in Japan (Shibata *et al.*, 1988; Koyama *et al.*, 1996). It will be valuable to reveal differences in floral development of both SA and A types under high temperature conditions for breeding programs of heat tolerant year-round producible spray chrysanthemums.

This study was intended to elucidate the causes of flowering delay under high temperature conditions in terms of floral initiation and development. In this study, both SA and A genotypes were grown under high temperature conditions. Their respective floral development characteristics were compared at various developmental stages.

Materials and methods

Cultivation outline: This study used four genotypes of summer-to-autumn flowering type (SA) and five genotypes of autumn flowering type (A) spray chrysanthemums. Mother plants were grown in a greenhouse maintained at a minimum temperature of 15°C under long day conditions (night break). Compost with a mixture of Masa soil (granite) and manure (3:1) was used for this study. All plants were fertilised with 1,000-times diluted Hyponex® (a complete soluble fertiliser, $N:P_2O_5:K_2O$=6:10:5, Hyponex Co. Ltd., Japan, Osaka) once a week during the experiment. For daylength regulation, the short-day treatment (light period 8:00–18:00) (SD) was given by blacking-out to extend the daily dark period; the long-day treatment (LD) was made by a 4 h night break (22:00-2:00) with incandescent lamps at 3 μmol m^{-2} s^{-1}. Budding was defined as visible terminal flower bud appearance. Bud break was defined as a developmental stage of inflorescence in which the top of involucres opened with 3-mm

diameter in terminal inflorescence. The vertical petal was defined as a state in which petals of ray florets extended a vertical state. Flowering was defined as the state in which petals of ray florets opened completely to a horizontal state.

Effects of temperature on floral initiation and development (Experiment I): Two genotypes of spray chrysanthemum were used in this experiment: 'Sei-Monako' (SA type) and 'Sei-Maria' (A type). Cuttings were provided from these mother plants on 26 April in 2001. Eleven rooted cuttings of each genotype were planted in containers (20 x 60 x 15 cm); they were decapitated at the uppermost leaf on 14 May. All lateral shoots were allowed to grow. On 15 June, the plants were transferred to growth chambers that were controlled at a constant 20 or 30°C; SD was given until flowering. For scanning electric microscopic observation (SEM, S-2150; Hitachi Ltd., Tokyo), four shoot apexes of each treatment (20 or 30°C) were collected at 5, 10, 15, 20, 25, 30 and 45 days after the start of SD. Collected samples were fixed immediately with FAA (formalin: acetic acid: 70% ethanol, 5: 5: 90). After fixation, leaves and bracts were removed from the shoot apex under a binocular microscope. The samples were then dehydrated in the ethanol – acetone – isoamyl acetate series and dried in a critical point drier (HCP-1; Hitachi Ltd.). After coating with Pt, the samples were observed using SEM. The remaining shoots (5 shoots per treatment) were allowed to continue cultivation until flowering to provide a measurement of the number of florets.

Effects of high temperature exposed immediately after the start of SD on flowering (Experiment II): Rooted cuttings of 'Sei-Monako' and 'Sei-Maria' were transplanted on 15 July in 2002 in 9 cm-diameter plastic pots. They were decapitated at the uppermost leaf on 29 July. After 4 weeks of growth in a greenhouse under LD, the plants were planted into 21 cm-diameter clay pots (five plants per pot, two replications in each treatment). One lateral shoot per plant was allowed to grow; the others were removed. Then SD treatment started in the growth chambers that were controlled at a constant 20 or 30°C. Treatments consisted of five groups: plants were exposed to 30°C in the period from the start of SD to 5, 10 or 15 days, and were then transferred and grown at 20°C until flowering; plants of the other two groups were grown at 20 or 30°C from the start of SD to flowering. The number of days from the start of SD to budding and flowering, the number of leaves and florets, and the diameter of inflorescence at flowering were recorded.

Genotype related difference in development of inflorescence (Experiment III): This experiment used eight genotypes of spray chrysanthemum: 'Sei-Monako', 'Sei-Snow', 'Yellow-Shoes' and 'Sei-Suffle' (SA type), 'Sei-Alps', 'Sei-Liese', 'Chatoo' and 'Sei-Pino' (A type). Rooted cuttings of eight genotypes were transplanted on 5 May in 2003 in 9 cm-diameter plastic pots. They were decapitated at the uppermost leaf on 19 May. After 4 weeks of growth under LD, the plants were planted into 21 cm-diameter clay pots (five plants per pot) on 19 June. Then SD was given until flowering in the growth chambers controlled at 20°C. One lateral shoot per plant was allowed to grow. For five plants of each genotype, the days from the start of SD to budding, bud break and anthesis were determined, as were the respective diameters of inflorescence at budding, bud break and flowering.

Effects of high temperature on flowering after the visible bud stage (Experiment IV): This experiment used four genotypes of spray chrysanthemum: 'Sei-Snow' and 'Sei-Suffle' (SA type), and 'Sei-Alps' and 'Sei-Pino' (A type). Rooted cuttings of four genotypes were planted on 7 April in 2004 in 9-cm-diameter plastic pots. They were decapitated at the uppermost leaf on 21 April. One lateral shoot per plant was allowed to grow. After 4 weeks of growth in a greenhouse that was maintained at minimum temperature of 15°C under LD, SD was begun on 22 May. They were transferred to growth chambers controlled at 20 or 30°C, and kept under SD until flowering when the plants began the visible bud stage. The number of days from the start of SD to budding, bud break, vertical petal and anthesis, and the diameter of inflorescence at the flowering of five plants per genotype were recorded. The size of inflorescence in terminal inflorescence was measured every day for a period of budding to vertical petal.

Results

Effects of temperature on floral initiation and development (Experiment I): Both 'Sei-Monako' and 'Sei-Maria' showed the same sequence of events in floral initiation and development at 20 and 30°C (Fig. 1). No genotypic differences were apparent in the time required from dome formation to complete floret formation. In both genotypes, however, inflorescences developed slowly at 30°C in comparison with those at 20°C. Each developmental stage of chrysanthemum inflorescence was defined according to Fukai *et al.* (1997). Shoot apexes were vegetative in both genotypes and temperatures until 5 days after start of SD (DASD). Shoot apexes of both genotypes at 20°C reached to the latter stage of involucre formation in 5-10 DASD. They reached the latter stage of floret formation, and corolla formation started in the florets at the bottom of the dome in 15 DASD. Shoot apexes of both genotypes at 20°C completed floret formation by 25 DASD. Flowering of 'Sei-Monako' and 'Sei-Maria' at 20°C was observed at 48 and 55 DASD, respectively. On the other hand, the shoot apexes of both genotypes at 30°C did not produce floret primordia until 15 DASD. Floret formation started in 15-20 DASD at 30°C, and a corolla appeared in the florets at the bottom of dome at 25 DASD. Shoot apexes of both genotypes at 30°C finished floret differentiation by 55 DASD. Flowers of 'Sei-Monako' and 'Sei-Maria' at 30°C were observed at 62 and 94 DASD, respectively.

Table 1. Effects of temperature on the number of florets in spray chrysanthemum

Types	Cultivars	Treatments	Number of florets[z]	
			Ray florets	Disk florets
Summer-to-autumn flowering type	'Sei-Monako'	20°C	23.5 ± 2.5**	253.5 ± 16.4**
		30°C	33.8 ± 0.4	455.6 ± 47.2
Autumn flowering type	'Sei-Maria'	20°C	21.6 ± 1.1 *	152.0 ± 7.1**
		30°C	23.3 ± 1.0	360.0 ± 33.3

[z]Mean ± SD. ** and * denote significant difference by t-test at $P < 0.05$ and $P < 0.01$, respectively.

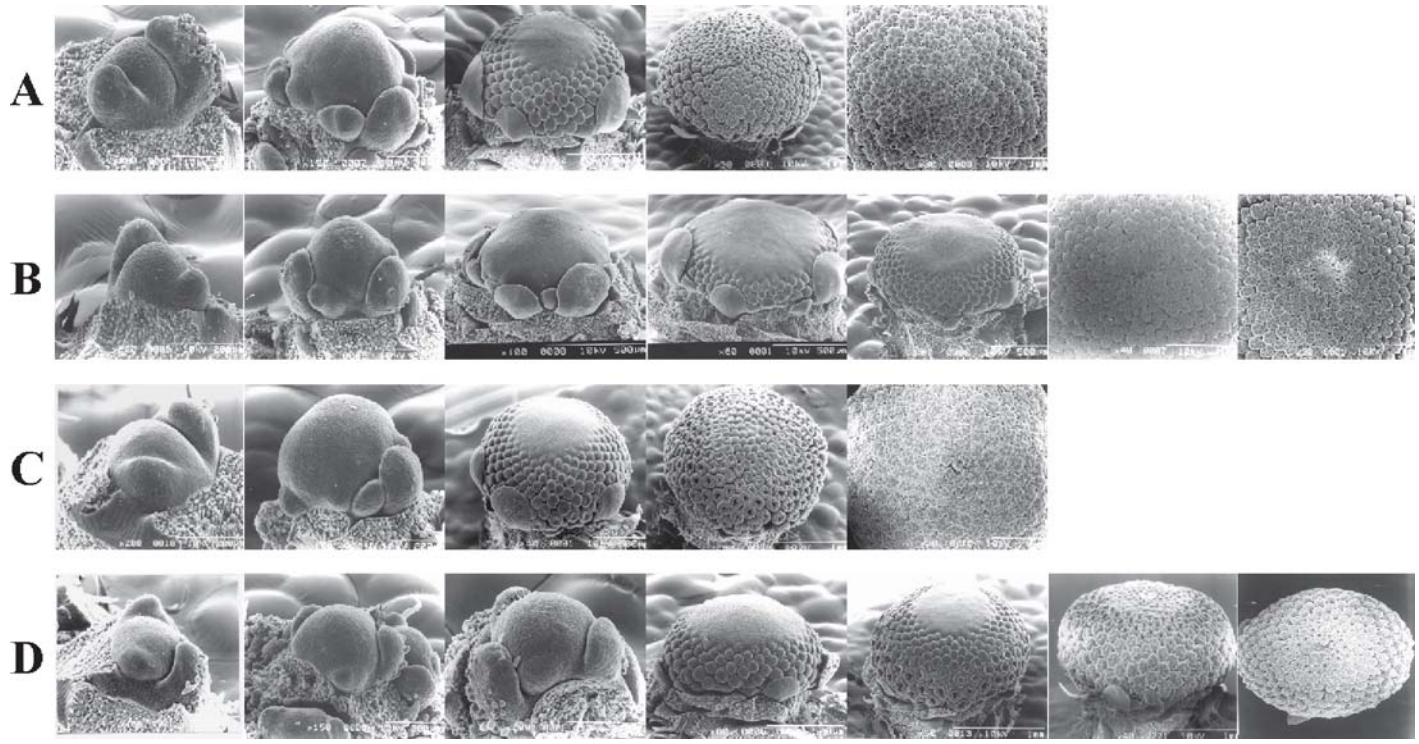

Fig. 1. Effects of temperature on morphological changes in the shoot apex. A: 20°C 'Sei-Monako', B: 30°C 'Sei-Monako', C: 20°C 'Sei-Maria', D: 30°C 'Sei-Maria'. These photographs represent the 5, 10, 15, 20, 25, 30 and 45 days after SD from the left of the line.

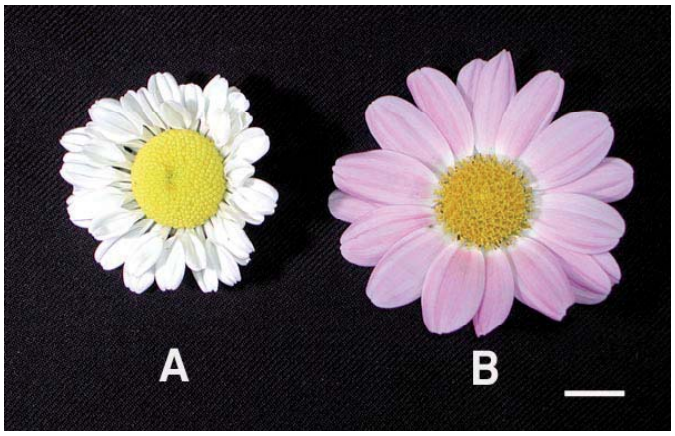

Fig. 2. Effects of temperature on the aspect of inflorescence at the flowering. A: 30°C 'Sei-Monako', B: 20°C 'Sei-Monako'. This photograph was taken 68 days after SD.

The size of inflorescence at 20°C was much larger than that at 30°C in both genotypes. Inflorescences at 20°C had shorter petals of ray florets with pale colour and a larger central part consisting of disk florets (Fig. 2). Considerably more ray florets and disk florets were apparent in plants grown at 30°C than in those grown at 20°C in both genotypes (Table 1).

Effects of high temperature exposed immediately after start of SD on flowering (Experiment II): The numbers of days to budding and flowering were slightly influenced by high temperature exposure immediately after the start of SD, whereas the number of florets increased considerably as a result of exposure to high temperature (Table 2). In 'Sei-Monako', no significant differences in the number of days to budding and flowering were apparent among the three treatments in which 5-15 days of high temperature were given during floral initiation to early development of inflorescence. All parameters of the three

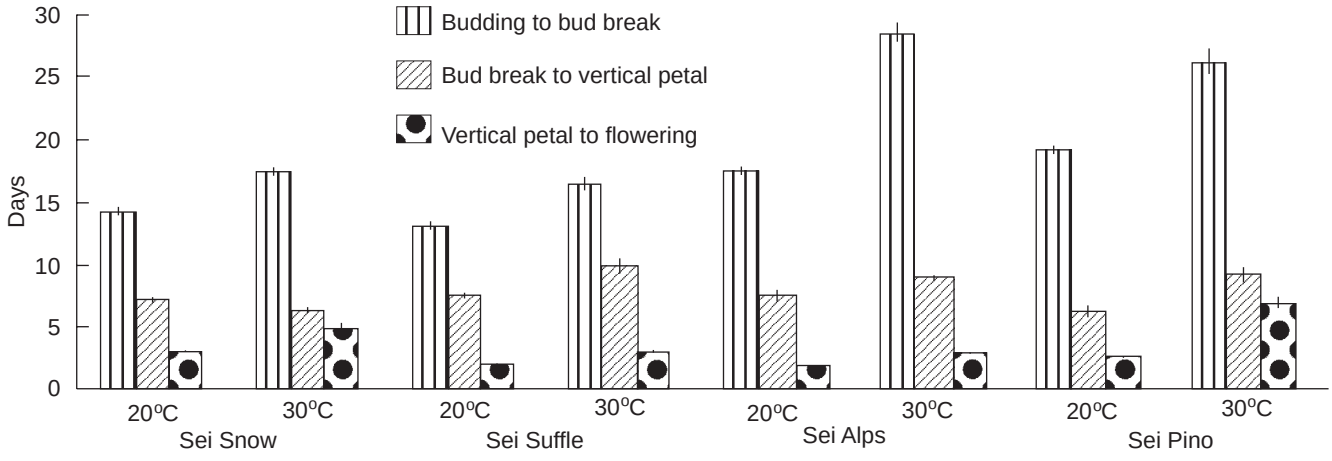

Fig. 3. Effects of temperature after budding on the days to flowering. 0 of the vertical axis denotes the budding day. Vertical bars represent SD.

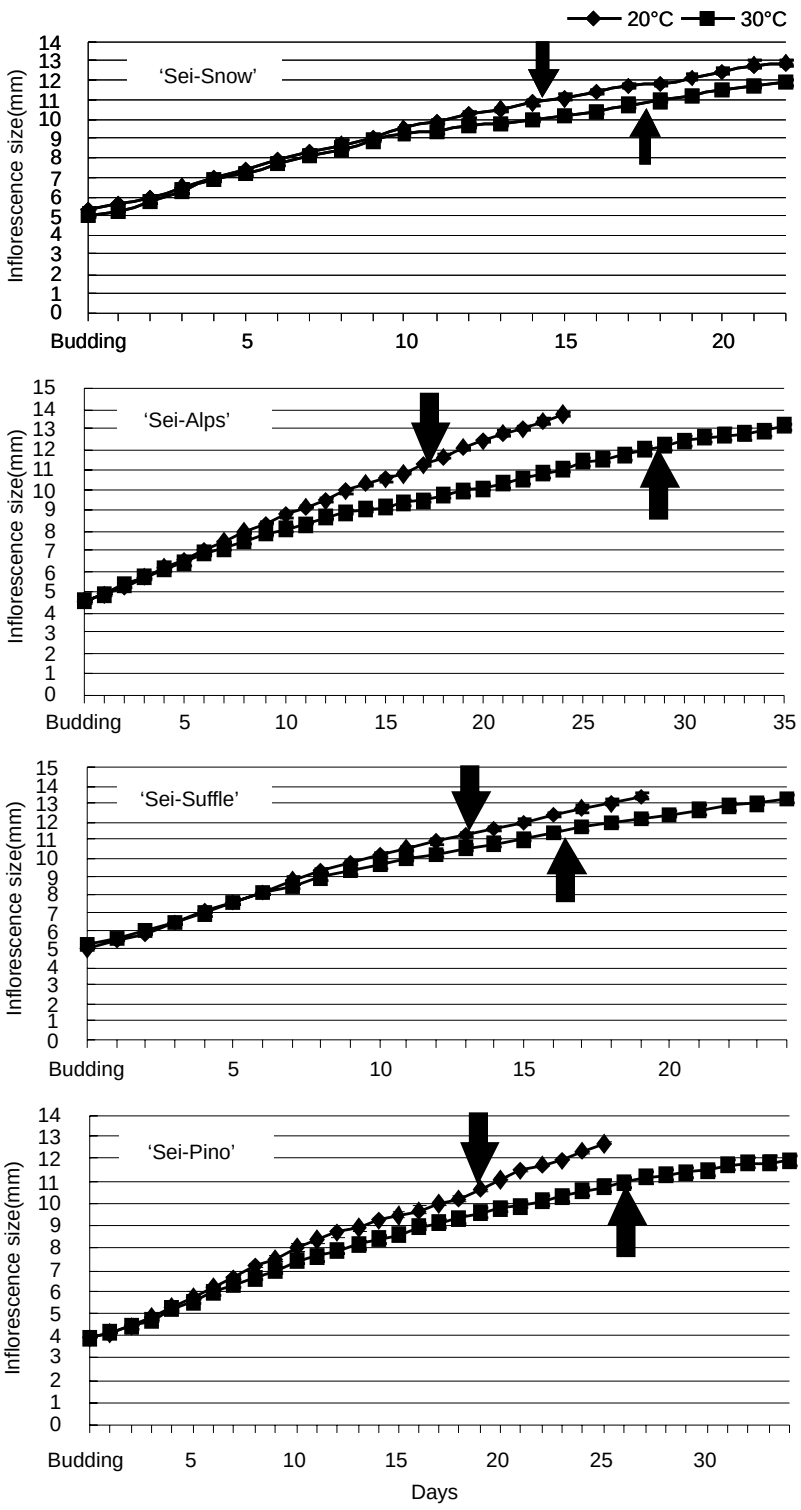

Fig. 4. Development situation of inflorescence from budding to vertical petals in the terminal flower bud. Arrows show the bud break period for each temperature.

treatments, except for the number of florets, were nearly equivalent to those of plants at 20°C. 'Sei-Maria' showed identical tendencies to those of 'Sei-Monako', but the days to flowering were slightly more in plants of the 15-day treatment. On the other hand, at 30°C, the days to budding were significantly more than those of other treatments in 'Sei-Maria'. In addition, the days to flowering at 30°C were many more than those of other treatments in both genotypes. No significant difference was found in the number of leaves, indicating that high temperatures had no effect on the transition from the vegetative phase to the reproductive phase. The inflorescence diameter was significantly smaller at 30°C in both genotypes. The number of disk florets increased in both genotypes when high temperatures were applied longer.

Genotypes difference in the development of inflorescence (Experiment III): All genotypes reached the budding stage in about 20 DASD, irrespective of flowering type SA and A (Table 3). On the other hand, the number of days to bud break varied from 29 days in 'Sei-Monako' and 'Chatoo' to 43 days in 'Sei-Alps'. 'Sei-Alps' and 'Sei-Liese' required more days to budding and showed more days to flowering. Diameters of inflorescences at bud break and flowering stage were significantly different depending on genotypes (Table 4). No constant relationship was observed in the diameter of inflorescence between the bud break and flowering stages.

Effects of high temperature after visible bud stage on flowering (Experiment IV): Plants grown at 30°C from budding to flowering showed delayed flowering compared with plants grown at 20°C. The flowering delay was remarkable in A genotypes (Fig. 3). The time required from budding to bud break at 30°C was much longer than that at 20°C in A genotypes, but the difference was small in SA genotypes. The time from bud break to the vertical petal stage and from vertical petal to flowering at 30°C was slightly longer than that at 20°C, except for 'Sei-Snow', for which the time from vertical petal to flowering at 30°C was longer than that at 20°C.

The time course of inflorescence development revealed a difference in the inflorescence size between at 20 and 30°C from 5 days after the budding stage in all genotypes. Differences between temperatures were small in SA genotypes and were large in A genotypes, in which the speed of inflorescence development slowed around 10 days after budding at 30°C. However, no big differences were apparent in the inflorescence diameter at the bud break stage in all genotypes. Flowering of SA genotypes at 30°C was almost identical to that at 20°C, but flowering at 30°C was particularly delayed in A genotypes.

Discussion

Effects of high temperature on chrysanthemum flowering have already been studied in relation to floral initiation (phase transition), flower-bud development, and other environmental factors (Cockshull, 1979; Cockshull and Kofranek, 1994; Karlsson *et al.*, 1989; Whealy *et al.*, 1987; Wilkins *et al.*, 1990). However, it remains unclear how the delay manifests itself in the processes of floral initiation and development in chrysanthemum. This study revealed that two independent phenomena occur in inflorescences under high temperature conditions: quantitative increase of florets and slow inflorescence development. Additionally, we emphasized the importance of the time required for development to a specific stage of inflorescence for flowering delay in chrysanthemum.

No difference was found in the sequence of floral initiation and development between the two genotypes at either 20 or 30°C in this study. The morphological changes in the process of floral development were almost

Table 2. Effects of high temperature during flower-bud initiation and development period on flowering

Cultivars	Treatments	Days to budding from the start of SD	Days to anthesis from the start of SD	Number of leaves	Diameter of inflorescence (mm)	Number of florets	
						Ray florets	Disk florets
'Sei-Monako'	20°C	20.7ab[z]	46.1a	19.1NS[y]	48.9b	27.5a	310.6a
	5 days	21.2ab	47.4a	18.1NS	49.8b	28.8a	316.3a
	10 days	20.1a	46.7a	17.7NS	49.4b	32.4b	386.7b
	15 days	21.9ab	48.8a	18.2NS	49.4b	32.9b	489.8c
	30°C	22.9b	60.5b	17.9NS	35.1a	33.8b	519.0c
'Sei-Maria'	20°C	20.8a	57.5a	23.2NS	60.7b	25.1NS	267.3ab
	5 days	21.4a	58.8ab	23.4NS	64.4b	25.6NS	258.1a
	10 days	21.1a	59.8ab	23.9NS	60.2b	23.3NS	262.6ab
	15 days	22.5a	61.8b	23.3NS	62.2b	25.4NS	287.2b
	30°C	25.6b	90.1c	23.3NS	35.9a	25.1NS	348.7c

z Different letters among treatments represent significant difference by Tukey's multiple range test ($P < 0.05$).
y NS: Not significant.

Table 3. Number of days from the start of SD to each development stage of inflorescence

Types	Cultivars	Days to budding from start of SD	Days to bud break from start of SD	Days to anthesis from start of SD
Summer-to- autumn flowering type	'Sei-Monako'	21.0ab[z]	29.0a	45.3ab
	'Sei-Snow'	19.6a	36.2b	46.4ab
	'Yellow-Shoes'	22.5b	34.0b	43.8a
	'Sei-Suffle'	19.3a	33.5b	45.5ab
Autumn flowering type	'Sei-Alps'	21.0ab	43.0c	51.8c
	'Sei-Liese'	19.4a	40.6c	50.6c
	'Chatoo'	20.8ab	29.0a	45.8ab
	'Sei-Pino'	19.2a	36.0b	48.0bc

Table 4. Diameter of inflorescence at different stages

Types	Cultivars	Diameter of inflorescence (mm)		
		Budding	Bud break	Flowering
Summer-to- autumn flowering type	'Sei-Monako'	5.23ab[z]	9.70ab	53.40bc
	'Sei-Snow'	5.24ab	11.24bc	41.72a
	'Yellow-Shoes'	5.93b	12.90cde	41.13a
	'Sei-Suffle'	5.48ab	13.10de	49.03b
Autumn flowering type	'Sei-Alps'	5.10a	14.37e	52.13bc
	'Sei-Liese'	5.42ab	14.48e	59.30c
	'Chatoo'	5.04a	8.76a	49.64b
	'Sei-Pino'	4.80a	12.20cd	38.52a

consistent with those of previous reports on floral development of chrysanthemums (Fukai *et al.*, 1997; Lee *et al.*, 2001; Okada, 1963; Yulian *et al.*, 1996; Zhang *et al.*, 1998). Present results showed that a morphological transition from a vegetative to reproductive shoot apex, *i.e.* dome formation, occurred during 5-10 DASD. Inflorescence development at 30°C was slower than that at 20°C, as described by Whealy *et al.* (1987). The period required from dome formation to completion of floret differentiation on the inflorescence was almost equal in both SA and A genotypes under identical temperature conditions. However, the number of days to flowering at 30°C in A genotype increased more than 30 days in comparison with the SA genotype. Results indicate that no direct relationship exists between flowering and completion of floret differentiation in inflorescence, suggesting that flowering delay is the result of high temperature effects on the latter developmental stages of the inflorescence.

Increased number of florets, especially disk florets, were observed in both SA and A genotypes at 30°C. Furthermore, increase was recognised when high temperatures were applied for only 5-15 days immediately after the start of SD. These facts indicate that the high temperature given in the early developmental stage of inflorescence determines the direction of differentiation in the inflorescence apex. In all cases, the increase of ray florets occurred even though a flowering delay did not take place, indicating that the increase of floret number and the delay of flowering under high temperature conditions are independent phenomena. Cockshull and Kofranek (1994) also described the increased floret number in chrysanthemum under high temperature conditions, but they did not recognize the independency of the increased flower number and delayed flowering. The reduced number of florets on the inflorescence or floral organs, particularly in petals, are recognised in many plants under high temperature conditions (Chimenti and Hall, 2001; Mito *et al.*, 1980). The reduced numbers of florets is inferred to have occurred because of faster growth under high-temperature conditions. In case of chrysanthemums, the increased number of florets can be attributed not only to the decreased development speed of inflorescence under high temperature conditions, but also some physiological changes in the inflorescence shoot apex.

The days to budding and flowering in both genotypes were almost equal for those grown at 20°C when high temperature was given only 5-15 DASD (Table 2). That fact implies that inhibition of inflorescence development in early stages that is caused by exposure to high temperature recovered rapidly after transferral to 20°C. These results indicate that the developmental speed of inflorescence is temperature dependent. In the same experiment, the number of leaves did not change among all temperature treatments. The high temperature given immediately after the start of SD did not greatly affect the phase transition from vegetative to reproductive. Observation by SEM, which showed that floral initiation occurred in the period of 5-10 DASD at 30°C, also supported this inference. Therefore, the delay of phase transition under high temperature conditions is very small even if it existed in genotypes used in this study. Nishio *et al.* (1988) reported no changes in the number of leaves in two genotypes of chrysanthemum when maintained at 27-30°C during the day time and at a range of 20-35°C night temperature in the first three weeks after start of SD. On the other hand, Wilkins *et al.* (1990) reported decreased leaf numbers from lower night temperatures when chrysanthemums were maintained at a minimum temperature of 21°C during the day and in a range of 13-21°C night temperatures in the first three weeks after starting SD. Cockshull and Kofranek (1994) also showed that high night temperatures of 32°C in the first 1-3 weeks after the start of SD increased the chrysanthemum leaf number. The differences of these results can be attributed to the fact that the phase transition from vegetative to reproductive phase is delayed under higher temperature conditions, but it is less delayed at night temperatures below 20°C (or mean daily temperature). Genotype differences in response to high temperature in terms of phase transition might also exist.

Inflorescence sizes at the budding stage were similar among genotypes used in this study when grown at 20°C, whereas differences in inflorescence size of genotype at the bud break stage were clear. The genotypes required a longer time to bud break at 20°C and required a longer time to flowering. In a genotypes, showing great delay of flowering under high temperature conditions, the time from budding to bud break stage was remarkably long at 30°C compared to that at 20°C (Fig. 3). The developmental speed of inflorescence in A genotypes at 30°C after the budding stage decreased considerably compared to those at 20°C, whereas the size of inflorescence at the bud break stage was not greatly different (Fig. 4). These results show that the inflorescence size at the bud break stage is genotype-dependent and that the time to reach this specific stage (size) is important to determine the flowering time. The time to reach the bud break stage was longer at 30°C, especially in A genotypes, showing the delay of flowering under high temperature conditions. The physiological explanation for such a phenomenon remains unclear. High photosynthetic capacity of SA genotypes under high temperature and high light intensity, as shown by Koyama *et al.* (2001), might be one reason for that difference. It can be concluded that high temperatures decreased the developmental speed of inflorescence in chrysanthemum after the budding stage. The time to reach the bud break stage was prolonged, engendering flowering delay.

Results of this study revealed that two independent events caused by high temperature occurred in the shoot apex of spray chrysanthemum under a short-day condition. First, the high temperatures decreased the floral development speed in inflorescence, thereby increasing the number of florets in both SA and A chrysanthemum genotypes. Secondly, as high temperatures decreased the developmental speed of inflorescence after budding stage, the time to reach the bud break stage was prolonged, thereby delaying flowering, especially in A chrysanthemum genotypes.

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Factors affecting fruit abortion in a gynoecious cucumber cultivar

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Abstract

Fruit growth of the gynoecious cucumber 'NK × AN8' was measured non-destructively to clarify whether the presence of fruit at lower nodes caused the abortion of fruit at upper nodes. When only one fruit per plant was allowed to grow, fruit growth could be divided into two phases: slow exponential and fast exponential. Phase change from slow to fast occurred when cumulative temperatures (CTs) after anthesis reached 38 and 54ºC d for pollinated and parthenocarpic fruit, respectively. The CT was calculated as the sum of the differences between daily temperatures and 5ºC. When fruit at nodes 4 and above were allowed to grow, the first growth phase was prolonged. Furthermore, parthenocarpic fruit aborted frequently when the sum of the relative growth rate (RGR) with respect to the CT (the sum of RGRs) for fruit at lower nodes exceeded 0.1 g g^{-1} (ºC d)$^{-1}$. Pollination with pollen of the monoecious cucumber '028' strongly suppressed fruit abortion; a large number of fruits could develop to a commercial size even when the sum of RGRs for fruit at lower nodes exceeded 0.1 g g^{-1} (ºC d)$^{-1}$. These results suggested that fruit abortion is more related to the existence of actively growing fruit than to the absolute amount of dry mass accumulation in the fruit.

Key words: Cucumber, gynoecious cultivar, fruit growth, fruit abortion, pollination, relative growth rate

Introduction

A large number of pistillate flowers are formed within a short period in gynoecious cucumber cultivars. Although the number of pistillate flowers is about two times greater in gynoecious cultivars than in monoecious cultivars, the yield is only 10-20% higher in the former than in the latter cultivars. A higher percentage of flowers abort in gynoecious cultivars compared with monoecious cultivars, leading to a lower yield than expected from the number of flowers (Hikosaka and Sugiyama, 2004). Marcelis (1994) proposed a simulation model of cucumber fruit growth, but his model is not satisfactory for the precise estimation of fruit abortion.

Fruit abortion being an important factor determining fruit production, it has been studied extensively in many crops. However, an unequivocal conclusion has not yet been attained. Some authors have considered that the abortion of reproductive organs was affected by plant hormones. For example, ethylene production is suggested to cause the abortion of *Hibiscus* flower buds (Van Meeteren and Van Gelder, 1995) and pepper fruit and flower buds (Huberman *et al.*, 1997; Wien *et al.*, 1989). On the other hand, the inhibition of auxin transport is related to the abortion of pepper fruit and flower buds under high temperature (Huberman *et al.*, 1997). Bangerth (1989) hypothesized that auxin exported from early-developed fruit inhibits auxin exported from later-developed fruit, causing fruit abortion.

In contrast, Marcelis *et al.* (2004) emphasized the importance of the source–sink relationship in the fruit abortion of sweet pepper. In tomato, fruit set was related to the source–sink ratio (Bertin, 1995). Guinn (1974) pointed out the importance of photoassimilate supply in preventing fruit abortion in cotton. Turner and Wien (1994a, b) considered that cultivar differences in the abortion of pepper fruit and flower buds can be ascribed to differences in photosynthetic activity, respiratory activity, and photoassimilate partitioning.

In monoecious cucumber cultivars, nodes with pistillate flowers are distributed at random on vines, while pistillate flowers are formed regularly at each node in gynoecious cultivars. Therefore, it is possible that the rate of flower formation and increases in the fresh mass of the fruit (crop load) can be estimated precisely in gynoecious cultivars, but not in monoecious cultivars. In the present experiment, we grew gynoecious parthenocarpic cucumbers to conduct a systematic growth analysis of the fruit. From the analysis of growth data, we tried to clarify the factor that was dominant in fruit abortion, that is, the existence of fruit or the crop load.

Materials and methods

Gynoecious parthenocarpic 'NK × AN8' cucumbers were sown on 5 April, 2004. Seeds were obtained from the Nihon Horticultural Production Institute (Matsudo, Chiba, Japan). On 30 April, plants with three fully expanded leaves were transplanted individually into plastic containers (20 L) filled with a growth medium (Soil Mix, Sakata Co., Yokohama, Japan) containing starter fertilizer (0.4 g kg^{-1} N, 0.9 g kg^{-1} P, and 0.5 g kg^{-1} K). Nodes were numbered acropetally, with the cotyledonary node designated as node 0. All lateral shoots were removed, while the apical portions of the main vines were not pinched. For all experiments, ten plants were used; five plants were allocated to the parthenocarpic treatment and the other five to the pollination treatment. In the parthenocarpic treatment, petals were clipped before anthesis to prevent pollination. In the pollination treatment, hand pollination was carried out using pollen from staminate

flowers of the monoecious cultivar '028'. In Experiment 1, only one fruit per plant was allowed to grow (node 10) and the diameter and length of the fruit were measured every day. Flower buds at nodes 4–9 and above 11 were removed just after their appearance. In Experiment 2, fruit at nodes 4 and above were allowed to grow. The length and diameter of all fruit (ovaries) at nodes 4–15 were measured daily from anthesis to harvest using a digital calliper. In both experiments, the fresh weight (FW) of an 'NK × AN8' fruit (g) could be estimated precisely from fruit (ovary) length (FL, mm) and diameter (D, mm) as follows: $FW = 8.09 \times 10^{-4} \times (D/2)^2\pi \times (FL) + 0.732$ ($n = 241$, $r^2 = 0.993$) (Hikosaka and Sugiyama, 2004). All fruit were harvested when they reached a commercial size, *i.e.*, 150 mm in length.

The maximum and minimum temperatures in the glasshouse were controlled at 28/15ºC. Temperature and photosynthetically active radiation inside the greenhouse were monitored every minute with a datalogger (CR10X; Campbell Scientific Inc., Logan, UT, USA). Plants were irrigated with 1-2 L of nutrient solution every day to maintain sufficient water and nutrient supply. The concentrations of ions in the nutrient solution were as follows: NO_3^-, 16 mM; $H_2PO_4^-$, 4 mM; Ca^{2+}, 4 mM; Mg^{2+}, 2 mM; K^+, 8 mM; and NH_4^+, 1.3 mM. The CT after anthesis was calculated as the sum of the differences between daily temperatures and 5ºC. This was because the RGR of the fruit of a typical Japanese cucumber cultivar is almost zero at 5ºC and increases linearly to 30ºC (Tazuke and Sakiyama, 1986). The RGR with respect to the CT (g g^{-1} (ºC d)$^{-1}$) and the growth rate (GR, g d^{-1}) of a fruit between n and n+1 days after anthesis (DAA) was calculated as:

$$RGR = (\ln FW_{n+1} - \ln FW_n) / (T_n - 5)$$

and

$$GR = FW_{n+1} - FW_n,$$

where, FW_n and FW_{n+1} are the estimated FWs of fruit at n and n+1 DAA, respectively, and T_n is the daily average temperature at n DAA.

Results

Fruit growth in abscence of competing fruit (Experiment 1): When only one parthenocarpic fruit was allowed to grow on a plant, increases in the logarithms of fruit FW ($\ln FW_n - \ln FW_0$, where FW_n and FW_0 are the FWs of fruit at n and 0 DAA, respectively) against the CT after anthesis can be depicted by two lines (Fig. 1a). These two lines intersected at 54ºC d, where FW reached about 3 g. In pollinated fruit, it appears that increases in the logarithm of fruit FW increased linearly with an increase in the CT. An exception was for fruit smaller than 3 g (fruit at 0-38ºC DAA). When the relationship between increases in the logarithm of fruit weight and the CT was calculated after fruit FW reached 3 g, no difference was found in the slopes between pollinated and parthenocarpic fruit. Therefore, the regression line in Fig. 2 was calculated for pooled data of both pollinated and parthenocarpic fruit. The relationships between increases in the logarithm of fruit FW and the number of days were diverse among fruit (data not shown), suggesting the rationality of expressing RGR based on CT.

Growth of parthenocarpic fruit in presence of competing fruit (Experiment 2): An example of the growth of parthenocarpic fruit on a plant is shown in Fig. 3a, along with the sum of RGRs (Fig. 3b), GRs (Fig. 3c), and the number of fruit per plant (Fig. 3d). Fruit at node 4 usually grew almost exponentially, but several fruit at nodes 5 and above did not grow immediately. Fruit growth usually resumed thereafter. In severe cases, however, fruit growth did not resume, leading to fruit abortion. When the growth of fruits at nodes above 9 was completely suppressed at 130ºC d, the sum of RGRs and the number of fruit were very high: 0.1 g g^{-1} (ºC d)$^{-1}$ and six fruits, respectively. However, the sum of GRs remained quite low.

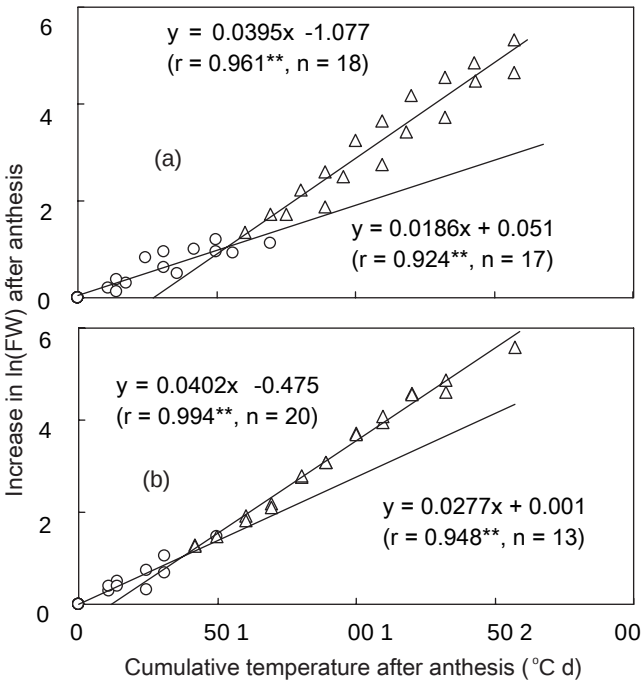

Fig. 1. Growth of cucumber 'NK × AN8' fruit in absence of competing fruit. Increases in the logarithm of fruit fresh weight (FW) after anthesis were plotted against cumulative temperature after anthesis. The cumulative temperature was calculated as the sum of the differences between daily average temperatures and 5ºC. Circles, fruit whose FW is less than 3 g; triangles, fruit whose FW weight is greater than 3 g. (a) parthenocarpic fruit, (b) pollinated fruit.

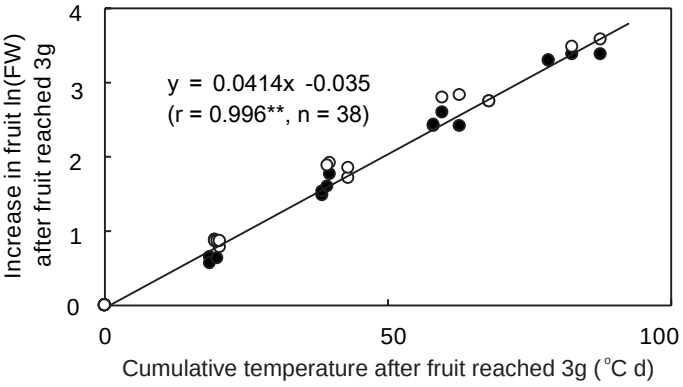

Fig. 2. Growth of cucumber 'NK × AN8' fruit in absence of competing fruit after the onset of rapid exponential growth. Increases in the logarithm of fruit fresh weight (FW) were plotted against cumulative temperature after fruit reached 3 g. Closed circles, pollinated fruit; open circles, parthenocarpic fruit. The regression line was calculated by pooling the data of both pollinated and parthenocarpic fruits.

Growth of pollinated fruit in presence of competing fruit (Experiment 2): When the pistillate flowers were pollinated, the growth pattern of fruit changed markedly (Fig. 4a). A few fruit ceased growing for a while after anthesis. When fruit at nodes 5

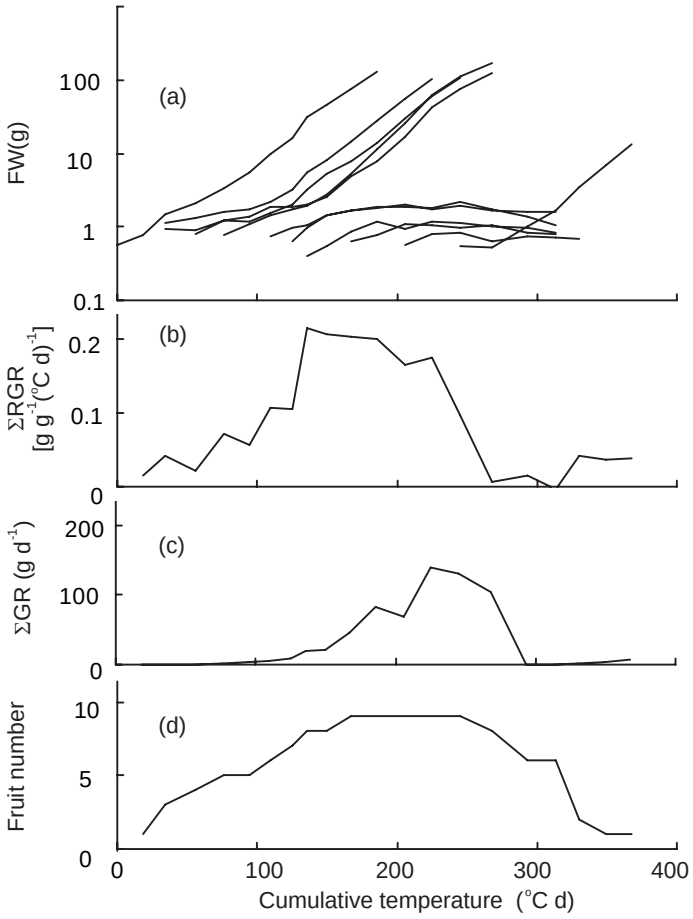

Fig. 3. Relationship between fruit growth and the sum of relative growth rates (∑RGRs), the sum of growth rates (∑GRs), and the number of fruit summed for all fruit from plants of the parthenocarpic cucumber 'NK × AN8'. Data of a plant are shown. All fruits at nodes 4-15 were allowed to grow and were harvested once they reached a marketable size. The data for the 14th node are missing because as a very rare case, male flower flowered on the 14th node. The sum of RGRs was calculated based on cumulative temperatures. Data from one plant are shown as an example.

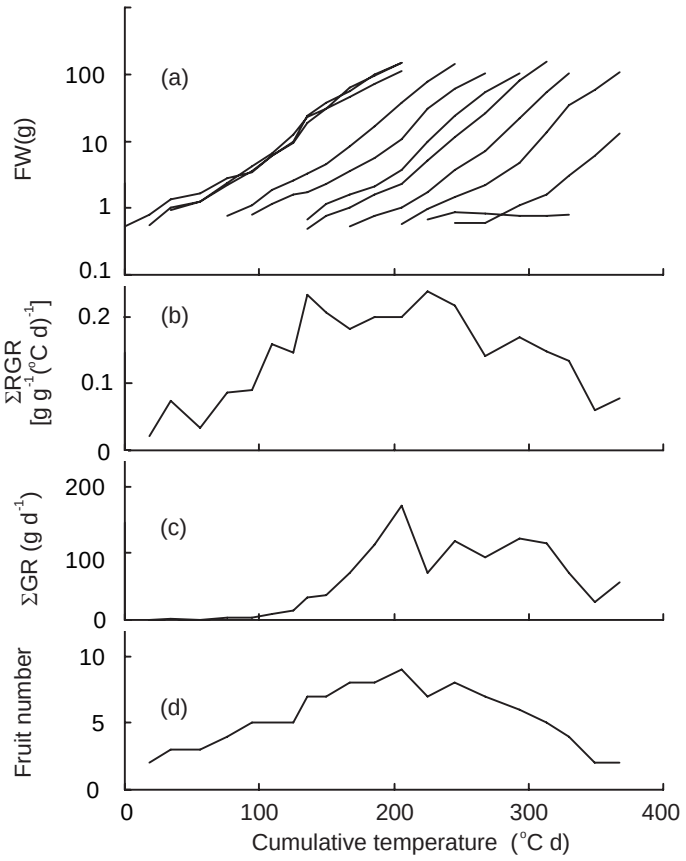

Fig. 4. Relationship between fruit growth and the sum of relative growth rates (∑RGRs), the sum of growth rates (∑GRs), and the number of fruit summed for all fruit from plants of the pollinated cucumber 'NK × AN8'. Data of a plant are shown. All fruits at nodes 4-15 were allowed to grow and were harvested once they reached a marketable size. Pollen was obtained from plants of the monoecious cultivar '028'. ∑RGRs was calculated based on cumulative temperatures. Data from one plant are shown as an example.

and above were compared, the period from anthesis to harvest was found to be shorter in pollinated fruit than in parthenocarpic fruit (Figs. 3 and 4). The competing potential of fruit at lower nodes against fruit at upper nodes, *i.e.*, the sum of RGRs, the sum of GRs, and the number of fruit, did not differ between plants with pollinated fruit and those with parthenocarpic fruit until CT reached 250°C d (Figs. 4b-d *vs.* Figs. 3b-d, respectively).

Evaluation of the initial period of slow growth (Experiment 2): To evaluate the effect of the sum of RGRs, the sum of GRs, and the number of competing fruit on the period of initial slow growth, these values were plotted against the reciprocal of the CT (1/CT) necessary for the development of fruit weighing 2, 3, 4, or 5 g. The CT was infinite (1/CT = 0) for fruit that did not reach 2, 3, 4, or 5 g. In the parthenocarpic treatment, some fruits reached 2-4 g despite the sum of RGRs being greater than 0.1 g g^{-1} (°C d)$^{-1}$ (Fig. 5). On the other hand, all fruits reached 5 g when the sum of RGRs was smaller than 0.1 g g^{-1} (°C d)$^{-1}$, but no fruit reached 5 g when the sum of RGRs was 0.1 g g^{-1} (°C d)$^{-1}$ or higher (Fig. 6a). In many pollinated fruits, however, the values of 1/CT were maintained at about 0.01 (°C d)$^{-1}$ even when the sum of RGRs was greater than 0.1 g g^{-1} (°C d)$^{-1}$ (Fig. 6a). In contrast, there was no relationship between 1/CT and the sum of GRs for parthenocarpic fruits (Fig. 6b). Relationships between 1/CT and the number of competing fruit were similar to those between 1/CT and the sum of RGRs, although they were not as clear (Fig. 6c).

Discussion

Potential growth curve of a fruit: Marcelis (1994) estimated the 'potential growth rate' of a fruit by monitoring fruit growth when there was no competition for assimilates from other fruit. He found that fruit growth fitted a Richards' equation and that the potential growth rate could be estimated from this equation. We also assumed that potential fruit growth could be attained when only one fruit was left on a plant to avoid competition from other fruit. In the present experiment, pollinated and parthenocarpic fruit grew exponentially at the same RGR, which started from 38°C d and 54 °C d of CT after anthesis, respectively (Figs. 1 and 2). This result suggests that potential fruit growth is independent of pollination, but that pollination hastens the start of rapid exponential growth. It is interesting to note that fruit cells began to enlarge 2 DAA in pollinated fruit and 4 DAA in parthenocarpic fruit of this cultivar (Boonkorkaew, pers. comm.). As 2 and 4 d roughly corresponded to 35 and 70°C d, respectively, it is likely that the potential growth rate could be accomplished in both parthenocarpic and pollinated fruit when fruit cells begin to enlarge. Furthermore, an initial slow growth period exists after anthesis even when only one fruit is allowed to grow on a

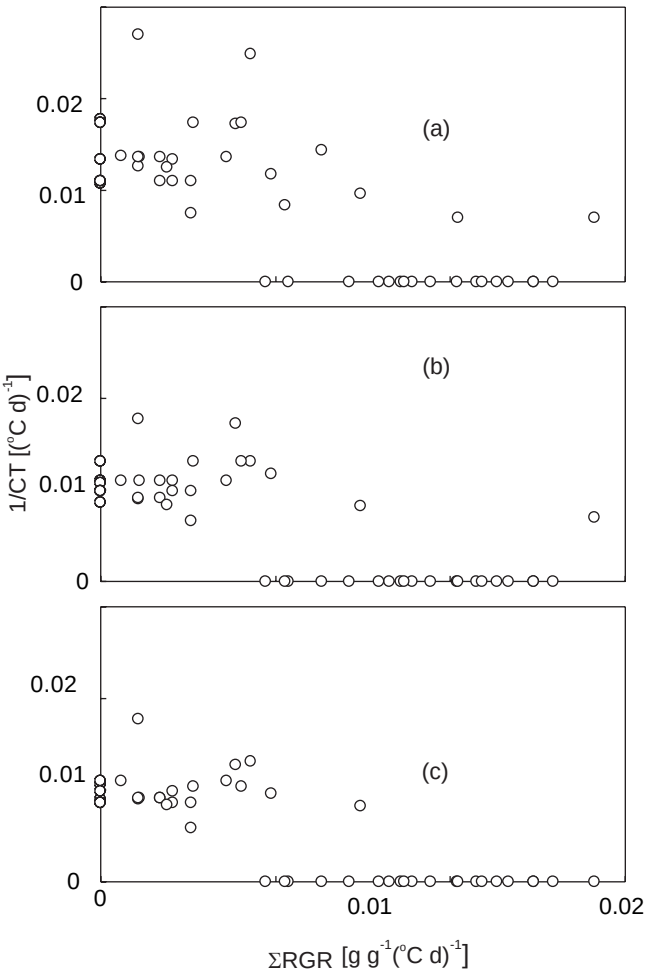

Fig. 5. Relationships between the reciprocal of the cumulative temperatures (1/CT) necessary for the development of fruit weighing (a) 2, (b) 3, and (c) 4 g and the sum of relative growth rates (∑RGRs) of fruit at lower nodes of the parthenocarpic cucumber 'NK × AN8'. ∑RGR was calculated on the day of anthesis of the fruit for which 1/CT was calculated.

plant. The discrepancy from the sigmoidal Richards' equation in the present experiment can be ascribed to the fact that fruit was harvested much earlier in the present experiment than in the experiment of Marcelis (1994).

Fruit abortion and competition between fruits: In absence of competing fruits, fruit FW reached 3 g at 54ºC d of the CT. However, the existence of competing fruits extended the initial slow growth period of parthenocarpic fruit from 54 to more than 100ºC d (Fig. 3). Furthermore, it appears that fruit FW at the end of the initial slow growth phase varied from 2 to 5 g when there was competing fruit. This makes the relationship between 1/CT and the sum of RGRs unclear if we assume that fruit FW reached 2-4 g at the end of the slow growth phase (Fig. 5). When the sum of RGRs was smaller than 0.1 g g^{-1} (ºC d)$^{-1}$, all fruits reached 5 g by about 100ºC d. However, all fruits aborted when the sum of RGRs was larger than 0.1 g g^{-1} (ºC d)$^{-1}$. These results suggested that (1) fruits that are not exposed to severe competition can complete the slow growth phase, (2) fruit FW at the end of the slow growth phase differs between individual fruits (2-5 g) and (3) fruits of which FW at the end of the slow growth phase was 2-4 g cease to growth and finally abort (1/CT=0). On the other hand, it is likely that fruit that can enter the fast growth phase do not abort because all fruit larger than 5 g would develop to

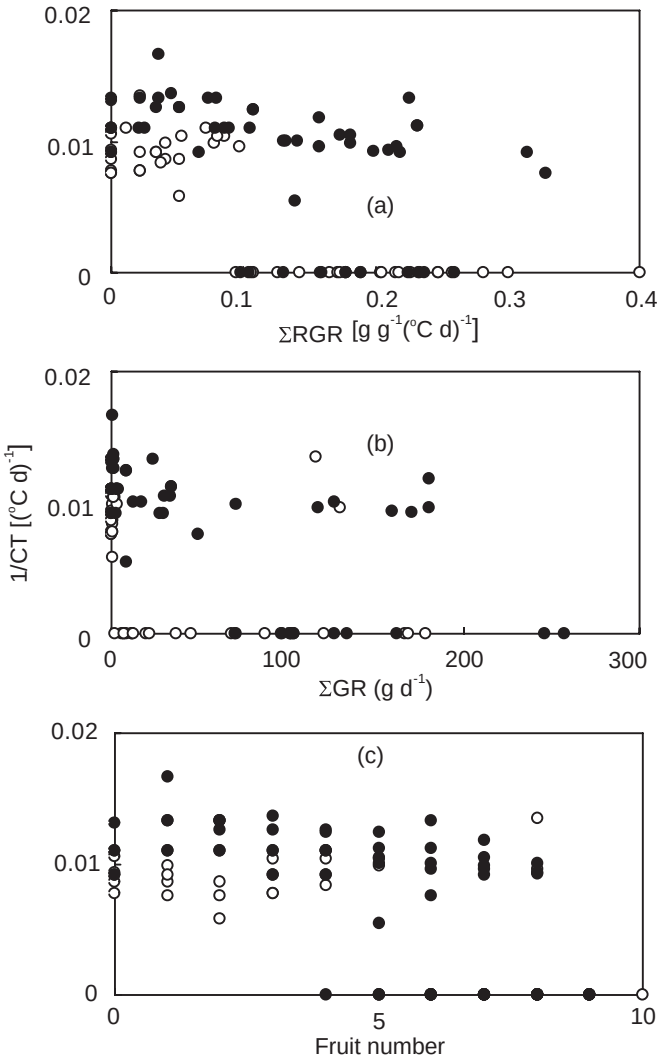

Fig. 6. Relationships between the reciprocal of the cumulative temperatures (1/CT) necessary for the development of fruit weighing 5 g and (a) the sum of relative growth rates (∑RGR), (b) the sum of growth rates (∑GR), and (c) the number of fruit at lower nodes of both the parthenocarpic and the pollinated cucumber 'NK × AN8'. In pollinated fruit, pollen was obtained from plants of the monoecious cultivar '028'. ∑RGR, ∑GR, and the number of fruit were calculated on the day of anthesis of the fruit for which 1/CT was calculated. Open circles, parthenocarpic fruit; closed circles, pollinated fruit. ∑RGRs was calculated based on cumulative temperature.

a commercial size. Takeno and Ise (1992) reported that in a gynoecious cucumber cultivar, fruit that reached 6 cm in length never aborted.

In cucumber fruits, RGRs were almost the same from the start of rapid exponential growth to harvest. Therefore, the sum of RGRs would be proportional to the total number of growing fruits, whereas the sum of GRs would be dependent on the number of large fruits. In the present study, the sum of GRs and the number of fruit did not relate closely with 1/CT; fruit FW did not reach 5 g even when the sum of GRs was very low. This suggests that growth suppression or fruit abortion is determined by the existence of the actively growing fruit, not by the size of the competing sink. Schapendonk and Brouwer (1984) reported that severe defoliation did not inhibit the normal development of cucumber fruit when only one fruit was allowed to grow. Thus, they hypothesized that factors other than the shortage of photoassimilates could cause fruit abortion. Stephenson *et al.* (1988) reported that seed number

in the first fruit determines whether later-developed zucchini fruit abort or not. The removal of old fruit decreased the abortion of young fruit by decreasing the ABA content of young fruit (Tamas *et al.*, 1979). Unlike parthenocarpic fruits, many pollinated fruit reached 5 g even when the sum of RGRs was larger than 0.1 g g^{-1} (ºC d)$^{-1}$. This suggests that pollination nullified the effect of the sum of RGRs on fruit abortion. It is well known that pollination stimulates the synthesis of plant hormones such as auxin.

Although Marcelis *et al.* (2004) emphasized the importance of the source–sink relationship in the abortion of flowers or fruit of sweet pepper, they also admitted the involvement of some dominance effect. It is well known that actively growing fruit produce plant hormones that might be involved in fruit retention (Huberman *et al.*, 1997; Takeno and Ise, 1992; Wien *et al.*, 1989). It is reported, however, that the availability of photoassimilates is associated with the occurrence of fruit abortion in plants other than cucumber (Aloni *et al.*, 1996; Guinn, 1974; Kinet, 1977; Marcelis *et al.*, 2004; Wien *et al.*, 1989). Therefore, although the relationship between 1/CT and the sum of the GR was not clear, the possibility that fruit abortion is determined by the availability of photoassimilates could not be ruled out.

In parthenocarpic fruit that reached 5 g, 1/CT ranged from 0.0058 to 0.0134 (Fig. 6a). This indicates that fruit that did not reach 5 g by 74-171ºC d of the CT, aborted in the parthenocarpic treatment. Hikosaka and Sugiyama (2005) reported that parthenocarpic fruit whose growth was suppressed by up to 10 d (ca. 175ºC d) usually aborts. As such, it is likely that the value of 1/CT changed abruptly from 0.0058 to zero in the present experiment.

In conclusion, the initial slow growing period of cucumber can be completed if the competing potential of fruit that flowered early was low. Also, the competing potential can be evaluated by the sum of RGRs of fruit at lower nodes, not by the sum of fruit weight.

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Genetics of corolla colour in periwinkle: relationship between genes determining violet, orange-red and magenta corolla

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Abstract

In periwinkle (*Catharanthus roseus*), pink, white and red-eyed (white corolla with red centre) are three common corolla colours, which are due to epistatic interaction between two genes *R* and *W*. Violet corolla, which is not found commonly in natural populations, is reported to be due to another gene *B* which blues the pigment in pink genotypes (*R- W-*). Recently, another gene *O* and its allele O^m have been reported to determine two uncommon corolla colours, orange-red and magenta corolla, respectively. Since, the relationship between genes determining violet, orange-red and magenta corolla was not known, a strain possessing violet corolla and white eye (VI) was crossed with strains possessing orange-red corolla and white eye (OR) and magenta corolla and white eye (MJ-1) to study: (i) the relationship between genes involved in the production of violet, orange-red and magenta corolla, (ii) to study the possibility of producing novel corolla colours and (iii) to determine the validity of the gene interaction models proposed earlier. The F_1 plants of both crosses, VI x OR and VI x MJ-1, had violet corolla. The F_2 generation of the cross VI x OR segregated into plants with (i) violet corolla, (ii) pink corolla, (iii) orange-red corolla, and (iv) white corolla in the ratio of 45:12:3:4, while the progeny of the backcross F_1 x OR segregated into three types of plants, (i) violet corolla, (ii) pink corolla, and (iii) orange-red corolla in the ratio of 2:1:1. The F_2 generation of the cross VI x MJ-1 segregated into five kinds of plants *viz.*, (i) violet corolla, (ii) pink corolla, (iii) magenta corolla, (iv) rose corolla, and (v) white corolla in the ratio of 144:48:12:36:16, while the progeny of the backcross, F_1 x MJ-1 segregated into four types of plants *viz.*, (i) violet corolla, (ii) magenta corolla, (iii) rose corolla and (iv) pink corolla in the ratio of 1:1:1:1. The results suggested that genes involved *B, R, W,* O/O^m and *J* were inherited independently and that the gene *B* blued the corolla pigment in *B-RRwwO-* genotypes but not in *B-RRwwO*^{m}*-jj* and *B-RRwwO*^{m}*-JJ* genotypes. No new corolla colours were observed in the studied crosses due to the interaction between genes governing violet, orange-red and magenta corolla. The observed segregation for different corolla colours in the studied crosses was same as that expected from independent segregation and known interactions between the genes involved, validating the earlier proposed models.

Key words: *Catharanthus roseus*, ornamental plant, medicinal plant, corolla colour, inheritance

Introduction

Periwinkle [*Catharanthus roseus* (L.) G. Don] is commercially grown for the extraction of highly valued anti-cancer and anti-hypertension alkaloids, which are present in its leaves and roots, respectively. It is also widely grown as a flowerbed plant in gardens because of its coloured flowers and hardy nature. The most commonly observed corolla colours in periwinkle are pink, red-eyed (white corolla with red eye), and white. Apart from these common corolla colours, uncommon corolla colours *viz.*, violet, orange-red, scarlet-red, magenta and rose corolla colours have also been reported. These corolla colours pink, red-eyed, pale pink, white, violet, orange-red, scarlet-red, magenta and rose have been found to be determined by interactions between alleles at seven loci, *A*, *R*, *W*, *B*, *I,* O/O^m, and *J* (Flory, 1944; Simmonds, 1960; Milo *et al.*, 1985; Kulkarni *et al.*, 1999; Sreevalli *et al.*, 2002; Kulkarni *et al.*, 2005). Genes *A* and *R* were reported to be basic complementary genes without both of which flowers would have white corolla (Simmonds, 1960). The genotypes *A-R-W-* produce pink corolla. The *R* allele produces anthocyanin pigments in the center of the corolla and is epistatic to the *W* and *I* alleles, *i.e.* they function only in the presence of *R* allele. In the presence of the *W* allele, the pigment in the eye region spreads into the corolla limb. The *I* allele also produces anthocyanin pigments but in smaller quantities, and produces pale pink corolla with red eye in the absence of the *W* allele (Milo *et al.*, 1985). The gene *B* is a copigmentation gene which blues the pigment in *R-W-* genotypes resulting in violet corolla with purple eye and, probably, also in *R-ww* genotypes resulting in white corolla with purple eye (Simmonds, 1960). A newly identified gene *O* was found to be responsible for the production of orange-red corolla even in the absence of *R* allele, the basic gene required for the production of coloured corolla. The gene *O*, however, did not express in the presence of the *W* allele. Another gene O^m, allelic to the gene *O*, was found to be responsible for the production of magenta corolla. An inhibitory gene *J*, which by itself did not produce any corolla colour, partially inhibited gene O^m to produce rose corolla. The allele O^m, like the *O* allele, does not express in the presence of the *W* allele, but unlike the *O* allele produces magenta corolla only in the presence of the *R* allele. Heterozygotes O/O^m were found to produce scarlet-red corolla (Kulkarni *et al.*, 2005). Another gene *E* was found to determine the presence or absence of pigmentation in the eye region *i.e.*, in the center of the corolla (Sreevalli *et al.*, 2002).

Periwinkle plants with violet corolla colour are not common

in naturally growing populations of periwinkle. Although the genetics of violet corolla colour has been reported earlier (Simmonds, 1960), its relationship with the recently reported genes determining novel corolla colours *viz.*, orange-red and magenta (Kulkarni *et al.*, 2005), is not known. A knowledge of genes and gene interactions involved in the production of different corolla colours in ornamental plants is essential for understanding the biochemical basis of different corolla colours as well as for producing novel corolla colours either by conventional or molecular breeding. (Meyer *et al.*, 1987; Forkmann, 1991; Davies *et al.*, 2003; Zufall and Rausher, 2003). We recently obtained a strain, designated as VI, possessing violet corolla and white eye from a local dealer in horticultural plants and crossed it with strains possessing orange-red corolla and white eye (OR) and magenta corolla and white eye (MJ-1) to study (i) the relationship between genes involved in the production of violet, orange-red and magenta corolla, (ii) to study the possibility of producing novel corolla colours and (iii) to determine the validity of the gene interaction models proposed earlier.

Materials and methods

Three strains, with orange-red, magenta and violet corolla colours, were procured from a local dealer in horticultural plants and were designated as OR, MJ-1 and VI, respectively. All the three strains had white eye and were found to be true-breeding for their respective corolla colours and white eye. The strain VI was crossed reciprocally with strains OR and MJ-1 to determine the relationship between genes determining violet, orange-red and magenta corolla colours. Parental plants were raised in a glass house from seeds obtained by artificial self-pollination. Reciprocal crosses were also made as described earlier (Kulkarni *et al.*, 2001). The F_1 plants were selfed and also backcrossed to both the parents. Seedlings of parental, F_1, F_2 and backcross generations were raised from their respective seeds, in perforated plastic trays filled with sterilized soil. Three months old seedlings of all six generations were transplanted into perforated polythene bags containing a mixture of soil, sand and farm yard manure in the ratio of 1:1:1 and placed on the benches in the glass house. The plants were scored for corolla colour after they flowered. Chi-square and G tests were used for testing the goodness of fit of observed and expected frequencies of different phenotypic classes in the F_2 and backcross generations (Sokal and Rohlf, 1981).

Results and discussion

Cross: **VI (violet corolla and white eye) x OR (orange-red corolla and white eye)**: Since both parental strains had white eye, all plants of F_1, F_2 and backcross generations also had white eye. Therefore, only corolla colours of plants of these generations are mentioned in the following. Also since both parental strains had coloured corolla they are expected to be homozygous for both *A* and *R* alleles and therefore, the genotype at *A* locus of different phenotypes observed in the F_2 and backcross generations is not mentioned in the following.

The flowers of F_1 plants had violet corolla resembling the parent VI. There were no differences in the corolla colour of F_1 plants of reciprocal crosses. The progeny of the F_2 generation could be classified into four classes based on their corolla colour, namely, (i) violet corolla, (ii) pink corolla, (iii) orange-red corolla, and (iv) white corolla. The observed ratio of these four corolla colours fitted a ratio of 45:12:3:4 both by Chi-square and G tests (Table 1).

The progeny of the backcross, F_1 x OR, produced three types of plants namely those with (i) violet corolla, (ii) pink corolla, and (iii) orange-red corolla and their observed frequencies fitted a ratio of 2:1:1 both by Chi-square and G tests (Table 1). The progeny of the backcross, F_1 x VI, produced all plants with violet corolla.

From an earlier study, it was known that the genotype of the parental strain OR with orange-red corolla and white eye is *eeRRwwOO* and that the *O* allele produces orange-red corolla even in the absence of *R* allele but does not express in the presence of *W* allele (Sreevalli *et al.*, 2002). According to Simmonds (1960), violet corolla is due to the interaction between *R, W,* and *B* genes *i.e.*, the gene *B* blues the pink corolla colour in plants with *R-W-* genotypes. Therefore, the genotype of the strain VI with violet corolla and white eye should be *eeRRBBWW.* Thus, the genotypes of parental strains OR and VI with respect to *B, R, O, W* and *E* would be *eebbRRwwOO* and *eeBBRRWWoo*, respectively. With these genotypes of the parental strains OR and VI and above mentioned interactions between these genes and their independent segregation, the expected frequencies of different genotypes and their phenotypes in F_2 and backcross generations are given in Table 1. The observed frequencies of plants with different corolla colours in both F_2 and backcross generations fitted the expected frequencies (Table 1). It was not known if the *B* gene would blue the orange-red corolla of *B-RRwwO-* genotypes. If the gene *B* did not blue the pigment to produce violet corolla colour in *B-RRwwO-* genotypes, then the expected ratio of different corolla colours in the F_2 generation would be 36 violet: 12 pink: 12 orange-red: 4 white. This ratio, however, did not fit the observed ratio of plants with these corolla colours ($\chi^2_{(d.f.3)}$ =13.573, P = <0.01, $G_{(d.f.3)}$ = 17.962, P = <0.01). If the gene *B* did produce violet corolla in *B-RRwwO-* genotypes, the expected ratio of corolla colours in the F_2 generation would be 45 violet: 12 Pink: 3 orange-red: 4 white. The observed ratio of corolla colours in the F_2 generation, as already mentioned above, fitted this expected ratio (Table 1).

Cross: **VI (violet corolla and white eye) x MJ-1 (magenta corolla and white eye)**: Since the parental strains had white eye, all plants of F_1, F_2 and backcross generations also had white eye. Therefore, only corolla colours of plants of these generations are mentioned in the following. Also since both parental strains had coloured corolla they are expected to be homozygous for both *A* and *R* alleles and therefore, the genotype at *A* locus of different phenotypes in the F_2 and backcross generations is not mentioned in the following.

The F_1 plants of this cross also had violet corolla. There were no differences in the corolla colour of F_1 plants of reciprocal crosses. In the F_2 generation, plants could be classified into five classes, namely, (i) violet corolla, (ii) pink corolla, (iii) magenta corolla, (iv) rose corolla, and (v) white corolla. The observed frequencies of these five kinds of plants fitted a ratio of 144:48:12:36:16 (Table 2).

The progeny of the backcross, F_1 x VI had violet corolla while the progeny of the backcross F_1 x MJ-1 segregated into four types of plants *viz.*, those with (i) violet corolla, (ii) magenta corolla, (iii) rose corolla and (iv) pink corolla in the ratio of 1:1:1:1 (Table 2).

Table 1. Phenotypes, genotypes, expected proportions, and observed and expected frequencies of plants with different corolla colours in the parental, F_1, F_2 and backcross generations of the cross VI (violet corolla and white eye) x OR (orange-red corolla and white eye) in periwinkle, according to the proposed model

Generation	Phenotype	Genotype	Expected proportion	Observed frequency	Expected frequency
OR	Orange-red corolla and white eye	*eebbRRwwOO*	1	All	All
VI	Violet corolla and white eye	*eeBBRRWWoo*	1	All	All
F_1	Violet corolla and white eye	*eeBbRRWwOo*	1	All	All
F_2	Violet corolla and white eye	*eeB-RRW-O-*	27/64		
		eeB-RRW-oo	9/64		
		eeB-RRwwO-	9/64		
		Total	45/64	70	71.7
	Pink corolla and white eye	*eebbRRW-O-*	9/64		
		eebbRRW-oo	3/64		
		Total	12/64	19	19.1
	Orange-red corolla and white eye	*eebbRRwwO-*	3/64		
		Total	3/64	5	4.8
	White corolla	*eeB-RRwwoo*	3/64		
		eebbRRwwoo	1/64		
		Total	4/64	8	6.4
Total			1	102	102
$\Sigma\chi^2_{(d.f.\ 3)}$ for the expected ratio of 45:12:3:4 = 0.448 (P = 0.95 – 0.90), $G_{(d.f.3)}$ = 0.422 (P = 0.95 – 0.90)					
Backcross					
F_1 x OR	Violet corolla and white eye	*eeBbRRWwO-*	1/4		
		eeBbRRwwO-	1/4		
		Total	2/4	6	6.5
	Pink corolla and white eye	*eebbRRWwO-*	1/4		
		Total	1/4	3	3.25
	Orange-red corolla and white eye	*eebbRRwwO-*	1/4		
		Total	1/4	4	3.25
Total			1	13	13

$\Sigma\chi^2$2(d.f. 2) for the expected ratio of 2:1:1 = 0.230 (P = 0.90 – 0.80), G(d.f. 2) = 0.216 (P=0.90-0.80)

From an earlier study, it was known O^m is allelic to *O* and does not express in the presence of *W* allele. Further, it was also known that unlike its allele *O,* it produces magenta corolla only in the presence of *R* allele, and that an independent gene *J,* which does not produce any corolla colour on its own, partially inhibits the gene O^m to produce rose corolla (Kulkarni *et al.*, 2005). Therefore, the presence of plants with rose corolla in the F_2 and backcross (F_1 x MJ-1) generations suggested the presence of gene *J* in the parent VI. Thus, the genotypes of parental strains VI and MJ-1 would be *eeBBRRWWooJJ* and *eebbRRwwO^{m}O^{m}jj*, respectively. With the already known interactions between genes *R, W, B,* O^m and *J* mentioned above and their independent segregation, expected frequencies of different genotypes and their phenotypes in F_2 and backcross generations are given in Table 2. The observed frequencies of plants with different corolla colours in both F_2 and backcross generations fitted the expected frequencies (Table 2). The allele *B,* however, did not appear to blue the magenta corolla colour in *B-RRwwO^m-jj g*enotypes. If it were to produce violet corolla colour in genotypes *B-RRwwO^m-jj*, the expected ratio of different corolla colours in F_2 would have been 153 violet: 48 pink: 3 magenta: 36 rose: 16 white, which however did not fit the observed ratio ($\chi^2_{(d.f.\ =4)}$=80.756, $P \leq 0.001$; $G_{(d.f.\ =4)}$=39.732 $P \leq 0.001$).

The results of the present study revealed that genes *B, R, W, O/* O^m and *J* involved in the production of violet, pink, orange-red, magenta, rose and white corolla were inherited independently and that only corolla colours, expected on the basis of the gene interaction models proposed earlier (Simmonds 1960, Kulkarni *et al.*, 2005) for the production of these corolla colours were observed in the crosses VI (violet corolla) x OR (orange-red corolla), and VI (violet corolla) x MJ-1 (magenta corolla), validating the proposed genetic models. It was also evident that the gene *B* blued the corolla pigment in *B-RRwwO-* genotypes but not in *B-RRwwO^m-jj* and *B-RRwwO^m-JJ* genotypes and that no new corolla colours were produced due to the interactions between *B, R, W, O* and O^m genes.

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Table 2. Phenotypes, genotypes, expected proportions, and observed and expected frequencies of plants with different corolla colours in the parental, F_1, F_2 and backcross generations of the cross VI (violet corolla and white eye) x MJ-1 (magenta corolla and white eye) in periwinkle, according to the proposed model

Generation	Phenotype	Genotype	Expected proportion	Observed frequency	Expected frequency
VI	Violet corolla and white eye	*eeBBRRWWooJJ*	1	All	All
MJ-1	Magenta corolla and white eye	*eebbRRwwO^{m}O^{m}jj*	1	All	All
F_1	Violet corolla and white eye	*eeBbRRWwO^{m}oJj*	1	All	All
F_2	Violet corolla and white eye	*eeB-RRW-O^m-J-*	81/256		
		eeB-RRW-oo J-	*27*/256		
		eeB- RRW-O^m-jj	27/256		
		eeB-RRW-oojj	9/256		
		Total	144/256	156	165.4
	Pink corolla and white eye	*eebbRRW-O^m-J-*	27/256		
		eebbRRW-ooJ-	9/256		
		eebbRRW-O^m-jj	9/256		
		eebbRRW-oojj	3/256		
		Total	48/256	56	55.1
	Magenta corolla and white eye	*eeB-RRwwO^m- jj*	9/256		
		eebbRRwwO^m-jj	3/256		
		Total	12/256	20	13.8
	Rose corolla and white eye	*eeB-RRwwO^m-J-*	27/256		
		eebbRRwwO^m-J-	9/256		
		Total	36/256	40	41.3
	White corolla	*eeB-RRwwooJ-*	9/256		
		eeB-RRwwoojj	3/256		
		eebbRRwwooJ-	3/256		
		eebbRRwwoojj	1/256		
		Total	16/256	22	18.4
	Total		1	294	294
$\Sigma\chi^2_{(d.f.4)}$ for the expected ratio of 144:48:12:36:16 = 4.077 (P = 0.50 – 0.30), $G_{(d.f.\ 4)}$ = 3.706 (P=0.50 – 0.30).					
Backcross					
F_1 x MJ-1	Violet corolla and white eye	*eeBbRRWwO^m-Jj*	2/16		
		eeBbRRWwO^m-jj	2/16		
		Total	1/4	13	15
	Pink corolla and white eye	*eebbRRWwO^m-Jj*	2/16		
		eebbRRWwO^m-jj	2/16		
		Total	1/4	12	15
	Magenta corolla and white eye	*eebbRRwwO^m-jj*	2/16		
		eeBbRRwwO^m-jj	2/16		
		Total	1/4	16	15
	Rose corolla and white eye	*eebbRRwwO^m-Jj*	2/16		
		eeBbRRwwO^m-Jj	2/16		
		Total	1/4	19	15
Total			1	60	60
$\Sigma\chi^2_{(d.f.3)}$ for the expected ratio of 1:1:1:1 = 1.998 (P = 0.70 – 0.50), $G_{(d.f.\ 3)}$ =1.970 (*P*=0.70 – 0.50)					

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Internal quality characterization and isolation of lycopene specific genes from tomato

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Abstract

Tomato (*L. esculentum* Mill), a popular vegetable in tropics is an excellent source for vitamin A, C, carotenoids and other health related components. It tops the list of industrial crops because of its outstanding processing qualities. It is valued for both its fresh and processed forms. Biochemical analysis in different wild species, varieties and hybrids of tomato showed the wild species, *Lycopersicon pimpinellifolium* LA 1593 to be a rich source for lycopene specific genes. Partial cDNA of lycopene specific *Phytoene desaturase* gene TNAU P was isolated from *L. pimpinellifolium* LA 1593 by RT-PCR technique. Sequence analysis of the partial cDNA showed 99.6% similarity with already available *Phytoene desaturase* gene from *L. esculentum*. Also, the sequence showed considerable homology with *Phytoene dehydrogenase*, *Zeta carotene desaturase* and *Phytoene desaturase* genes from Gentian, *Oryza, Momardica,* citrus and pea. The high intensity of the amplified product in *L. pimpinellifolium* coupled with 99.6 % homology to *L. esculentum* inferred that the level of expression of *Phytoene desaturase* is more in *L. pimpinellifolium*. Isolation of *Phytoene desaturase* genes can be further exploited to produce transgenic plants with increased content of lycopene by transferring the genes from wild species to cultivars.

Key words: *Lycopersicon pimpinellifolium, Phytoene desaturase,* RT-PCR, lycopene

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops grown throughout the world for fresh consumption as well as for processing. In terms of human health, tomato is a major component of daily meals in many countries and constitutes an important source of minerals, vitamins and antioxidant compounds. Tomato fruit quality for fresh consumption is determined by appearance (color, size, shape), firmness, texture, dry matter, organoleptic (flavor) and nutraceutical (health benefit) properties. The organoleptic quality of tomato is mainly attributed to its aroma volatiles, sugar and acid content, while the nutraceutical quality is defined by its mineral, vitamin, carotenoid and flavonoid content.

Several works have established the role of soluble solid content (SSC), acids and sugars in the taste and flavour intensity of tomato fruits. It is well known that sweetness has a high correlation with SSC, pH and reducing sugars, and sourness has a high correlation with pH and, to a lesser degree with titrable acidity (TA) (Baldwin *et al.*, 1998). This happens because the major constituents of SSC content in *Lycopersicon* fruits are all soluble sugars. The pH and TA are good measures of free acid and H-ion concentrations, responsible for the sourness of a solution (Stevans *et al.*, 1977). Moreover SSC, sugars and TA highly contribute to overall flavour intensity (Baldwin *et al.*, 1998). Nevertheless, SSC, pH and TA are ambiguous variables since the profile and content of the substances that contribute to them can vary greatly between accessions. These variations can be large if accessions of different species related to cultivated and wild tomato are characterized.

The most abundant phytonutrients in tomatoes are the carotenoids (Gann *et al.*, 1999). Lycopene is the most prominent carotenoid followed by gamma carotene, zeta-carotene, phytofluene, phytoene, neurosporene and lutein (Leonardi *et al.*, 2000; Clinton, 1998). Research indicates that lycopene supplements and lycopene-rich foods such as tomato sauce support fertility, promote heart health, protect against Alzheimer's disease and several types of cancer (Hadley *et al.*, 2002). Tomato also contain other components that are beneficial to human health including vitamin A, trace elements, flavonoids, phytosterols, several water soluble vitamins and two other cancer-fighting phytochemicals, courmaric and chlorogenic acids (Campbell *et al.*, 2004).

Although tomato is a source of phytonutrients, the levels are not sufficient to meet out the daily requirements. Also, compared to wild varieties of tomato, current tomato cultivars are relatively low in the important phytochemical, lycopene. Among wild species of tomato *L. pimpinellifolium* is reported to be a potential source of genes for specific antioxidants like lycopene, total phenolics and beta carotene. *L. pimpinellifolium,* also known as the currant tomato, produces tiny fruits which contain over 40 times more lycopene than domesticated tomatoes (Grolier and Rock, 1998).

The objective of this work is to characterize and classify different tomato genotypes according to their usefulness for internal quality breeding programs of fresh tomato. The variables considered in this study are related to the nutritional and qualitative aspects, emphasizing the sugar, acid composition and carotenoids of the samples. Also isolation and characterization of lycopene specific genes was carried out from a wild species *L. pimpinellifolium.*

Materials and methods

Plant materials: The plant materials used in the present study includes young fruits of *Lycopersicon spp*. Wild species used in the study included *L. peruvianum* - EC 52071 obtained from NBPGR, New Delhi and *L. pimpinellifolium* – AC No LA 1593, LA 1582, LA 1586 obtained from AVRDC, Taiwan. Other local cultivars used in the study included CO 3, PKM 1 and hybrids, Ruchi, COLCRH 1, COLCRH 2, COLCRH 3 collected from Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore.

Analytical methods: Fresh fruits were homogenized in a laboratory blender. Aliquots were taken to analyse pH, total soluble solids, titrable acidity, vitamin C, total sugars, carotenoids and lycopene. pH was determined by using ELICO LI 127 pH meter. Total soluble solids content was determined by using a 'Zeiss' hand refractometer and the results are reported as ^{o}Brix. Total sugars and reducing sugars were quantified spectrophotometrically of soluble sugars and available carbohydrates, after hydrolysis of complex carbohydrates with hydrochloric acid using Nelson's arseno molybdic reagent (Somogyi, 1952). Titrable acidity was measured by titration with 0.1N NaOH and phenolphthalein, and the results were expressed as percent citric acid (A.O.A.C, 1975). Ascorbic acid was measured by titration with 4% oxalic acid and 2,6, dichlorophenol indophenol dye solution and the results were expressed as mg/100g (A.O.A.C, 1975). Total carotenoids and lycopene were quantified spectrophotometrically and expressed as mg/100 g of sample (Ranganna, 1979).

Statistical analysis: An analysis of variance (ANOVA) was performed on the data for different genotypes related to quality parameters, followed by mean separation with Fisher's protected least significant difference test (PLSD, at P=0.05). All the statistical work were analyzed using AGRES Version 7.01 software to determine differences between means.

Gene isolation studies: For RNA isolation, the frozen pericarp tissues of different wild species/ cultivars/ hybrid tomato genotypes were ground to a fine powder in liquid nitrogen, homogenized and total RNA was isolated as described by Chomczynski and Sacchi (1987) using TRIzol reagent. mRNA was isolated separately from total fruit RNA using GenElute™ mRNA Miniprep Kit (Sigma Aldrich Chemicals Pvt. Ltd., USA).

For cloning the cDNA and determining the expression levels, reverse transcription of the isolated fruit mRNA was performed in a 20µl reaction mixture containing: 50 ng of mRNA, RNAse inhibitor 1.0 µL, 0.1 M DTT 1.0 µl, RT buffer (5X) 4.0 µL, 30 mM dNTP mix 2.0 µL, AMV reverse transcriptase 0.5 µL (Bangalore Genei). The samples were incubated at 42°C for 1 h and thereafter the reverse transcriptase was inactivated by heating at 97°C for 5 min.

Gene specific primers were designed based on the already available sequence for *Phytoene desaturase* gene from *L. esculentum* (Acc. No. X59948). The primer sequences were as follows:

TNAU P F : 5' TGGAGGCAAGGGATGTTC 3'
TNAU P R : 5' GCTTCACCTCGCACTCTTCTTC 3 '
PCR reactions were performed in a total volume of 50µL containing: First strand cDNA 3.0 µL, 10X PCR buffer 5.0 µL, 30 mM dNTP mix (7.5 mM each) 1.0µL, forward gene specific primer (10 pmol/µL) 1.5 µL, reverse gene specific primer (10 pmol/µl) 1.5 µL, *Taq* DNA polymerase (3 U/µL) (Genei) 1.0 µL, sterile water 37.0 µL in 0.2mL thin walled tubes, using thermal cycler (Epppendorf). The PCR cycling profile was denaturation at 92°C for 1 min, annealing at 60°C for 1 min and an extension at 72°C for 1.5 min for 30 cycles. The amplification products were separated by electrophoresis on 1% agarose gel containing ethidium bromide and photographed using Alpha Imager TM 1220 Documentation and Analysis Systems. The amplified cDNA products obtained in PCR reactions were loaded separately on 1.5% low melting agarose gel, electrophoresed and eluted using GenElute™ Gel Extraction Kit (Sigma Aldrich Chemicals Pvt. Ltd., USA).

Cloning and sequencing of TNAU P cDNA: The eluted 1400 bp cDNA product obtained from RT-PCR using the above gene-specific primers were cloned in pTZ57R/T vector (InsT/A clone™ PCR Product Cloning Kit, MBI Fermentas Inc., USA) using T/A cloning strategy. Sequencing of the clone was done using the M13 primer sequences present in the pTZ57R/T vector in a automated sequencer model 3100 version 3.0 (ABI PRISM) at the DBT- supported DNA sequencing facility at University of Delhi South Campus, New Delhi.

Analysis of the sequence data of the cDNA clone was performed using various bioinformatics tools *viz.*, BLAST, BLASTp, CLUSTALW, FASTA, GeneRunner and Treetop.

Table 1. Quality parameters in different wild species/varieties/hybrid tomato

Sl. No.	Species	pH	Total soluble solids (°Brix)	Titrable acidity (%)	Ascorbic acid (mg 100g^{-1})	Total sugars (mg 100g^{-1})	Reducing sugars (mg 100g^{-1})	Carotenoids (mg 100g^{-1})	Lycopene (mg 100g^{-1})
1.	COLCRH 1	4.18	4.7	0.51	23.38	4.14	3.96	3.91	6.39
2.	COLCRH 2	4.29	4.69	0.52	23.29	4.18	3.43	3.72	6.84
3.	COLCRH 3	4.28	4.65	0.54	22.68	4.16	3.20	3.94	6.61
4.	Ruchi	4.18	4.9	0.52	23.84	4.22	3.06	3.52	6.54
5.	CO 3	4.16	4.74	0.51	23.29	4.32	4.29	3.78	6.42
6.	PKM 1	4.26	4.70	0.54	23.74	4.07	3.34	3.70	6.53
7.	*L. pimpinellifolium* LA 1593	5.26	5.32	0.30	25.48	3.69	3.87	4.47	7.12
8.	*L. pimpinellifolium* LA 1582	5.28	5.33	0.32	25.42	3.44	3.62	4.36	7.05
9.	*L. pimpinellifolium* LA 1586	5.22	5.36	0.33	24.80	3.45	3.75	4.42	7.07
10.	*L. peruvianum* EC 52071	5.38	5.28	0.34	25.97	3.86	3.79	4.54	6.85
Grand Mean		4.65	4.97	0.44	24.19	3.93	3.63	4.04	6.74
SEd		0.03	0.02	0.02	0.02	0.10	0.03	0.03	0.03
CD (P=0.05)		0.06	0.03	0.04	0.05	0.02	0.06	0.08	0.06

Results and discussion

Fruits of different wild species, varieties and hybrids were utilized for estimating different biochemical parameters and the results of the analysis are shown in Table 1.

The pH and titrable acidity (TA) are good measures of free H^+ ion concentration responsible for the sourness of a solution (Stevans *et al.*, 1977). A great pH variability was observed (4.16-5.38) and the results clearly separate *L. peruvianum* from *L. pimpinellifolium* accessions. Lower pH values were observed for cultivar CO 3 (4.16). Hybrids selected in this study showed pH values in the range of 4.18 - 4.29. Thangam (1998) reported pH values of 4.18 and 4.29 for hybrids Iswaraya Lakshmi and Sadabahar, respectively. However the wild species, *L. peruvianum* and *L. pimpinellifolium* species showed a higher pH range (5.22-5.38). Based on the pH values, the wild species, varieties and hybrids can be classified for their acidic nature. All the varieties and hybrids were grouped under 'acid I' category (pH 4.6-3.7), as they had pH values between 4.3-4.2. The wild species *L. pimpinellifolium* was grouped under 'semi acid' (pH 5.3-4.6) as all the accessions studied had pH values in the range of 5.3-5.2. *L. peruvinaum* was grouped under 'non-acidic' pH 7.0-5.3 as it had a pH value of 5.4.

The total soluble solids (TSS) is an important trait for processing tomato. The flavour of the tomato products depends upon the TSS content of the fruits (Ruiz *et al.*, 2004). The TSS of different varieties and hybrids chosen for this study ranged from 4.65- 4.90 °Brix. Raghupathy (2004) reported a TSS of 4.7 °Brix in PKM 1. Thangam (1998) reported a TSS of 4.65 °Brix in the hybrid, IHR-709 and 4.90 in the hybrid, Avinash. Higher mean value of 5.36 °Brix were observed in the small fruited wild species *L. pimpinillifolium* LA 1586. Other wild species chosen in the study recorded a TSS in the range of 5.28 - 5.33 °Brix.

The nutritive quality of the tomato fruit is also assessed by the acidity and ascorbic acid content. A hybrid with low acid content is preferred for fresh market. But for processing purpose, varieties with high acid content (0.50 %) is desirable. In the present study all varieties and hybrids gave a titrable acidity greater than 0.50 %. Thamarai Selvan (2004) reported acidity values of 0.5 and 0.54 % for CO 3 and PKM 1, respectively.

Minimum level of ascorbic acid needed for processing is 23mg $100g^{-1}$ and for canning purposes is 17mg $100g^{-1}$. The varieties and hybrid chosen in the study recorded ascorbic acid contents in the range of 24–26 mg $100g^{-1}$ indicating their suitability for processing purposes.

Apart from nutritional benefits, ascorbic acid is reported to have a role in nematode resistance. The role of ascorbic acid, ascorbate oxidase, hydroxyproline containing proteins imparting root-knot resistance has been reviewed by Arrigoni (1979). It has been reported that increase in the concentration of ascorbic acid leads to enhanced synthesis of hydroxyl proline rich proteins which in turn is reflected as resistance expression. In the present study, *L. peruvianum* had the highest ascorbic acid content of about 25.97 mg $100g^{-1}$. Also *L. peruvianum* was found to be resistant to root knot nematode. The increased ascorbic acid content of *L. peruvianum* may attribute to its increased nematode resistance.

The sugars make an important contribution to the flavour of

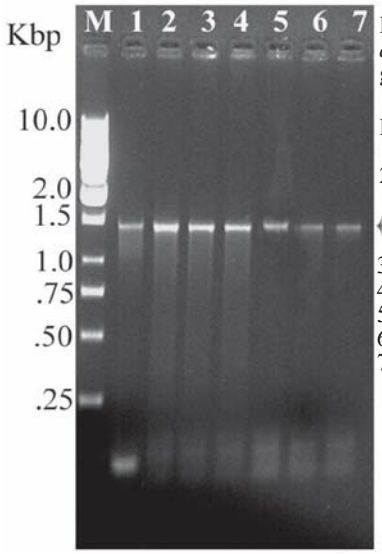

Fig. 1. Amplified *Phytoene desaturase* cDNA in different genotypes of tomato

M- 1 Kb DNA ladder,
1- *L. peruvianum* EC 52071,
2- *L. pimpinellifolium* LA 1593,
3- *L. pimpinellifolium* LA 1586,
4- *L. pimpinellifolium* LA 1582,
5- CO3,
6- PKM 1,
7- Ruchi

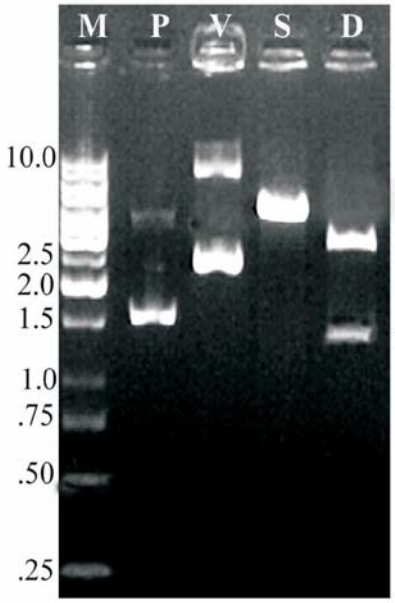

Fig. 2(a). Confirmation of recombinant clones by restriction digestion

M- 1 Kb DNA ladder
P- Undigested plasmid with insert
V- pTZ 57R/T vector
S- Restricted plasmid digested with *Xba* I
D- Restricted plasmid digested with *Xba* I and *BamH* I
Arrows indicate released cDNA fragments after digestion

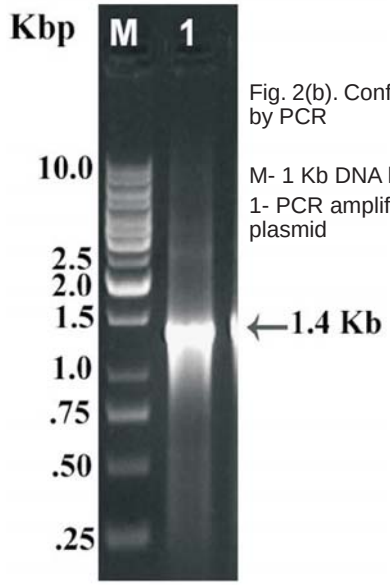

Fig. 2(b). Confirmation of recombinant clones by PCR

M- 1 Kb DNA ladder
1- PCR amplified products from recombinant plasmid

tomato and generally the reducing sugars contribute at least to 50% of total sugars. In the present study, a total sugar content of 4.32% and a reducing sugar content of 3.96% were observed in cultivar CO 3. High total sugar of 3.94% was reported by Khandaker *et al.* (1994) in a tomato variety, Pelican. There was not much difference between wild species and other cultivars with respect to total and reducing sugar contents.

Bright red colour of the fruit is one of the quality factors which is due to lycopene content. It is an important attribute for fresh market as well as for processing (Nicolle *et al.*, 2004; Fraser *et al.*, 1994; Ronen *et al.*, 2000). In the present study, the wild species, *L. pimpinellifolium* LA 1593 had the highest lycopene content (7.12 mg) among all the samples studied. Besides imparting colour, lycopene is also reported to be an antioxidant which protects against several types of cancer (Hadley *et al.*, 2002; Canene-Adams *et al.*, 2005).

Using first strand cDNA obtained from fruit mRNA of wild species, variety and hybrid fruits as template and with phytoene desaturase specific forward (5') and a reverse (3') primers the second strand cDNA synthesis was carried out. A cDNA fragment of 1.4 Kb were obtained in all the samples analyzed (Fig. 1). The cDNA fragments were intense in all the *L. pimpinellifolium* accessions when compared to the varieties CO 3, PKM 1 and hybrid Ruchi lanes and the wild species *L. peruvianum* EC 52071. The cDNA fragment of 1.4 Kb obtained by RT-PCR using the gene specific primers from *L. pimpinellifolium* LA 1593 was cloned in pTZ57R vector (Invitrogen) using T/A cloning strategy. The recombinant cDNA clone was confirmed for the presence of insert using restriction

Fig. 3. Multiple sequence alignment of TNAU P with other carotenoid genes

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TNAU P                          ------------SRMHLDLEARDVLGGKVAAWKDDDGDWYETGLHIVFGAYPNIQNLFG 47
PHYTOENE DEHYDROGENASE          LGGLSTAKYLADAGHKPILLEARDVLGGKVAAWKDDDGDWYETGLHIFFGAYPNIQNLFG 180
GENTIAN PDS                     LAGLTTAKYLADAGHKPILLEARDVLGGKVAAWKDDDGDWYETGLHIFFGAYPNVQNLFG 176
CITRUS PDS                      LAGLSTAKYLADAGHKPLLLEARDVLGGKVAAWKDGDGNWYETGLHIFFGAYPNIQNLFG 149
ORYZA PDS                       LAGLSTAKYLADAGHKPILLEARDVLGGKIAAWKDEDGDWYETGLHIFFGAYPNIQNLFG 161
MOMARDICA PDS                   LAGLSTAKYLADAGHKHVLLEARDVLGGKVAAWKDNDGDWYETGLHIFFGAYPNLQNLFG 173
CAROTENE 7,8 DESATURASE         LAGLSCAKYLADAGHTPFVYEARNVLGGKVAAWKDDDGDWYETGLHIFFGAYPNMLQLFK 69
PEA PDS                         ------------------------------------------------------------
ZETA CAROTENE DESATURASE        VAGLSAAIELVDRGHTVELYEKRKVLGGKVSVWKDSDGDSIESGLHIVFGGYTQLQKYLD 69

TNAU P                          ELGINDRLQWKEHSMIFAMPSKPGEFSRFDFSEALPAPLNGILAILKNNEMLTWPEKVKF 107
PHYTOENE DEHYDROGENASE          ELGINDRLQWKEHSMIFAMPSKPGEFSRFDFSEALPAPLNGILAILKNNEMLTWPEKVKF 240
GENTIAN PDS                     ELGINDRLQWKEHSMIFAMPNKPGEFSRFDFAEVLPAPLNGIWAILKNNEMLTWPEKVKF 236
CITRUS PDS                      ELGINDRLQWKEHSMIFAMPNKPGEFSRFDFPEVLPAPLNGILAILRNNEMLTWPEKVKF 209
ORYZA PDS                       ELGINDRLQWKEHSMIFAMPNKPGESSRFDFPETLPAPLNGIWAILRNNEMLTWPEKVKF 221
MOMARDICA PDS                   ELGINDRLQWKEHSMIFAMPNKPGEFSRFDFPEVLPAPINGIWAILRNNEMLTWPEKVKF 233
CAROTENE 7,8 DESATURASE         ELDIEDRLQWKSHSMIFNQPEEPGTYSRFDFPD-LPAPINGVAAILSNNDMLSWPEKISF 128
PEA PDS                         ----------KEHSMIFAMPSKPGQFSRFDFLEVLPSPLNGIWAILRNNEMLTWPEKIKF 50
ZETA CAROTENE DESATURASE        KIGAGDNYLWKDHSLIYAESD--GKQSFFKKAN-LPSPWAEVVGGLQADFLTMW-DKISL 125

TNAU P                          AIGLLPAMLGGQSYVEAQDGISVKDWMRKQGVPDRVTDEVFIAMSKALNFINPDELSMQC 167
PHYTOENE DEHYDROGENASE          AIGLLPAMLGGQSYVEAQDGISVKDWMRKQGVPDRVTDEVFIAMSKALNFINPDELSMQC 300
GENTIAN PDS                     AIGLVPAILGGQPYVEAQDGITVKDWMRKQGVPDRVTEEVFIAMSKALNFINPDELSMQC 296
CITRUS PDS                      AIGLLPAIIGGQAYVEAQDGLTVQEWMRKQGVPDRVTTEVFIAMSKALNFINPDELSMQC 269
ORYZA PDS                       ALGLLPAMVGGQAYVEAQDGFTVSEWMKKQGVPDRVNDEVFIAMSKALNFINPDELSMQC 281
MOMARDICA PDS                   AIGLLPAMLGGQPYVEAQDGLTVQEWMRNRGVPDRVTTEVFIAMSKALNFINPDELSMQC 293
CAROTENE 7,8 DESATURASE         GLGLVPAMLRGQNYVEDCDKYSWTEWLKKQNIPERVNDEVFIAMSKALNFIGPDEISSTV 188
PEA PDS                         AIGLLPAILGGQAYVEAQDGVSVKEWMRKQGIPERVTDEVFIAMSKALNFINPDELSMQC 110
ZETA CAROTENE DESATURASE        IKGLWPALAGNEEYFRSQDHMTYSEWHRLHGASEHSLQKLWRAIALAMNFIEPNVISARP 185

TNAU P                          ILIALNRFLQEKHGSKMAFLDGNPPERLCMPIG-------------------------- 200
PHYTOENE DEHYDROGENASE          ILIALNRFLQEKHGSKMAFLDGNPPERLCMPIVEHIESKGGQVRLNSRIKKIELNEDGSV 360
GENTIAN PDS                     ILIALNRFLQEKHGSKMAFLDGNPPERLCMPIADHIQSRGGEVRLNSRIQRIELNEDGSV 356
CITRUS PDS                      ILIALNRFLQEKHGSKMAFLDGNPPERLCLPIVEHIQSLGGEVRLNSRVQKIELNDDGTV 329
ORYZA PDS                       ILIALNRFLQEKHGSKMAFLDGNPPERLCMPIVDHVRSLGGEVRLNSRIQKIELNPDGTV 341
MOMARDICA PDS                   ILIALNRFLQEKHGSKMAFLDGNPPERLCEPVVEHIRSLGGEVRLNSRIQKIELNNDGTV 353
CAROTENE 7,8 DESATURASE         LLTALNRFLQEKNGSKMAFLDGAPPERLCQPIVDHIRTLGGDVFLNSPLKKINLKEDGSV 248
PEA PDS                         ILIALNRFLQEKHGSKMAFLDGNPPERLCMPIVDHIQSLGGEVHLNSRIKSIELNDDSTV 170
ZETA CAROTENE DESATURASE        ILTIFKYFGTDYAATKFAFFRKNPGDSMIEPMRQYIQSKGGRIFIDARLSRFELNDDKTI 245
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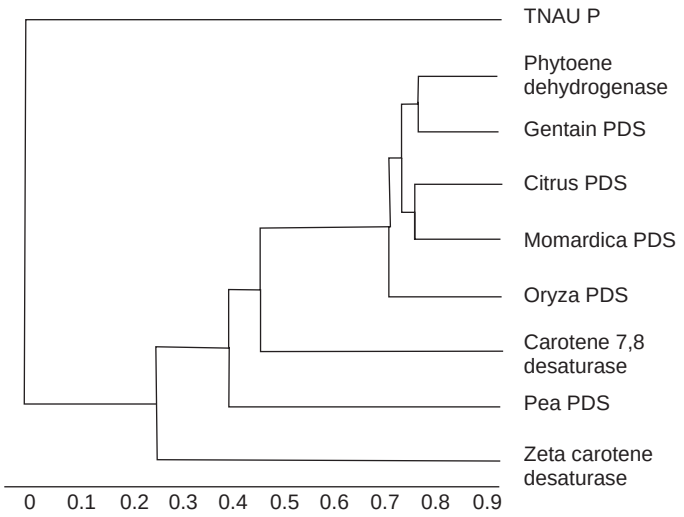

Fig. 4. Phylogenetic grouping of TNAU P with other carotenoid genes
TNAU P – *Phytoene destaurase* gene from *L. pimpinellifolium* LA 1593

Phytoene dehydrogenase	*Phytoene dehydrogenase* from *L. esculentum*
Gentian PDS	*Phytoene desaturase* gene from *Gentian lutea*
Citrus PDS	*Phytoene desaturase* gene from *Citrus sinensis*
Oryza PDS	*Phytoene desaturase* gene from *Oryza sativa*
Momardica PDS	*Phytoene desaturase* gene from *Momardica charantia*
Carotene 7,8 desaturase	Carotene 7,8 desaturase

digestion and PCR analysis (Fig. 2a, 2b). The confirmed recombinant clone was sequenced and named as TNAU P. The sequences were submitted in National Centre for Biotechnology Information (NCBI), New York, USA. The NCBI genebank accession number for the sequence corresponding to *Phytoene desaturase* gene, named TNAU P from *L. pimpinellifolium* is DQ911639. The high level of lycopene in the wild species, *L. pimpinellifolium* may be due to increased expression of the *PDS* gene. This view was also supported by the fact that the amplified product of *Phytoene desaturase* gene on agarose gel was more intense in the wild species, *L. pimpinellifolium* compared to that of other varieties, hybrids and *L. peruvianum*.

The sequence annotation results and BLAST analysis of cDNA clone TNAU P from *L. pimpinellifolium* LA 1593 revealed 99.6% homology to that of the already reported sequence of *L. esculentum, Phytoene desaturase* gene using BLAST software.

The nucleotide sequence of TNAU P was translated to protein sequence. The protein sequence of TNAU P showed 99% identity with already available *Lycopersicon esculentum Phytoene desaturase* gene using NCBI-BLASTp software. The multiple sequence alignment of TNAU P showed considerable homology with *Phytoene dehydrogenase*, *carotene 7, 8 desaturase*, *zeta carotene desaturase* and other *Phytoene desaturase* genes from Gentian, *Oryza*, *Momardica*, citrus, pea using DBClustal software (Fig. 3). The results are in agreement with Li *et al.* (1996), Linden *et al.* (1994), Pecker *et al.* (1996) and Bartley (1991) who independently reported the homology and identity of available dicot and monocot *Phytoene desaturase* gene sequences. Phylogenetic tree constructed using Treetop software with the multiple sequence alignment data showed two clusters with grouping of TNAU P as one cluster and other desaturase genes in another cluster (Fig. 4). The phylogenetic relationships between various carotene desaturases has been extensively reviewed by Sandmann (1994). Li *et al*. (1996) reported a similar phylogenetic relationship of maize *PDS* with available monocot and dicot amino acid sequences and concluded that they have some evolutionary relationships.

Through quality parameters and gene isolation studies, it could be concluded that *L. pimpinellifolium LA* 1593 is a potential source for lycopene and it could be further exploited for isolation of lycopene specific genes.

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Flowering time and concentration of secondary metabolites in floral organs of *Hypericum perforatum* are affected by spectral quality

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Abstract

Hypericin and pseudohypericin are the major bioactive constituents of floral parts of *Hypericum perforatum* L., mainly used for the treatment of neurological disorders and depression. The principle objective of the current study was to evaluate the effect of blue, blue and red mixed, and red light on flowering time and concentration of hypericin, pseudohypericin and hyperforin in the floral tissues of *H. perforatum* plants. The results revealed that red light promoted flowering and production of the three major medicinal components, indicating the influence of spectral characteristics of light on flowering of *H. perforatum* plants. Spectral quality of light was found to be an important factor in controlling the flowering of *H. perforatum* plants.

Key words: *Hypericum perforatum* L., artificial light, controlled environment, hyperforin, hypericin, long-day plant, St. John's wort

Introduction

Plants recognize changes in their light environment by sensing light quality using signal-transducing photoreceptors. Light signals detected by the photoreceptors directly or indirectly affect physiological, morphological, and anatomical features in plants (Goto, 2003). Light quality has been reported to influence the flowering time of many long-day plants (Runkle and Heins, 2006). Under different light-quality cues the proportion of total phytochrome in the active form (phytochrome photoequilibrium) has been reported to regulate floral morphogenesis (Weinig, 2002) and flowering time of several long-day plants (Downs and Thomas, 1990). In other studies, the ratio of R/FR has been shown to alter the flowering time of many plant species including *Antirrhinum majus, Campanula carpatica, Coreopsis grandiflora, Petunia x hybrida,* and *Hyoscyamus niger* (Runkle and Heins, 2006, and references therein).

Hypericum perforatum L. (St. John's wort) has been used medicinally for thousands of years. It is a long-day flowering plant as it flowers in Europe around St. John's Day (June 24) and its flowering was promoted under 16-18 h d^{-1} light period in controlled environments (S.M.A. Zobayed, unpublished data). The major medicinally important metabolites, hypericin, pseudohypericin and hyperforin, are located mainly in the floral tissues of this plant. The current experiments were conducted to evaluate the influence of spectral characteristics of light on flowering time and production of major metabolites in the floral tissues of *H. perforatum* plants.

Materials and methods

Plant material: Seeds of *H. perforatum* (cv. 'Standard', Murakami seed Co., Ltd., Yokohama, Japan) were sown in 128-cell plug seedling trays (Takii Seed Co., Ltd., Kyoto, Japan) filled with a commercial soil mixture (Yanmar Agricultural Equipment Co., Ltd., Osaka, Japan). Twenty-one days after sowing, plants were transplanted to individual pots (bore diameter, 7.5 cm; capacity, 250 mL), filled with the commercial soil mixture. Plants were supported with commercial wires and threads. The plants were cut at the eighth node of the main stem 31 days after sowing to produce lateral branches. Thirty-six days old plants (fresh weight, 498.2 ± 23.0 mg; dry weight, 97.0 ± 5.1 mg) were used as the plant materials. The plants were grown in a controlled environment room with air temperatures of 27/24°C (light/dark period), relative humidity of 60%, and a 16 h d^{-1} light period provided by a combination of blue fluorescent lamps (FLR40S-EB/M, Matsushita Electric Industrial Co., Ltd., Osaka, Japan) and red fluorescent lamps (FLR40S-ER/M, Matsushita Electric Industrial Co., Ltd.). The CO_2 concentration was 1000 μmol mol^{-1} and the photosynthetic photon flux (PPF) measured at the soil surface was 250 μmol m^{-2} s^{-1}.

Treatments and growing conditions: The experiment was designed to evaluate the effects of different light qualities: blue light (B), mixture of blue and red light (BR), and red light (R). Blue and red fluorescent lamps were used as light sources. The spectral characteristics of each light source are listed in Table 1. There was a notable difference in the red/far-red ratio (R/FR) for the three light sources; however the phytochrome photoequilibrium (Pfr/P) value, which is an indicator of phytochrome response to R/FR, was almost the same. The experiment was conducted in a closed transplant production system (Nae terasu, Taiyo-Kogyo Corp., Tokyo, Japan). Environmental conditions common to all treatments were 27/24°C air temperatures (light/dark period), a 16 h d^{-1} light period, 60% relative humidity, and 1000 μmol mol^{-1} CO_2 concentration. As the seedlings grew, the distance between the lamps and the growing points of the plants was adjusted to maintain a constant PPF (250 μmol m^{-2} s^{-1}). The positions of the seedlings within the treatments were rearranged to minimize the variation of PPF. Subirrigation with a nutrient solution (Otsuka hydoponic composition adjusted to EC 1.2 dS/m and pH6.0, Otsuka Chemical Co., Ltd., Osaka, Japan; 4.2 mmol L^{-1} NO_3^-, 1.3 mmol L^{-1} $H_2PO_4^-$, 1.0 mmol L^{-1} Ca^{2+}, 0.38 mmol L^{-1} Mg^{2+}, 2.2 mmol L^{-1} K^+, 0.4 mmol L^{-1} NH_4^+) was applied once a day after germination.

Fig. 1. Developmental stages of *H. perforatum* flowers. At stage 1, flower buds were entirely green and about 1-3 mm in length. Stage 2 buds had the first visible yellow petals, 3-4 mm. Stage 3 buds had exposed yellow petals with small dark glands on the exposed surface, 4–6 mm. Stage 4 buds were slightly larger with yellow petals and dark glands clearly visible, 6-8 mm. Stage 5 buds were the most mature closed buds with dark glands visible on petals and anthers, over 8 mm. Stage 6 buds consisted of newly open flowers. Stage 7 flowers withered.

Table 1. Spectral characteristics of each fluorescent lamp

	B	BR	R
Photon flux (μmol m^{-2} s^{-1})			
300-400 nm (UV)	1.4	1.4	1.3
400-500 nm (B)	198.0	102.1	17.4
500-600 nm (G)	41.8	46.2	50.2
600-700 nm (R)	10.2	101.7	182.4
700-800 nm (FR)	2.1	12.5	22.4
B/R (400-500 nm/600-700 nm)	19.5	1.0	0.1
R/FR (600-700 nm/700-800 nm)	4.9	8.1	8.1
Phytochrome photoequilibrium (Pfr/P)*	0.8	0.8	0.8

*The value of phytochrome photoequilibrium was calculated using the equation proposed by Hanyu *et al.* (1996).

Table 2. The number of flower buds of *H. perforatum* plants grown under different light quality for 33 days (69 days after sowing)

Light quality	Flower developmental stages						
	1	2	3	4	5	6	7
B	4.6 a	3.1 a	3.0 b	1.5 a	0.8 b	0.3 a	1.0 b
BR	6.3 a	4.0 a	5.1 a	2.3 a	2.1 a	0.8 a	2.1 b
R	6.1 a	1.4 b	2.9 b	2.2 a	1.9 ab	0.8 a	5.1 a

The data are means from 29-31 plants and different letters indicate significant difference at $P<0.05$ level according to Tukey-Kramer test.

Flowering: The definition of floral developmental stages was modified from Murch *et al.* (2002) (Fig. 1). The time courses of the percentage of plants with flower buds (stage 1) and the percentage of plants with open flowers (stage 6) 69 days after sowing were observed. The days from bearing of the flower bud (stage 1) to flowering (defined as bud opening) (stage 6) were measured. The number of the flower buds, and the fresh and dry weights of flower buds were measured 69 days after sowing.

Extraction and determination of hypericin, pseudohypericin, and hyperforin concentrations: Samples representing seven distinct stages of flower development in BR treatment and stages 3 and 6 in B and R treatments were collected 69 days after sowing and stored at -85°C until used for the analysis of hypericin, pseudohypericin, and hyperforin as described by Mosaleeyanon *et al.* (2005). The concentrations of the medicinal components in the sampled flower buds were expressed as mg g^{-1} flower bud DW, and the total medicinal content of each flower bud (g/flower bud) was calculated by multiplying the concentrations of the medicinal components by bud dry weight. The medicinal content of total flower buds of each plant (g/plant) was calculated by multiplying the concentrations of medicinal components in the sampled flower buds in BR treatment by the number of flower buds per plant in each treatment, assuming that the total medicinal contents of each flower bud (g/flower bud) during each treatment were same.

Statistical analysis: The experiment was conducted twice with 18 replications. Means and standard errors obtained from replicate experiments were subjected to an analysis of variance (ANOVA). The means were compared using the Tukey-Kramer test at a 5% level of significance. The means of the percentage of plants with flowers were compared using Fisher's exact test with Bonferroni correction.

Results

Effects of light quality on flowering, concentrations of medicinal components and medicinal content of flower buds: The percentages of plants with flower buds in all light quality treatments rapidly increased from 3 days after observation of the first bud and were saturated within 9 days (Fig. 2). Plants grown in R light produced buds earlier than those grown in B or BR, while plants in the two latter treatments produced their first flower buds on the same day. Fifty percent of plants grown in B, BR, and R had buds by 56, 54, and 52 days after sowing, respectively. The time between appearance of the first flower bud and flowering in B, BR, and R treatments was 13, 12, and 12 days, respectively. The percentage of plants with flowers, 69 days after sowing was highest in R treatment, 1.2 and 2.0 times that of plants grown in BR and B (Fig. 3). The total number of flower buds per plant was almost the same under different light conditions (Table 2). At 69 days after sowing, the number of flower buds at stages 1, 4, and 6 was similar among the treatments; however the largest number of buds at stages 2, 3, and 5 was found in plants with BR treatment, and stage 7 buds were most abundant in R treatment.

Hypericin concentrations in stage 3 and 6 flower buds were similar under different light treatments (Fig. 4). Pseudohypericin concentration in stage 3 flower buds was significantly higher in the B treatment than in BR and R treatments, but was similar among the treatments in stage 6 buds. Hyperforin concentration in stage 3 flower buds was higher in R treatment than B and BR treatments, but like pseudohypericin was almost the same among the treatments by stage 6.

Dry weight of stage 3 flower buds in R treatment was greater than that in B treatment, and almost the same as that in BR treatment

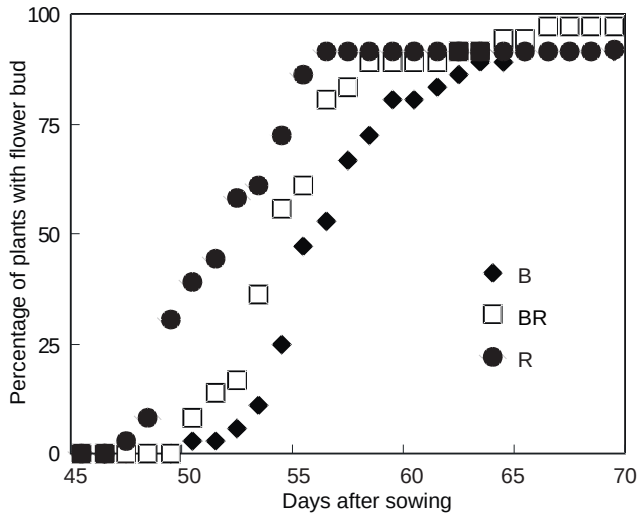

Fig. 2. Time courses of the percentage of plants with stage 1 flower buds, n=36.

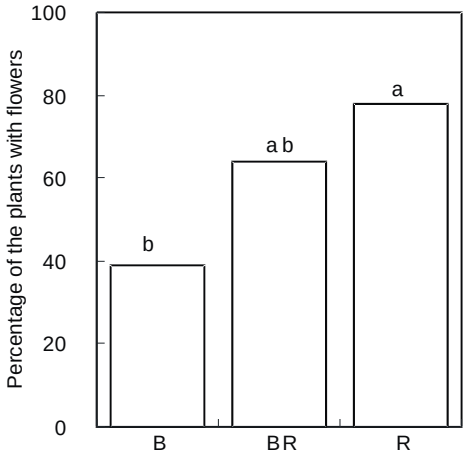

Fig. 3. Percentage of plants with flowers grown under different light conditions for 33 days (69 days after sowing). Different letters indicate significant differences between the treatments at $P \leq 0.05$, determined by the Fisher's Exact test with Bonferroni correction. Each bar represents mean ± S.E. of 36 replicates.

(Fig. 5). Dry weight of stage 6 flower buds was highest in R treatment. Total content of hypericin and pseudohypericin in stage 3 flower buds was almost the same among the treatments (Fig. 6). The hyperforin content in R treatment was higher than that in B treatment, and was almost the same in BR treatment. The contents of the three medicinal components in stage 6 flower buds in R treatment were significantly higher than those in B and BR treatments.

The total content of hypericin, pseudohypericin, and hyperforin in flower buds of plants 69 days after sowing (μg/plant) was highest in R treatment (Fig. 7).

Quantity and concentration of medicinal components in flower buds at different stages: The concentrations of major metabolites in floral tissues were explored in plants grown under BR. In this treatment, the dry weight of flower buds increased from stage 1-6, and then decreased (Fig. 8). The concentrations of the medicinal components in flower buds of plants 69 days after sowing are shown in Fig. 9 (A). Hypericin and pseudohypericin concentrations were highest in stage 2 flower buds, and subsequently declined. Hyperforin concentration was highest in stage 2 buds and subsequently decreased, was constant during

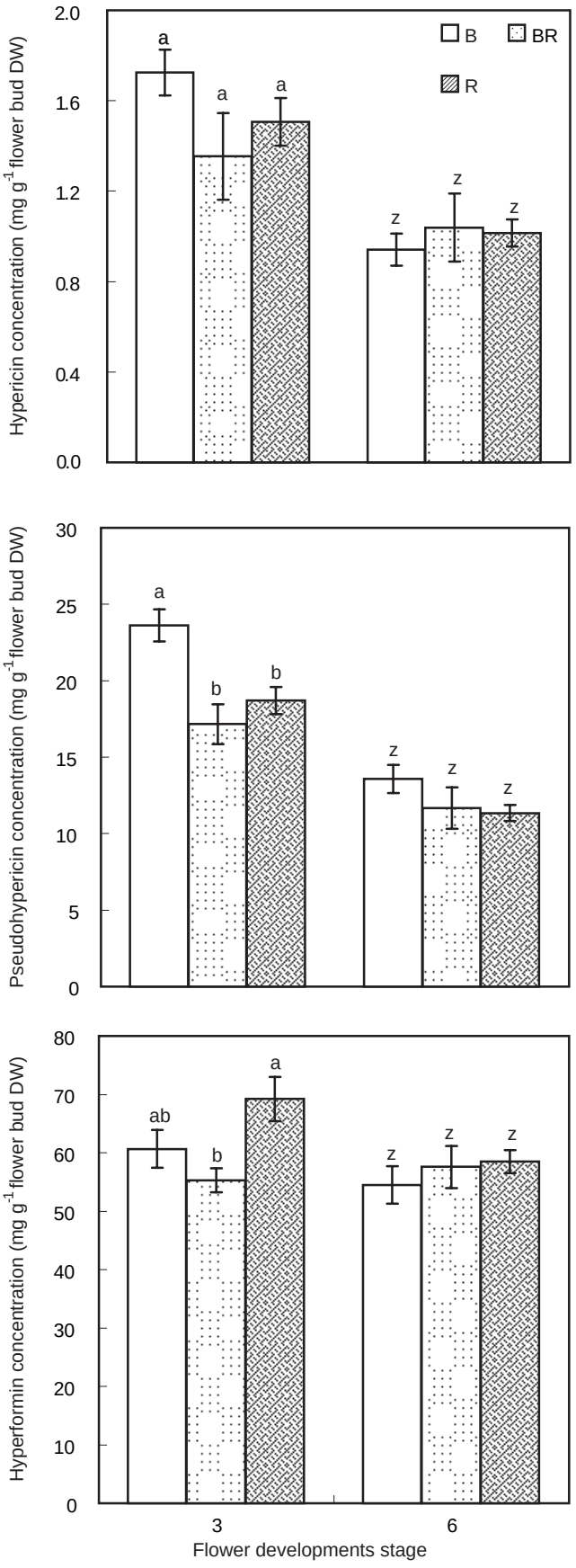

Fig. 4. The hypericin, pseudohypericin, and hyperforin concentrations of stage 3 and 6 flower buds of plants grown under different light conditions for 33 days (69 days after sowing). Different letters indicate significant differences between the treatments at $P < 0.05$, determined by the Tukey-Kramer test. Each bar represents mean ± S.E. of 11-12 (stage 3) and 6-14 (stage 6) replicates.

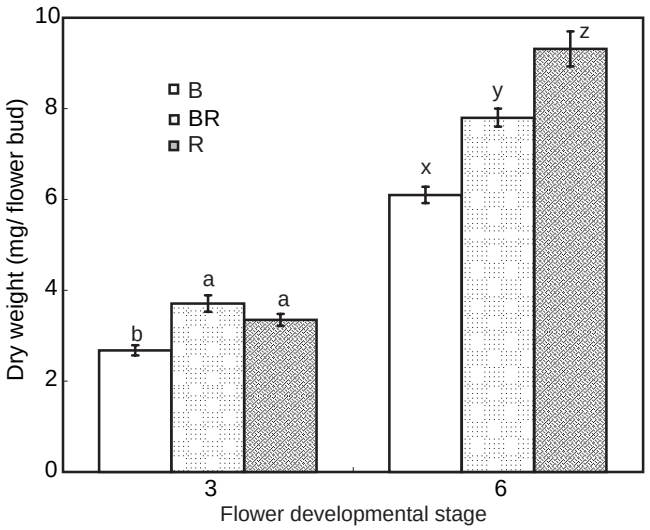

Fig. 5. Dry weights of stage 3 and 6 flower buds of plants grown under different light conditions for 33 days (69 days after sowing). Different letters indicate significant differences between the treatments at $P < 0.05$, determined by the Tukey-Kramer test. Each bar represents mean ± S. E. of 24-27 (stage 3) and 8-19 (stage 6) replicates, respectively.

stages 3-6, and then increased again. The medicinal contents 69 days after sowing in BR treatment are shown in Fig. 9 (B). Hypericin and pseudohypericin contents in stage 1-4 flower buds increased during flower development, and remained high in stages 4-7. Hyperforin content increased throughout flower development.

Discussion

The present experiments were undertaken to evaluate the influence of spectral characteristics of light on flowering of *H. perforatum* plants without altering the phytochrome photoequilibrium values, or the PPF at the growing points of plants. We found that R light promoted flowering in *H. perforatum*: plants grown in R light developed flower buds earlier than those grown in B or BR, and the percentage of plants with flowers in R treatment was higher than that in B and BR treatments. The number of days from appearance of the first flower bud to flowering in B, BR, and R treatments were almost the same; therefore we concluded that the pace of flower development was not influenced by light quality.

The floral transition in *Arabidopsis*, a quantitative long-day plant, is regulated by at least four flowering pathways: the photoperiod response, the vernalization response, the autonomous pathway, and the gibberellin (GA)-dependent pathway (Mouradov *et al.*, 2002). *H. perforatum* may be a long-day plant because it flowers around St. John's Day (June 24) in Europe, and its flowering was promoted under a 16-18 h d^{-1} light period in a controlled environment (S.M.A. Zobayed, unpublished data). Furthermore, flowering of *H. perforatum* plants does not require vernalization. Therefore, phytochrome may have a role in the flowering time of *H. perforatum* plants. Many studies on the control of flowering by light quality focus on promotion of flowering by a decrease in R/FR, which in turn decreases the value of Pfr/P. In the current study, although R/FR was different among the three light quality treatments, the value of Pfr/P was the same; therefore the results of the current study cannot be explained by any flowering pathway known to date. Therefore we deduced that proportions of blue

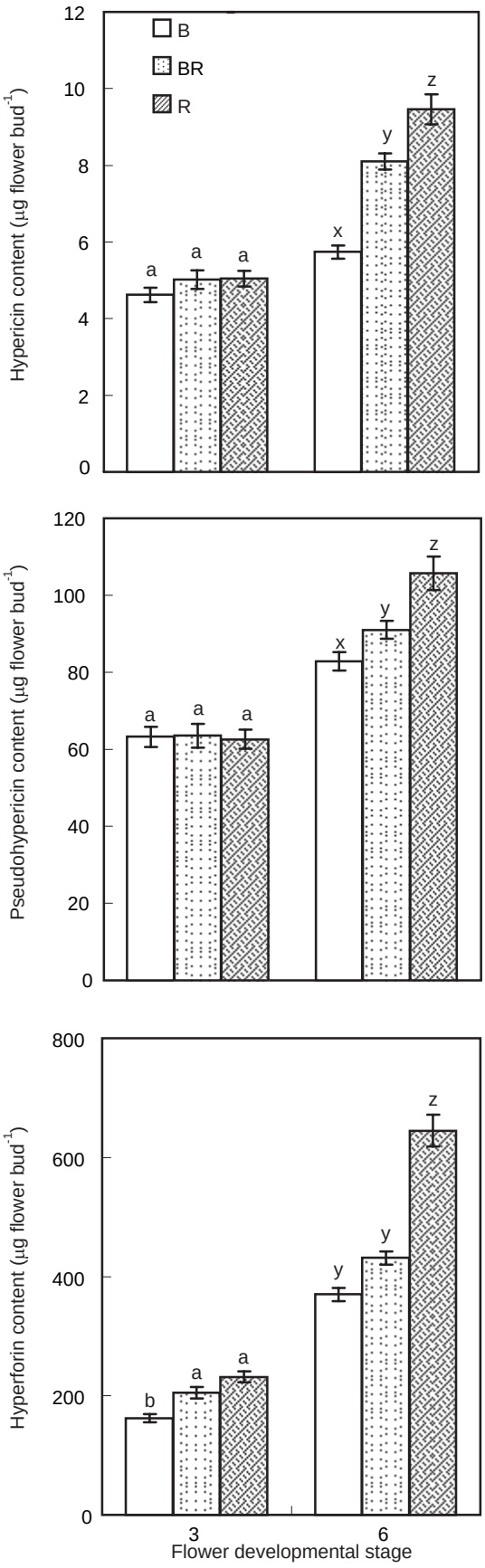

Fig. 6. The hypericin, pseudohypericin, and hyperforin contents of stage 3 and 6 flower buds of plants grown under different light conditions for 33 days (69 days after sowing). Different letters indicate significant differences between the treatments at $P < 0.05$, determined by the Tukey-Kramer test. Each bar represents mean ± S.E. of 24-27 (stage 3) and 8-19 (stage 6) replicates, respectively.

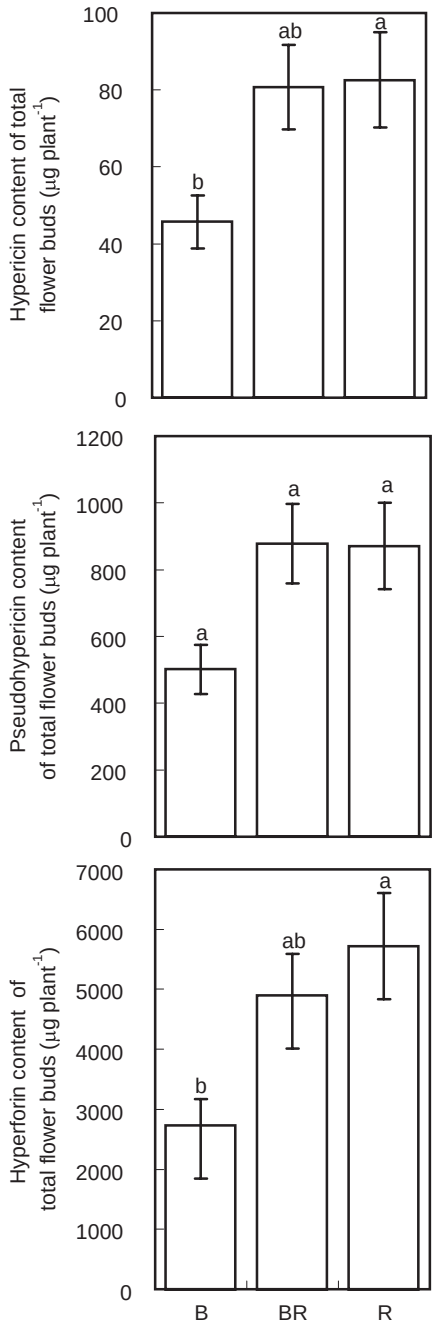

Fig. 7. The hypericin, pseudohypericin, and hyperforin contents of total flower buds of plants grown under different light conditions for 33 days (69 days after sowing). The contents of total flower buds were calculated by multiplying the medicinal content of each flower bud in BR treatment by the number of flower buds per plant in each treatment. Plants without flower buds were not included in the calculation. Different letters indicate significant differences between the treatments at $P < 0.05$, determined by the Tukey-Kramer test. Each bar represents mean ± S.E. of 29-31 replicates.

or red light to PPF and blue/red ratio (B/R) may affect several points in the flowering pathway.

The hypericin, pseudohypericin and hyperforin concentrations of *H. perforatum* grown in a greenhouse were previously reported to be highest in yellow flower buds about 3-4 mm (flower developmental stage 2) with a subsequent decline (Murch *et al.*, 2002). Our findings (Fig. 9) were in agreement with the previous results. Hypericin and pseudohypericin accumulate in significant quantities in dark glands. Stamens have higher concentrations of hypericin and pseudohypericin than any other organ of *H.*

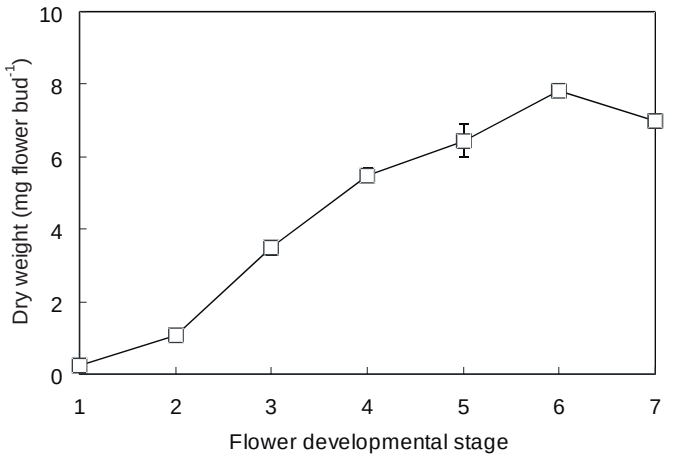

Fig. 8. Dry weight of flower buds of plants grown in BR treatment for 33 days (69 days after sowing). Error bars represents ± S.E. of 13–27 replicates.

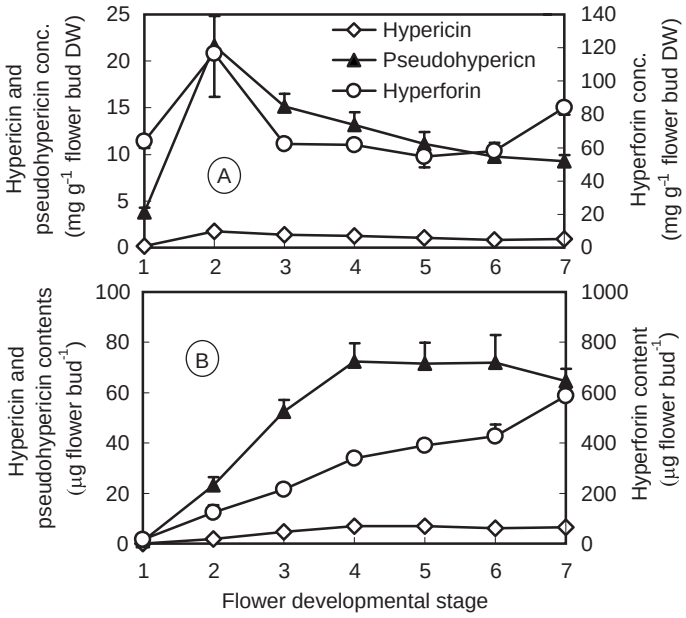

Fig. 9. Hypericin, pseudohypericin, and hyperforin concentrations (A) and contents (B) in the flower buds of plants grown in BR treatment for 33 days (69 days after sowing). Each bar represents mean ± S.E. of 7-13 replicates.

perforatum plants and also contain a significant number of dark glands (Zobayed *et al.*, 2006). The stamen anthers are formed during floral stages 1 to 2. The concentrations of hypericin and pseudohypericin in stage 2 flower buds were relatively high because the dry weight of other floral organs than stamens may be smaller at stage 2 than at stages 3 to 7. Therefore, decreasing hypericin and pseudohypericin concentrations from stages 2 to 7 is attributable to the increase in dry weight of the flower organs other than the stamens through flower development.

The stamens of flower buds develop during stage 1 to 4, and then stop developing (Zobayed *et al.*, 2006). Accordingly, the hypericin and pseudohypericin contents of flower buds increased during stages 1 to 4, and then were saturated. In contrast, the hyperforin content of flower buds continued to increase throughout flower development. The mechanism of hyperforin synthesis is little known, and differs from that of hypericin and pseudohypericin. Hyperforin may not accumulate in the dark glands; and hyperforin content of flower buds does not appear to be associated with stamen development.

Although the concentration of medicinal components in stage 6 flowers were almost the same under different light quality treatments; at stage 3, the hypericin and pseudohypericin concentrations were higher in B treatment, while the hyperforin concentration was higher in R treatment. The dry weight of stage 3 flower buds was higher in R treatment than in B treatment. The amount of anther tissue which accumulated hypericin and pseudohypericin in significant quantities may be relatively low, and the amount of any other floral organs which accumulated hyperforin may be relatively high. Calculating the medicinal contents in flower buds (g/flower bud) by multiplying the concentrations of medicinal components by flower bud dry weight, we demonstrated that the medicinal contents were highest in R treatment compared to those in B and BR treatments (Fig. 6).

The hypericin, pseudohypericin, and hyperforin contents of total flower buds of plants 69 days after sowing were higher in R treatment than those in B and BR treatments. Therefore, red light promoted the development of flower buds and produced flower buds with a higher content of secondary metabolites compared to blue light under constant Pfr/P values. We also conclude that spectral quality of light is an important factor in controlling the flowering of *H. perforatum* plants.

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Phenolics and parthenolide levels in feverfew (*Tanacetum parthenium*) are inversely affected by environmental factors

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Abstract

Feverfew (*Tanacetum parthenium* [L.] Schultz-Bip., Asteraceae) products have shown high variability in the market. The objective of this study was to determine whether environmental factors affect the composition of key phytochemicals in feverfew. Plants of feverfew were exposed to water stress in greenhouse and commercial field conditions. The highest yield of parthenolide (PRT) was found in plants that received reduced-water regimes. Phenolics concentration was higher in plants grown under adequate-water conditions. The effect of time of harvest on PRT concentration and phenolics content was also investigated. Increased PRT was found during afternoon hours whereas total phenolic compounds decreased during the photoperiod and increased at night. When plants were exposed to artificial light during night hours, the phenolics content remained low. Our results revealed that manipulating the environment to favour increased accumulation of PRT resulted in a decline of phenolics content in feverfew. These findings have implications on standardization of herbal products.

Key words: Asteraceae, feverfew, *Tanacetum parthenium*, time of harvest, water stress, parthenolide, phenolics

Introduction

Products of feverfew (*Tanacetum parthenium* [L.] Schultz-Bip., Asteraceae), an herb regarded as an effective prophylactic treatment of migraine, rheumatoid arthritis and menstrual cramps (Grauds *et al.*, 1995), have shown large variability of parthenolide (PRT) content, a sesquiterpene lactone commonly associated with the medicinal effects (Awang *et al.*, 1991).

In addition to PRT, certain phenolics, notably tanetin, a lipophilic flavonoid, contribute to the medicinal value of feverfew (Williams *et al.*, 1995). The major flavonols and flavone methyl ethers found in feverfew have been shown to inhibit the arachidonic acid pathway (Williams *et al.*, 1999). Phenolic content may be another criterion for assessment of commercial quality of feverfew.

Evidence indicating that pre-harvest factors influence final quality of herbs has been reported. In various plants, environmental stress has increased the accumulation of phenolics (Keinanen, 1999). Secondary metabolites are prone to diurnal fluctuation (Veit *et al.*, 1996), a response likely due to the effects of light intensity on carbon partition (Stoker *et al.*, 1998; Middleton *et al.*, 1994). It is uncertain whether PRT or any other secondary metabolite in feverfew fluctuates during the day. Water stress increases the production of jasmonic acid followed by increase of abscisic acid (ABA), which induces stomata closure (Wasttenack and Parthier, 1997) and sesquiterpene accumulation (Singh *et al.*, 1998), and may interfere with tannin yields (Horner, 1998). Our early work showed that ABA and PRT biosynthesis are connected and both increase during or after a wilt event (Fonseca *et al.*, 2005) prompted us to determine whether PRT and other secondary metabolites of interest fluctuate with environmental conditions in a similar fashion.

It is possible that pre-harvest conditions influence concentration of key metabolites in feverfew, however, research on PRT and phenolics dynamics as a response of changing environments is lacking. Thus, the objective of this study was to evaluate the effect of water stress and time of day of harvest on PRT and total phenolics content in feverfew.

Materials and Methods

Effect of water stress: Feverfew seeds (Richter's Seed Co. Ontario, Canada) were germinated in 60 mL-cell trays under an intermittent mist and were transferred to 4 L pots six weeks after germination. Four months after germination, plants were divided in two groups, one continuing with daily watering, and the other group receiving water only after a wilt event. Typically, plants were allowed to dry for 4-6 days until they wilted (-4.47 to –8.65 MPa). After wilt, plants were watered daily to run off as the controls (-1.94 to -2.07 MPa) for five days before exposing the plants to water withdrawal again. The plants were subjected to a total of three wilt events and harvested before the onset of the fourth water stress event. The experiment was repeated once and included treatments consisting of twelve plants, harvested during summer months. Maximum irradiance of visible light, measured with a LI-250 Quantum meter (Licor Inc. Lincoln, NE, USA), was 461.4 $\mu mol\ s^{-1}\ m^{-2}$.

The effect of water stress on PRT and total phenolics content in feverfew was also evaluated in a grower's field. Feverfew plants

Table 1. Conditions at 11 am in a commercial trial that evaluated the effect of water stress on parthenolide concentration in feverfew

Factors	Non-Irrigated	Irrigated
Temperature	26.00	26.00
Sunlight	1645.80	1645.80
Water potential soil	-70.46	-0.04
Water potential plant	-3.63	-1.11

Units are °C for temperature, μmol s^{-1} m^{-2} for sunlight intensity and P Ma for water potential.

were grown for 5 months with and without drip irrigation at a commercial production site near Kingsburg, SC, USA, from November to April. Plants were harvested when approximately 5 percent of the plants had begun to bloom. Batches of 8 non-flowering plants from each field (non-irrigated and irrigated) were randomly selected and harvested four times during daytime. Water potential in soil and leaves, photosynthetic radiation (PAR) and temperature at the harvest site are provided in Table 1.

Effect of time of day of harvest: Plants were grown in greenhouse following cultural practices described earlier. Five months after planting, plants were harvested at four different times of the day (5 AM, 9 AM, 1 PM, 5 PM) during January and February. To evaluate the effect of light during night hours plants harvested at 5 AM were exposed to light (8 μmol s^{-1} m^{-2}) for 8 hours (starting at 9 PM) and compared with non-irradiated plants. The trial included harvest of three plants per treatment per each of four different days. The experiment was arranged in a randomized complete block design and it was conducted twice.

Analysis of parthenolide and total phenolics: In preparation for PRT analysis, all plant tissue above ground were harvested and dried in a conventional oven at 50 °C until moisture content reached 4-6% and then ground with a coffee grinder. The powder was sieved and particles of <500 μm size were used for immediate analysis. Samples of 150 mg were combined with 10 mL 90% acetonitrile for 10 min and extracted using the bottle stirring method. Aliquots of each extraction solution were taken from supernatant, filtered through 0.45 PTFE μm membranes and 10 μL injected onto a RP-HPLC system (Waters ™ 1525 pump), equipped with a C-18, 5 μm column (Waters Symmetry ®) of 150 x 4.6 mm dimensions. The injections were performed in duplicate. Mobile phase was an isocratic 55% acetonitrile: 45% water per 8 min at 1.5 mL/min. The peaks were analyzed at 210 nm using an ultraviolet detector.

Total phenolic content was measured using the Folin Ciocalteau procedure (Singleton and Rossi, 1965) modified by Kähkhönen *et al.* (1999). The extraction was performed by combining 50 mg feverfew powder samples with 10 ml 80% methanol with stirring for 10 min. Samples were vortexed for 1 min before a 200 μL aliquot was taken from the supernatant**.** One mL of Folin-Ciocalteu reagent (2N, Sigma) was added to the 200 μL sample. After 3 minutes, 0.8 mL of sodium carbonate (7.5%) was added and the mixture was allowed to stand for 30 min. Absorption was measured at 765 nm using a Spec 20® spectrophotometer (Thermo Spectronic, Rochester, NY, USA). Total phenolics were expressed as gallic acid units. A multipoint linear curve was obtained with gallic acid standard (Sigma) ranging from 20 to 400 μg/mL. Two standards (20 and 100 μg) were included for comparison with each set of samples analyzed.

Statistical analysis: Experiments were arranged in a completely randomized design. Data were subjected to analysis of variance (ANOVA) at $P \leq 0.05$ to determine statistical significance. Mean comparisons were conducted using Fisher's Protected LSD Method at $P \leq 0.05$ (SAS Institute, Cary, NC).

Results and discussion

Plants under water stress had higher PRT levels than plants receiving water daily regardless of the growing environment, greenhouse or commercial field. Unlike the pattern observed with PRT, plants in greenhouse conditions under reduced-water conditions had lower phenolic content than plants that received continuous irrigation, but no difference was observed in plants grown in the field (Table 2).

Unlike our previous work (Fonseca *et al.*, 2005), in this study the plants were harvested after subjecting the plants for extended time to water stress, and the leaves were not fully turgid at the moment of the harvest. The results from this study revealed that water stress in commercial field conditions enhance PRT content in feverfew, however, the plants showed 20-35% reduced dry weight (data not shown), discouraging field production without irrigation systems. The dry weight of plants grown in pots, receiving only three wilt events, was reduced by less than 5% (data not shown) which encourages studies involving the evaluation of mild stress conditions. It is possible that regulated water stress programs increase metabolites concentration without changing quantities of phytochemicals per area. The total phenolics levels found in feverfew are similar to those reported for other herbs and medicinal plants (Kähkönen *et al.*, 1999), however, to our knowledge this is the first time that phenolics content variability in a medicinal plant is associated with a single agricultural practice.

Plants harvested during the afternoon contained significantly more PRT than plants harvested in the morning. Plants harvested at 5 PM had the highest PRT. The 5 AM harvest had the lowest PRT

Table 2. Effect of water stress on parthenolide and phenolics content in feverfew grown in greenhouse and field conditions

Environment	Irrigation regime	Parthenolide content (g kg^{-1} dry weight)	Phenolics content (g GAC kg^{-1} dry weight)
Greenhouse	Daily watering	0.47 a	64.77 a
	Reduced watering	1.89 b	33.45 b
	LSD ($P \leq 0.05$)	1.36	24.15
Field – commercial production	Drip irrigation	5.38 a	110.50
	No irrigation	6.76 b	93.85
	LSD ($P \leq 0.05$)	1.31	49.33

Values are the averages of 16 samples taken during the day of harvest. Different letters within the same column and environment indicate significant differences between treatments.

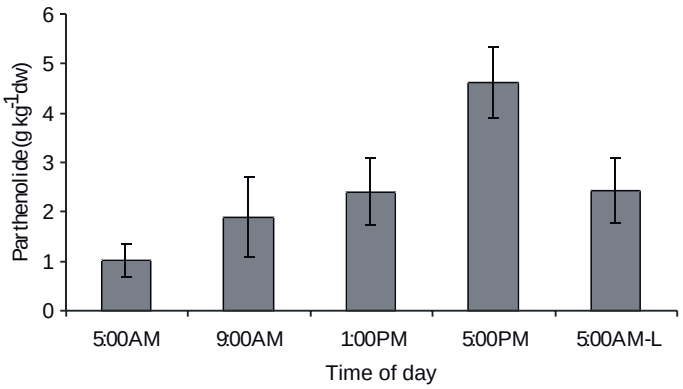

Fig. 1. Effect of time of day of harvest on parthenolide content in feverfew. "L" means plants exposed to artificial light during night hours (5 PM to 5 AM). Values are the average of 12 samples. Error bars indicate S.E.

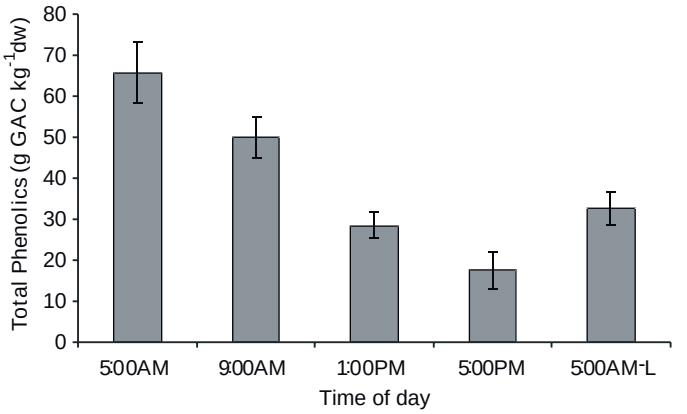

Fig. 2. The effect of time of day of harvest on total phenolics content in feverfew. "L" means plants exposed to artificial light during night hours (5 PM to 5 AM). Values are the averages of 12 samples. Error bars indicate S.E.

concentration. Although no significant difference was detected, when 5 AM plants were exposed to light PRT levels averaged two fold higher than those in non-irradiated plants (Fig. 1). In contrast, the phenolic concentration was higher during night time, decreasing during daylight. At 5 AM harvest, the plants exposed to light had significantly less concentration of phenolics (Fig. 2). These results show that light prior to harvest influences the levels of PRT and total phenolics in feverfew. The pattern observed with phenolic levels, increasing in the dark and decreasing during daylight or with artificial light irradiance, has been observed in other plants (Veit *et al.*, 1996; Burns *et al.*, 2002).

It is interesting that the concentration of PRT in plants grown during the winter (the field experiment and the study of time of day), which are normally shorter and bushy, was markedly higher than in plants grown in the summer (experiment of water stress in greenhouse conditions). Moreover, it has been observed that vegetative plants accumulate higher PRT levels than reproductive plants (Hendriks *et al.*, 1997), which normally bloom during the summer. There may be a synergistic interaction between long day conditions and environmental factors.

Overall results in this study showed that environmental factors may produce opposite effects among secondary metabolites in medicinal plants and this was clearly observed between PRT and phenolic content in feverfew. The accumulation of secondary metabolites is driven by the availability of excess carbon. When growth is reduced due to stress, more carbon becomes available for secondary metabolism and, as the plant adjusts to the specific stress the accumulation of some metabolites is favored over others (Tuomi *et al.*, 1998). Phenolics fluctuate daily in part because the plant produces them in preparation to periods of high UV sunlight irradiance (Middleton and Teramura, 1994). Moreover, our results with artificial light revealed that visible light functions as an external signal that turns on and off the phenolic production mechanism in feverfew, regardless of UV light incidence.

The results obtained in this study demonstrate that medicinal or "commercial" quality of feverfew is affected by environmental factors. The visible light prior to harvest and the water regime during the plant growth are crucial in altering the content of PRT and phenolics in feverfew. Our results revealed however, that practices that increased PRT result in lower total phenolic content. The many interactions among phytochemicals, and the rapid adjustment of the plant to changing environments, involve a major decision of which key metabolites to target, knowing that this will potentially carry a "trade off" including a decrease in the content of other metabolites. Clearly, manipulation of production and handling protocols can alter secondary metabolism of feverfew but studies are needed to verify if this holds true for other medicinal plants as well. Such studies are crucial for the development of appropriate regulations for herbal product quality.

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Improved plant regeneration in cowpea through shoot meristem

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Abstract

Cowpea is a highly recalcitrant nutrient-rich leguminous vegetable crop. Efforts to genetically transform cowpea with insect-resistant genes remains a challenging task due to lack of an efficient regeneration system. We have established an efficient regeneration system in cowpea through shoot meristem. Shoot meristems were isolated from embryos that were precultured for 3-5 days on Murashige and Skoog (MS) medium containing 8.9 µM benzylaminopurine (BA). The isolated shoot meristems were cultured on MS medium containing 0.89 µM BA. After 3-4 weeks, multiple shoots were separated from the explant and cultured on half-strength MS medium for elongation and rooting. More than 90% of the regenerants formed roots. The rooted plantlets were transferred first to peat pellets and subsequently to the greenhouse. The plants were allowed to flower and set seed. The efficiency of regeneration in all four cultivars ranged from 76-87%, demonstrating a significant improvement over the published protocols (1-32%). At least six to seven plantlets were obtained from each meristem. The protocol using shoot meristems is simple, efficient, rapid and genotype-independent and may be amenable for transformation through particle bombardment.

Key words: *Vigna unguiculata*, shoot meristem, regeneration, transformation, legumes

Introduction

Cowpea (*Vigna unguiculata* L. Walp.), an annual vegetable, is one of the world's important legume food crops. Cowpea grain contains about 25% protein, especially rich in folate, potassium, iron, magnesium, and the essential amino acids lysine and tryptophan. Cowpea is also rich in phytochemicals that may help prevent chronic diseases such as cardiovascular disease, cancer and diabetes. In addition, cowpea is a good source of fiber. A diet high in fiber can help lower blood cholesterol levels, which can reduce risk of heart disease (www.mayoclinic.com/health/legumes/NU00260)

Cowpea is severely infected by insects such as thrips (*Megalurothrips sjostedti*), aphids (*Aphis craccivora*), curculio (*Chalcodermus aeneus*), pod borer (*Maruca vitrata*), weevils (*Callosobruchus maculatus*) etc. that cause significant damage to crop production and yield (Singh *et al.*, 1990). However, current cultivars do not offer protection against insect damage. Efforts to develop durable insect resistance did not succeed because the genome of cowpea may be devoid of major resistance genes to many insect pests that attack cowpea. Also, attempts to bring insect resistance into cowpea from wild *Vigna* species have failed because of high genetic barriers between wild *Vigna* and cultivated cowpea (Singh *et al.*, 1997).

Cowpea is an ideal vegetable crop for the application of genetic engineering technologies for developing insect resistance. However, cowpea is highly recalcitrant to tissue culture and therefore genetic transformation is difficult to achieve. There have been a few reports of plant regeneration through organogenesis and somatic embryogenesis (Muthukumar *et al.*, 1995; Pellegrineschi, 1997; Brar *et al.*, 1999a, b; Anand *et al.*, 2000, 2001; Ramakrishnan *et al.*, 2005). The efficiency of regeneration in these reports is too low (1-32%) to reliably obtain transgenic plants. Consequently, efforts to transform cowpea were mostly unsuccessful or resulted in very few transgenic plants (Garcia *et al.*, 1986, 1987; Penza *et al.*, 1991; Muthukumar *et al.*, 1996; Ikea *et al.*, 2003). In recent years, regeneration of shoots from cotyledon nodes or from other meristematic explants has emerged as a rapid and relatively efficient method of transformation in a number of legumes that are highly recalcitrant in tissue culture (Oger *et al.*, 1996; Trieu and Harrison, 1996; Larkin *et al.*, 1996; Olhoft *et al.*, 2001). In cowpea, transgenic plants were regenerated using cotyledon nodes containing axillary meristems (Popelka *et al.*, 2006), although at low frequency (0.05-0.15%), demonstrating the feasibility of using meristems as an alternate source for genetic transformation.

In this report, we present a simple, efficient, rapid and genotype-independent regeneration system for cowpea plants from four cultivars by using shoot meristems. The regeneration method has potential use in transforming cowpea with insect resistant genes.

Materials and methods

Four cowpea cultivars (Early Scarlet, Coronet, Quick Pick and AR87-435-68) were selected for regeneration. Mature seeds (kindly provided by Dr. S. Okiror, University of Arkansas at Pine Bluff, USA) were surface sterilized in 70% alcohol for 5 minutes, rinsed in sterile water and placed in 0.2% sodium hypochlorite solution. After 1 h, the seeds were rinsed thrice with sterile water. Finally, the seeds were allowed to soak in sterile water overnight. Murashige and Skoog (1962) medium (MS) supplemented with 3% (w/v) sucrose (Sigma, USA) and various concentrations of growth regulators were used for tissue culture and regeneration. The media were adjusted to pH 5.8 with 1 N NaOH or 1 N HCl,

solidified with 3 g L^{-1} phytagel (Sigma, USA) and autoclaved at 1 kg cm^{-2} for 20 min. Media (50 mL) were dispensed into 20- by 100-mm sterile Petri dishes (Falcon, Becton Dickinson Labware, USA). The cultures were maintained at 25 ± 2 ^{0}C with a 16-h photoperiod (25-40 µmol cm^{-2} s^{-1}). All growth regulators were filter sterilized before adding to the media. Embryos were isolated and precultured either on MS basal medium or MS medium containing BA (8.9 µM) (Table 1). After 3-5 days, the shoot meristems (0.5-1 mm) were carefully isolated and cultured on MS media with different concentrations of BA (0.4-22.2 µM) for three week. After three weeks, the regenerated multiple shoots were transferred to elongation and rooting medium (half-strength MS with no growth regulators). Plantlets with well-developed roots were removed from the culture medium, the roots washed thoroughly with tap water, and transferred to peat pellets [Jiffy-7, Jiffy Products (N.B.) Ltd., Shippagan, Canada] for initial acclimatization. The plantlets were covered with plastic wrap to maintain high humidity for first few days. Gradually the humidity was reduced by slowly removing the plastic wrap and the hardened plants were transferred to the greenhouse [24-28°C, 16/8 h (day/night) photoperiod supplemented by sodium halide lights]. Plants were allowed to flower and set seed. The root tips of the regenerated plants were collected in cold distilled water, kept at 4^{0}C for 24 h and then fixed in Farmer's fixative (3:1 95% ethanol: glacial acetic acid). The root tips were squashed with carbol fuchsin and observed under a microscope.

Results

Shoot meristems (0.5-1 mm) were carefully isolated from precultured embryos and cultured on different concentrations of BA (Table 1). After 3-5 d of culture, newly formed multiple shoots could be seen from the cut end of the meristem. After 3-4 weeks, individual shoots were separated from each meristem and transferred to half-strength MS medium for elongation and rooting. Among the different concentrations tried, MS medium containing 0.89 µM BA produced more shoots (3.2 shoots per meristem) than other concentrations. To improve the number of multiple shoots, embryos were precultured on higher concentration of BA (8.9 µM) for 3-5 days and the isolated shoot meristems were cultured on different concentrations of BA (Table 1). Significantly, more plants were regenerated after transfer to MS medium containing 0.89 µM BA from all four cultivars tested, indicating the positive effect of preculture in inducing multiple shoots (Fig. 1A). More than six shoots per meristem were regenerated from all four cultivars with the preculture on BA (8.9 µM) medium. Increasing the concentration of BA beyond 0.89 µM in the culture media resulted in callus growth that significantly reduced the number of multiple shoots. The addition of other cytokinins such as zeatin and kinetin in the regeneration media also led to callus growth from shoot meristems, thereby significantly reducing the number of shoots (data not shown). Shoots were separated and transferred to half-strength MS medium for elongation (Fig. 1B) and rooting. More than 90% of the regenerants formed roots in the half-strength MS medium (Fig. 1C). The regenerated plants with roots were initially transferred to peat pellets for hardening. Only 50% of the plants survived during hardening. After 10-12 days of hardening, the surviving plants were transferred to the greenhouse (Fig. 1D). No phenotypic and chromosomal abnormalities (2n = 22) were noticed.

Table 1. Effects of benzylaminopurine (BA, µM) on shoot regeneration from cultured meristems in cowpea

Genotype	Preculture medium	Culture medium	Explants forming shoots (%)	Number of shoots per explant†
Early Scarlet	MS	MS	56.25	1.16 j
	MS	MS + 0.4 BA	59.37	2.62 f,g,h,i,j
	MS	MS + 0.9 BA	60.94	3.24 c,d,e,f,g
	MS	MS + 2.2 BA	59.37	2.83 e,f,g,h,i
	MS	MS + 4.4 BA	51.28	2.37 f,g,h,i,j
	MS	MS + 8.9 BA	69.58	2.16 g,h,i,j
	MS	MS + 13.3 BA	60.35	1.89 h,i,j
	MS	MS + 22.2 BA	68.71	1.22 j
	MS + 8.9 BA	MS + 0.4 BA	70.89	4.87 b,c
	MS + 8.9 BA	MS + 0.9 BA	72.33	6.73 a
	MS + 8.9 BA	MS + 2.2 BA	76.82	5.45 b
	MS + 8.9 BA	MS + 4.4 BA	74.02	4.21 c,d,e
Coronet	MS	MS	72.66	1.99 g,h,i,j
	MS	MS + 0.9 BA	73.08	3.34 d,e,f,g,h
	MS + 8.9 BA	MS + 0.9 BA	87.65	6.89 a
	MS + 8.9 BA	MS + 2.2 BA	86.23	5.32 b
Quick Pick	MS	MS	81.23	1.89 h,i,j
	MS	MS + 0.9 BA	83.78	3.87 c,d,e,f
	MS + 8.9 BA	MS + 0.9 BA	86.66	6.46 a
	MS + 8.9 BA	MS + 2.2 BA	85.62	5.12 b
AR87-43568	MS	MS	75.83	1.71 i,j
	MS	MS + 0.9 BA	79.21	3.43 c,d
	MS + 8.9 BA	MS + 0.9 BA	84.02	6.29 a
	MS + 8.9 BA	MS + 2.2 BA	82.17	5.18 b

† means followed by the same letters are not significantly different based on Duncan's multiple range test (P<0.05).

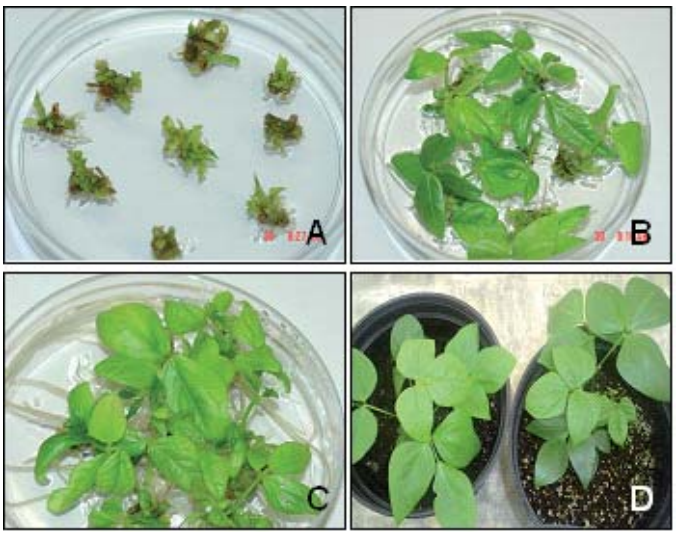

Fig.1. *In vitro* regeneration of cowpea plants cv Early Scarlet from shoot meristem. A. Development of multiple shoots from precultured shoot meristems on BA medium (0.89 µM); B. Multiple shoots, separated and transferred to rooting medium; C. Rooted plants in half-strength MS medium; and D. Fully established plants in the greenhouse.

Discussion

Cowpea is one of the most recalcitrant leguminous vegetable for regeneration and transformation (Popelka *et al.*, 2004). Consequently, well known insecticidal genes such as those for *Bacillus thuringiensis* (*Bt*) toxin, alpha-amylase inhibitor, plant lectins etc. could not be introduced into cowpea due to the lack of a simple, routine and reproducible regeneration system. Previously, regeneration of cowpea plants was achieved through organogenesis and somatic embryogenesis using explants such as cotyledons, hypocotyls, primary leaves or embryonal axes (Muthukumar *et al.*, 1995; Pellegrineschi, 1997; Brar *et al.*, 1999a, b; Anand *et al.*, 2000, 2001; Ramakrishnan *et al.*, 2005). However, the frequency of plant regeneration was too low (1-32%) to establish a routine and reproducible transformation system in cowpea.

In recent years, use of meristems as a source of totipotent cells for regeneration and transformation has emerged in a number of plants including legumes (Somers *et al.*, 2003). To date, the meristem-based transformation method has been successfully established in several species such as pea, sunflower, corn, tobacco, rice, and maize (Hussey *et al.*, 1989; Bidney *et al.*, 1992; Gould *et al.*, 1991a, b; Zimmerman and Scorza, 1996; Park *et al.*, 1996; Zhang *et al.*, 2002). Because inducing organogenesis or somatic embryogenesis is difficult, meristem-based direct regeneration may overcome bottlenecks in cowpea transformation.

In our study, shoot meristem culture was first established in the cultivar Early Scarlet. Embryos were cultured on MS basal medium for 3-5 days. Subsequently, shoot meristem was isolated and cultured on different concentrations BA. At least three shoots were regenerated from each meristem on medium containing 0.89 µM BA. Calli began to proliferate at the base of the meristems with increasing concentrations of BA (2.2-22.2 µM) thereby significantly reducing the number of shoots regenerated. Further, the number of multiple shoots significantly increased when shoot meristems were preconditioned on medium containing high BA (8.9 µM) before culturing on 0.89 µM BA, regenerating at least 6-7 shoots per meristem. The positive effect of preculturing explants on media containing cytokinins like BA on shoot regeneration has been reported in other legumes such as grain legume (*Vigna mungo*), *Phaseolus* sp. and *Cajanus cajan* (Shiv Prakash *et al.*, 1994; Santalla *et al.*, 1998; Saini and Jaiwal, 2005). Once the regeneration system for the cultivar Early Scarlet was optimized, the system was applied to the cultivars Coronet, Quick Pick and AR87-43568. The shoot meristems from these cultivars were isolated from the embryos precultured on 8.9 µM BA and cultured on 0.89 µM BA. All three cultivars produced six to seven shoots per meristem, demonstrating the applicability of the shoot meristem-based protocol to different genotypes. The regeneration efficiency of shoot meristems that produced multiple shoots ranged from 72-88% in the four cultivars compared to 1-32% obtained through organogenesis or somatic embryogenesis. In conclusion, we have successfully established a simple, rapid and efficient regeneration system in cowpea using shoot meristems. The regenerated plants exhibited no phenotypic and genotypic abnormalities. Moreover, the regeneration response of four cultivars to the shoot meristem based system was similar, demonstrating the genotype-independent nature and applicability of this protocol. The regeneration procedure developed from this study may be used to transform cowpea with insecticidal genes through biolistics transformation.

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Rapid *in vitro* propagation of grapevine cv. Crimson Seedless— Influence of basal media and plant growth regulators

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Abstract

Grapevine genotypes differ in tissue culture requirements and thus require optimized culture conditions for *in vitro* propagation. Single node segments of Crimson Seedless cultured on six different basal media *i.e.* Murashige and Skoog (MS), Eriksson (ER), Gamborg (B5), Nitsch and Nitsch (NN), Woody plant medium (WPM) and Chee and Pool (C_2d) showed different percentage of shoot initiation and morphogenetic responses. The maximum shoot initiation (90.0%) was observed in MS medium. Except ER, all other media induced rooting at the base of nodal segments in varying percentages though number and quality of roots and their establishment on transfer to pots varied greatly. WPM induced the maximum rooting in nodal segments (69.1%) with establishment rate of 100.0%. Induction of multiple shoots in nodal segments was achieved on inclusion of 6-benzyl adenine (BA) (8.87 µM) and indole-3-butyric acid (IBA) (1.48 µM) in the MS medium. In second sub-culture *i.e.,* at 90 days, shoot bud proliferation could be increased many fold on transfer of these initial shoot clumps to glass bottles instead of culture tubes. The maximum average number of primary shoots (19.5 per explant) was achieved on MS with BA (8.87 µM) and IBA (1.48 µM). Elongation of shoots was achieved on MS with BA (2.22 µM) + α-naphthalene acetic acid (NAA) (0.54 µM). Induction of *ex vitro* rooting and establishment of rooted shoots after transfer to pots was achieved in different efficiencies when shoots were given pulse treatment of indole-3-acetic acid (IAA) or IBA or NAA at 57.08, 49.0 and 53.71 µM, respectively, for 5 or 10 min. Survival of *in vitro* and *ex vitro*-rooted shoots on potting was 90.0 and 100.0%, respectively.

Key words: Crimson Seedless, grapevine, micropropagation, *Vitis.*

Introduction

In grapes, response of different cultivars to *in vitro* multiplication varies (Barlass and Skene, 1980; Botti *et al.*, 1993). Hence, this necessitates the optimization of micropropagation procedure for different cultivars, clones or newly introduced varieties. Shoot apex as an explant was commonly used for micropropagation of herbaceous species (Murashige, 1977; Abbot, 1978) but to a lesser degree in woody species. Grapevine was among the first woody plants, where the use of shoot apices and axillary buds for *in vitro* propagation of various species and cultivars of *Vitis* was reported. *In vitro* propagation of vines could be obtained by culture of shoot apices (Harris and Stevenson, 1979; Goussard, 1981) and adventitious shoot formation from fragmented apices (Barlass and Skene, 1978). Use of other explants like meristem in *Vitis rotundifolia* (Thies and Graves, 1992), microcutting and axillary buds in *Vitis* x *Muscadania* hybrids (Torregrosa and Bouquet, 1995) have also been documented. Despite a moderate multiplication rate, nodal segment remains a widely used explant in micropropagation of vines due to its operational feasibility and genotype stability (Torregrosa *et al.*, 2001). *In vitro* propagation could be obtained by axillary shoot initiation in nodal cuttings (Galzy, 1969). Mhatre *et al.* (2000) used nodal segments as explants to propagate three *vinifera* varieties. Studies on *in vitro* culture and propagation of vines have recently been reviewed by Torregrosa *et al.* (2001).

Crimson Seedless, a red table grape variety was developed by Ramming and Tarailo of the USDA, Fresno, California, USA as a result of cross between Emperor and C33-199 (Dokoozlian *et al.*, 1998). Retail trade over there has received the variety favorably due to its excellent eating characteristics like crisp and firm berries. *In vitro* propagation offers an advantage of clonal multiplication of desired material at faster rate and on a continuous basis. To the best of our knowledge, there is no report available for *in vitro* propagation of Crimson Seedless. The presernt paper deals with the Crimson Seedless specific micro propagation requirements.

Materials and methods

Plant material: Twigs of field grown vines of Crimson Seedless were collected from the vineyard of National Research Centre for Grapes, Manjri, Pune, India. Single node segments (1.5-2 cm long) were used as explant for culture. Nodal segments were surface sterilized by soaking them in liquid soap solution for 10 min followed by thorough rinses with running tap water. The explants were then submerged in 0.1% fungicide solution (Bavistin™, BASF, India) for one hr followed by 2-3 washes with sterile distilled water. Then the explants were treated with 0.1% (w/v) mercuric chloride for 10 min followed by several rinses with sterile distilled water in a laminar flow hood. Excess water was removed by blotting dry the explants on a sterile filter paper.

Shoot initiation: For shoot initiation, nodal segments were inoculated in glass culture tubes containing following six different basal media: MS (Murashige and Skoog, 1962), ER (Eriksson, 1965), B5 (Gamborg *et al.*, 1968), NN (Nitsch and Nitsch, 1969), WPM (Llyod and McCown, 1981) and C_2d (Chee and Pool, 1987). Different workers have reported *in vitro* propagation of grapevine

employing various basal media. Based on the earlier reports, these six media were selected to determine the optimum basal medium for shoot initiation and other morphogenetic processes for the cultivar Crimson Seedless.

To maximize the shoot initiation response, another experiment was set with MS basal medium supplemented with BA (0.44 µM-44.38 µM). Each culture tube had only single nodal segment.

Induction of multiple shoots: For induction of multiple shoots, primary nodal segments from field grown vines as well as secondary nodal segments excised from *in vitro* grown shoots from primary nodal segments were inoculated on MS basal medium supplemented with different growth regulators like BA (8.87 µM) alone or in combination with IAA (0.57-1.71 µM) or IBA (0.49-1.48 µM) or NAA (0.54-1.61 µM). Explants with induced multiple shoots were shifted to fresh media after every 4 weeks.

Elongation of multiple shoots: For elongation, shoots clumps were kept on MS basal medium supplemented with BA (2.22 µM) alone or in combination with IAA (0.57-1.71 µM) or IBA (0.49 -1.48 µM) or NAA (0.54-1.61 µM).

***In vitro* rooting of shoots**: Elongated shoots were transferred to culture tubes containing half strength semi-solid or liquid MS basal medium supplemented with IAA (0.57-1.71 µM) or IBA (0.49-1.48 µM) or NAA (0.54-1.61 µM). Agar (0.65%) or gelrite (0.2%) were used as gelling agents.

Sucrose (2%) as a carbon source was added to all the media and pH adjusted to 5.8 before autoclaving at 121^{0}C and 105 KPa for 20 min. All the growth regulators were added before autoclaving.

***Ex vitro* rooting**: Three auxins, IAA, IBA and NAA at a concentration of 57.08, 49.0 and 53.71 µM, respectively, were used for pulse treatment. Elongated shoots (4.5-5.0 cm) were given pulse treatment for 5 or 10 min. After pulse treatment, shoots were transferred to plastic cups consisting of a mixture of sterile peat: soil: vermiculite (1:1:1). Plants were irrigated with ¼ strength of MS salts medium without sucrose and covered with thin and transparent polythene sachets and placed in growth room with 24h light with an intensity of 24.4 µ mol m^{-2} s^{-1} at 25 ± 2^{0}C. Untreated shoots, which served as control, were also transferred to the same potting mixture and growth conditions.

Hardening of plants: Shoots rooted under *in vitro* conditions and nodal segments with direct rooting in basal media were transferred to plastic cups containing a mixture of soil and sand (1:1). Sachet technique followed by Ravindra and Thomas (1995) and Bharathy *et al.* (2003) was used for hardening of *in vitro* and *ex vitro*-rooted shoots and nodal segments with induced direct rooting in basal media. Plants were covered with thin, transparent polythene sachets and kept in growth room having 24h light with intensity of 24.4 µ mol m^{-2} s^{-1} at 25 ± 2^{0}C. After 2 weeks, plants were shifted to another room having ambient temperature. Here, sachets were cut at top corners and were removed completely after 2 weeks. After that plants were transferred to a polyhouse.

Observations of all the experiments were taken at 30 days interval. Data were analyzed using ANOVA (Spiegel, 1992).

Results and discussion

Shoot initiation: Shoot initiation in nodal segments commenced after 7 days of inoculation and continued up to 25 days. Out of six nutrient media tested, the maximum shoot initiation in explants was observed in MS basal medium (90.0%) followed by NN (89.4%) and WPM (87.2%) after 30 days of inoculation (Table 1). Percent of explants showing shoot induction was highest (85.7) in MS followed by 78.8 in NN and 78.7 in WPM.

Six different basal media had varying influence on induction of direct rooting in explants. With the exception of ER, all other basal media induced rooting at the basal end of nodal segments (Fig. 1A). The maximum response (69.1%) was observed in WPM followed by B5 (60.0%) though establishment of rooted nodal segments into plants on potting was 100.0 and 66.7%, respectively. Direct rooting in nodal segments has advantage in micropropagation, since explants rooted in this manner can directly be transferred to pots and hardened plantlets can be obtained after 2 months. Also use of single node cuttings in culture instead of 3-4 node cuttings used in vineyard can give larger number of plants if source of mother material is a serious limitation.

Induction of shoots in nodal segments could be increased to 100.0% on incorporation of BA at 4.44 µM in MS basal medium (data not shown). There was no rooting at the base of explants in any of the BA concentration unlike BA free MS basal medium. In a study with different grapevine cultivars and rootstocks, Roubelakis-Angelakis and Zivanovitc (1991) reported increased rhizogenic effect in single node segments on medium containing lower amounts of N, K, Ca and Mg. In our study, WPM contains

Table 1. Effect of different nutrient media on morphogenetic responses in Crimson Seedless

Nutrient Medium	Number of explants Inoculated	Explants showing shoot initiation (%)	Explants developed shoots (%)	Average length of shoots (cm) ± SD	Explants showing rooting at base (%)	Average number of roots per explant ± SD	Plants established on potting (%)
MS	70	90.0	85.7	1.36±0.26	25.0	3.67±0.26	33.3
ER	45	75.6	66.7	1.54±0.50	00.0	0.00±0.00	00.0
B5	69	60.9	53.7	1.75±1.00	60.0	6.03±0.67	66.7
NN	47	89.4	78.8	1.30±0.47	55.8	6.75±0.67	00.0
WPM	47	87.2	78.7	1.57±0.74	69.1	6.52±1.70	100.0
C_2d	46	84.8	78.3	1.36±0.16	22.0	2.11±0.08	25.0
LSD (*P*=0.05)		15.2	11.4	0.49	5.60	1.30	8.1
		*	**	**	**	*	**

* Significant at 5%; ** significant at 1%.

Explant: Primary single node segment. Observations recorded after 30 days of inoculation.

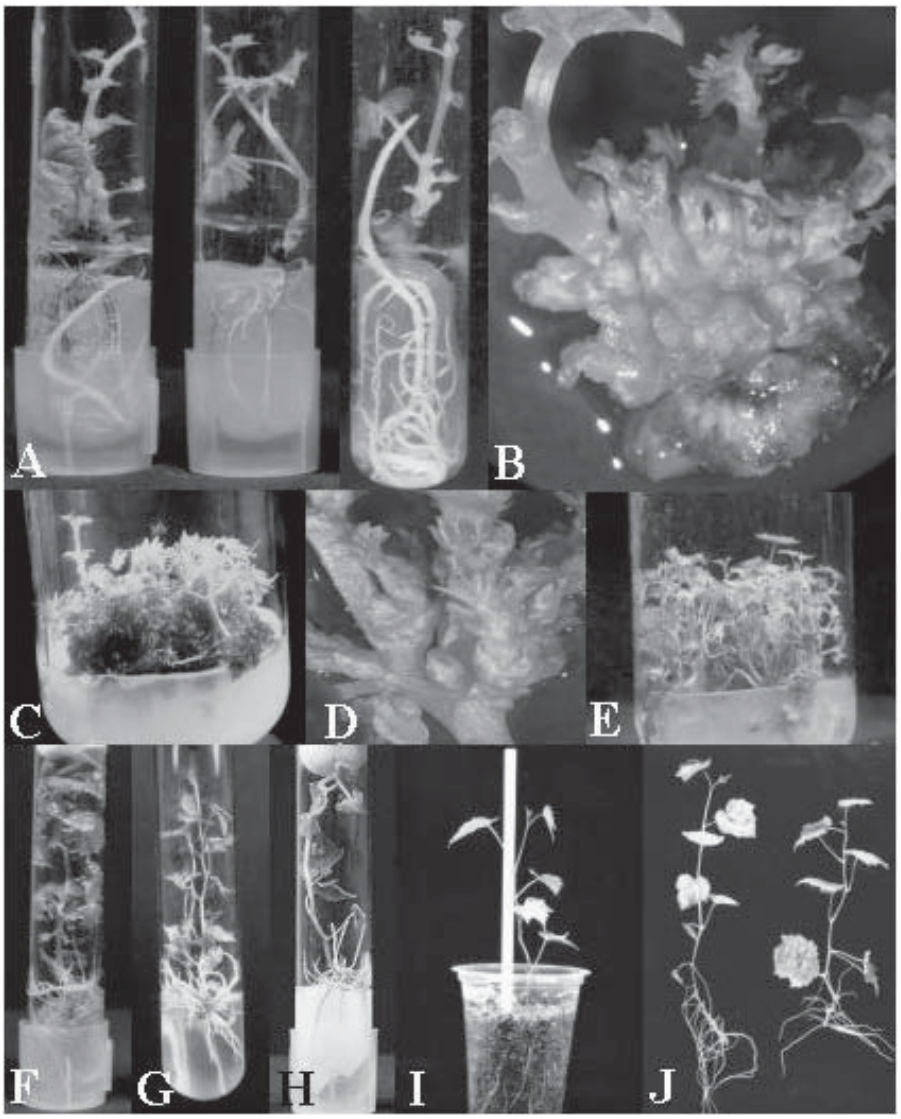

Fig. 1. Direct rooting in single node explants (A); Multiple shoots in secondary nodal segment (B); Proliferation of multiple shoots (C); Shoot buds in axils (D); Elongated shoots (E); Rooted shoot in medium with agar (F); Rooted shoot in medium with gelrite (G); Rooted shoot on filter paper bridge in liquid medium (H); *Ex vitro* rooted shoots (I & J).

lower amount of nitrogen compared to other five media tested and hence could be a reason for increased rooting response in nodal segments. Similarly, maximum amount of nitrogen present in ER medium compared to all other media tested could be a reason for absence of rooting in the ER medium.

Multiple shoot induction: In preliminary trials conducted to optimize the ideal concentration of BA for multiple shoot induction, BA concentration 8.87 µM was found to be better for inducing multiple shoots in higher percent of explants. In further experiments, various auxins were tried along with BA at this concentration to maximize multiple shoot induction. In two separate experiments; both primary as well as secondary nodal segments were used to induce multiple shoots. An average of 2.71 shoots per primary nodal segment could be induced on MS medium with BA (8.87 µM) and IBA (0.98 µM) in 37.0% of explants after 30 days of inoculation (data not shown). More or less similar observations were recorded for secondary nodal segments too (Table 2). Out of 3 auxins tested, IBA at 0.98 µM added to MS medium containing BA (8.87 µM) induced multiple shoots in 25.0% of the explants. Results with BA (8.87 µM) and IAA at all three levels varied marginally. Inclusion of NAA in the medium not only induced lower response of multiple shoots but also resulted into heavy callusing at basal end of explants and shoots were hyperhydric.

On further subculture of these explants onto their respective media in test tubes did not improve multiple shoot number in majority of the media. It was observed that node region swelled and enlarged in size. When these explants were subcultured to glass bottles instead of test tubes, a dramatic increase in number of multiple shoots was observed at the end of 30 days. On an average 19.5 shoots per explant were recorded on MS basal medium supplemented with BA (8.87 µM) and IBA (1.48 µM) (Table 2). Also, a large number of shoot buds were observed in axils of multiple shoots (Fig. 1B, C and D). The poorest response was recorded on MS supplemented with BA (8.87 µM). MS alone without any growth regulator (control) did not show multiple shoot induction.

Due to its favorable response, BA has been the most commonly used cytokinin in grape tissue culture. BA in the range of 5-10 µM was found to be an effective growth regulator for induction of

shoots in grapevine cultures (Harris and Stevenson, 1982; Mhatre *et al.*, 2000). Lee and Wetzstein (1990) reported that higher BA levels (20, 30 and 40 µM) strongly inhibited shoot elongation, with few or no larger shoots in *Vitis vinifera* cv. Summit. They also reported that proportion of small (<1 cm) shoots increased with increased BA levels and cultures at higher BA levels had dense, unexpanded shoots with high mortality. Thomas (1997) obtained multiple shoots with poor elongation at BA (5 µM) in *Vinifera* cultivar 'Arka Neelamani'. He also observed that BA at higher level (10-20 µM) resulted into condensed shoots or undifferentiated growth. Chee and Pool (1985) reported that shoot proliferation of *Vitis* hybrid Remaily Seedless increased with increased concentration of BA (0-80 µM) reaching maximum at 5 µM. Hence optimization of BA concentration in the present study was considered a necessary step.

Table 2**.** Effect of growth regulators and subculture on induction and proliferation of multiple shoots in secondary nodal segments of Crimson Seedless

Medium composition (concentration, µM)	Explants showing multiple shoots (%)	Number of shoots per explant ± SD	
		(30 days)[1]	(90 days)[2]
MS+BA (8.87)	18.8	2.33±0.20	2.33±0.00
MS+BA (8.87)+IAA (0.57)	18.8	2.50±0.25	13.00±6.02
MS+BA (8.87)+IAA (1.14)	18.8	2.50±0.17	15.20±5.35
MS+BA (8.87)+IAA (1.71)	15.6	2.00±0.00	11.80±6.83
MS+BA (8.87)+IBA (0.49)	9.3	2.00±0.00	8.67±6.65
MS+BA (8.87)+IBA (0.98)	25.0	2.13±0.07	16.17±8.92
MS+BA (8.87)+IBA (1.48)	15.6	2.60±0.50	19.50±4.94
MS+BA (8.87)+NAA (0.54)	9.9	2.00±0.00	9.50±3.62
MS+BA (8.87)+NAA (1.07)	9.4	2.33±0.17	6.33±4.93
MS+BA (8.87)+NAA (1.61)	12.5	2.00±0.00	8.60±2.07
MS	0.0	0.00	0.00
LSD (*P*=0.05)	2.16	0.27	8.17
	*	*	**

* Significant at *P*=0.05; ** significant at *P*=0.01. [1] Explants - shoot clumps in test tubes; [2] Shoot clumps in bottles.

Table 3. Effect of BA and auxins on elongation of multiple shoots in Crimson Seedless

Medium composition (concentration, µM)	Number of shoots elongated per clump	Average height of elongated shoots (cm)
MS	3.60±2.73	1.70±1.85
MS+BA (2.22)	8.10±2.91	3.28±1.69
MS+BA (2.22) + IAA (0.57)	6.70±2.76	3.70±1.38
MS+BA (2.22) + IAA (1.14)	4.30±2.15	4.20±1.72
MS+BA (2.22) + IAA (1.71)	6.00±1.98	3.50±0.81
MS+BA (2.22) + IBA (0.49)	6.50±3.90	2.99±0.90
MS+BA (2.22) + IBA (0.98)	5.85±2.81	3.27±1.81
MS+BA (2.22) + IBA (1.48)	5.14±3.79	3.22±0.99
MS+BA (2.22) + NAA (0.54)	7.85±4.93	4.53±1.26
MS+BA (2.22) + NAA (1.07)	3.43±3.56	4.71±2.01
MS+BA (2.22) + NAA (1.61)	3.00±2.36	4.97±2.86
CD (P=0.05)	5.64	2.32
	**	**

** Significant at *P*=0.01. Culture vessels used: Glass bottles. Observations recorded after 30 days of inoculation.

Elongation of multiple shoots: Since multiple shoots induced were stunted in growth and were in form of clumps, it was necessary to define a medium for shoot elongation. MS basal medium supplemented with lower concentration of BA (2.22 µM) alone or in combination with IAA (0.57-1.71 µM) or IBA (0.49-1.48 µM) or NAA (0.54-1.61 µM) was tested (Table 3). The least elongation was obtained on MS basal medium without any growth regulator (control). The maximum number of shoots (8.1 per clump) elongated (Fig. 1E) on MS with BA at 2.22 µM followed by 7.85 shoots per clump on MS with BA (2.22 µM) + NAA (1.61 µM). On these media, shoots grown could be separated easily from each other. Though comparatively higher elongation was achieved on media with NAA at all the three levels, however there was excessive callusing at base with adventitious roots, an effect undesirable for plant establishment. Similar to our findings, Mhatre *et al.* (2000) reported enhanced shoot elongation with addition of 1.14 µM IAA to the MS medium containing BA (2.22 µM) compared to the medium containing BA (2.22 µM) alone. From the published reports, it can be inferred that no fixed BA concentration was applicable for different stages of *in vitro* multiplication of different grapevine cultivars and concentrations need to be optimized for each cultivar.

***In vitro* rooting**: Since different auxins are documented to exhibit varying rooting response depending on cultivars, we investigated rooting response in shoots using MS half strength semi-solid medium gelled with agar (Fig. 1F) or gelrite (Fig. 1G) and also liquid medium with filter paper supports (Fig. 1H) with supplement of IAA (0.57-1.71 µM) or IBA (0.49-1.48 µM) or NAA (0.54-1.61 µM). Though rooting of *in vitro* shoots could be observed in varying percentages in agar or gelrite solidified or liquid media without auxins (control), however number of roots induced was fewer compared to media with auxins. It was also observed that with NAA, the number of roots was higher compared to media with IBA at same concentrations, which is in

Table 4. Effect of auxin pulse treatment on *ex vitro* rooting of shoots and plantlet establishment in Crimson Seedless

Treatment	Percentage of shoots rooted	Number of roots per shoot ± SD	Root length (cm) ± SD	Shoot length (cm) ± SD	Plants established (%)
Control	62.5	10.17±0.19	3.65±0.59	11.28±0.39	56.3
IAA 57.08 µM for 5 min	73.7	9.60±0.58	4.65±1.50	9.20±1.92	52.6
IAA 57.08 µM for 10 min	100.0	11.42±0.95	4.34±0.87	11.16±0.29	100.0
IBA 49.00 µM for 5 min	84.2	11.29±1.55	3.24±1.11	9.75±0.42	73.9
IBA 49.00 µM for 10 min	84.2	11.63±0.35	4.70±1.25	10.60±2.89	79.0
NAA 53.71 µM for 5 min	94.7	8.66±0.35	2.43±0.52	8.13±0.02	84.2
NAA 53.71 µM for 10 min	94.7	16.59±2.54	4.60±1.15	9.59±2.40	94.8
LSD (P=0.05)	17.8	1.86	1.70	2.60	12.4
	**	**	**	*	**

* Significant at *P*=0.05; ** significant at *P*=0.01. Observations recorded after 40 days of transfer to cups.

agreement with the findings of Helior *et al.* (1997). In the present study, the higher dose of auxin induced callusing and lead to poor establishment of the plants during hardening. Among the 3 auxins tested, NAA at 1.07 µM induced 100.0% rooting of shoots in all three conditions mentioned above and was found to be a better auxin for Crimson Seedless (data not shown). Survival of rooted shoots after hardening was 90.0%. Similar to our studies, Gray and Benton (1991) demonstrated that NAA at 1 µM incorporated in the media significantly increased the percentage of rooting, number of roots per shoot, and root length in three muscadine cultivars of grapevine. IBA is reported to be a better auxin for *in vitro* rooting of various clones of grapevine (Novak and Juvova, 1983) and in *vinifera* cv. Pinot noir (Helior *et al.*, 1997). Harris and Stevenson (1982) obtained better rooting with IAA (0.57 µM) in different clones of grapevines.

Though half strength MS medium devoid of growth regulators induced rooting in 84.1% shoots with an average of 5.2 roots per shoot, addition of auxin in the medium significantly improved the percent rooting, number of roots per shoot and survival percentage on potting.

***Ex vitro* rooting**: Experiments were carried out with *ex vitro* rooting to circumvent the *in vitro* stage, which takes about 3-4 weeks in culture. Elongated shoots were given pulse treatment of IAA (57.08 µM) or IBA (49.0 µM) or NAA (53.71 µM) for 5 or 10 min. Roots became visible through the transparent plastic cups (Fig. 1I) after 15 days of potting of shoots. Though rooting of shoots was observed in all the treatments, efficiency of response varied. The maximum response (100.0%) in terms of induction of roots and establishment of rooted shoots after potting was recorded with pulse treatment of IAA (57.08 µM) for 10 min (Table 4) (Fig. 1J). Gray and Benton (1991) in cultivars of muscadine grapes (*Vitis rotundifolia*) demonstrated that more shoots rooted *in vitro* than in *ex vitro* (77% vs. 46%), however, *ex vitro* technique was preferred, since acclimation of plants was achieved in lesser time and a major *in vitro* step was eliminated.

Hardening of plantlets: *In vitro* as well *ex vitro* rooted shoots and primary nodal segments with direct rooting could be hardened successfully by following Sachet technique. It was observed that covering of plantlets with polythene sachets for minimum of 4 weeks was very essential. Though top corners of bags could be cut after 2 weeks, however, complete removal of bags before 4 weeks caused scorching and drying of *in vitro* leaves.

From the present studies, it can be demonstrated that Crimson Seedless can successfully be propagated *in vitro* culturing single node segments on WPM without growth regulators where explants induced direct rooting at the base, initiated shoots and showed survival on potting. By this technique, hardened plantlets could be produced in 2 months. Another method is to induce multiple shoots in primary or secondary nodal segments and its proliferation on transfer to glass bottles after 60 days of culture in test tubes. By second route, plant production could be increased by many fold. Shoots could be rooted *ex vitro* by pulse treatment of auxins. This bypass one major *in vitro* stage and cuts down the cost and time of production. Hardening of plantlets could be achieved in a simple set up by Sachet technique. Thus, present procedure would be of immense help in commercial production of planting stock of this exotic variety of grapevine.

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Effect of heavy manuring of phosphorous and its toxicity on growth, photosynthesis and photosynthetic pigments in Zn-efficient genotype of spearmint MSS-5

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Abstract

Changes in growth attributes, photosynthesis (Pn), photosynthetic pigments with γ-Glu.cys peptidase peptide and Zn accumulation in a Zn-efficient genotype of spearmint MSS-5 were investigated. Effect of phosphorus toxicity on MSS-5 were significantly different than the other genotypes; Arka, Neera and control (the local strain), in terems of phenotypic changes in height and a decrease in chlorophyll contents and CO_2 exchange rate. Heavy P manuring lead to the tolerance of Zn accumulation in MSS-5 with γ-Glu.cys. peptidase peptide with high protein contents and Pn. Hence, the P toxicity induced a differential utilization of γ-Glu.cys.peptidase peptide for higher accumulation of Zn in MSS-5 spearmint with higher photosynthetic rate for increasing the height and essential monoterpene oil(s). The study also indicated that accumulation of toxic heavy metal-Zn with γ-Glu.cys.peptidase peptide made protein synthesis easier with antioxidants Zn cofactor enzymes.

Key words: Spearmint, *Mentha spicata*, Zn-efficient genotype MSS-5, protein, photosyntheis, photosynthetic pigments, Zn toxicity.

Introduction

The heavy and unmanaged fertilization of major nutrients *viz.*, N, P & K for the improvement of crop yields, leads to the deficiency of micronutrients. Heavy doses of P and its precipitation to phosphate salts makes Zn unavailable to the plant (Marschner, 1986). Zn shows the toxicity in medicinal and aromatic plants cultivation (Misra and Ramani, 1991). Furthermore, the acidic soil of hills bears the Fe toxicity in rose cultivation. One way or other, the toxicity of Zn and Fe especially in micronutrients usage affects the productivity and secondary plant products. The phytoremediation is a technique that plants uses to cleanup contaminated soil and water. No work has been reported so far on the cultivation of medicinal and aromatic plants on Fe efficient genotypes to cleanup the contaminated soil with heavy metal Zn.

Spearmint (*Mentha spicata* L.) is widely cultivated to obtain the essential monoterpene oil(s). Zn efficient genotype, MSS-5 has been taken up for the complete exploitation of monoterpene oil(s), with the aim of phytoremediation and to extract out Zn from Zn toxic fields. The aim to use this Zn efficient genotype MSS-5 was to know the physiology and growth behaviour in the wake of phytoremediation processes. In phytoremediation some plants tolerate high levels of toxic micronutrient metals by a variety of mechanisms such as reduced uptake, active efflux and intracellular and/or extracellular sequestration. The most predominant molecules in intracellular detoxification, widely prevalent in eukaryotes are thiol tripeptides, γ-Glu-Cys peptides or phytochelatins. These are synthesized by phytochelatin synthase which is activated by subleathal metal concentrations and play a crucial role in cytosolic metal detoxification (Steffens, 1990; Zenk, 1996). The physiological concentrations of these intracellular metal binding ligands have sometimes been used as a specific indicator of metal tolerance in plants (Grill *et al.*, 1988), and the role of heavy metals in decline and damage to forest ecosystem (Gawell *et al.*, 1996). Zinc is potentially toxic metal when transferred from plants via food chain to human. Agriculture system is also the principle sources of Zn, includes the heavy P fertilization for crops (Misra and Sharma, 1991).

Possibly Zn tolerant spearmint efficient genotypes MSS-5 cultivation in fields leads, for the removal of toxic heavy metal Zn, from heavily phosphorous fertilized soil, for the establishments of tolerant varieties capable of synthesizing more γ-Glu-cys peptides or phytochelatins. The essential monoterpene oil(s) of this efficient genotypes is commercially important for pharmaceutical and aromatic industries. The efficient genotype MSS-5 due to toxicity of heavy metals produces the Zn induced Fe deficiency symptoms. Therefore, a detailed study on the growth and physiology of an efficient genotype MSS-5 alongwith Arka and Neera was conducted to compare Zn accumulation and tolerance against the phosphorous toxicity and to show the Zn induce Fe deficiency in genotypes with primary plant products –photosynthetic pigments and Pn, and simultaneously the essential monoterpene oil(s)in spearmints, in heavly phosphorus fertilized crops.

Materials and methods

The experiment was conducted in controlled glasshouse condition from December to March at an ambient temperature of 27 ± 3^{o}C and with 11 h day length. Uniform suckers of spearmint cultivars *viz.*, MSS-5, Arka, Neera and a local strain (control) were grown in 5,000 cm^3 plastic containers containing nutrient solution (Hoagland and Arnon, 1950). Each 2.8 Fe µg mL^{-1} treatment and mint strain were replicated 6 times and put in completely randomized block design with complete 5.6 Fe µg mL^{-1} strength, nutrient solution, were taken under the existing

studies. The composition of nutrient solution was (as mg L^{-1}): 102 K, 100 Ca, 70 N-NO_3, 16 S, 12 Mg, 9 Cl, 5 P, 0.52 B, 0.33 Mn, 0.33 Mo, 0.10 Zn, 0.02 Cu and Fe was as Fe-EDTA (Ferric ethylinediamine tetraacetate). During the study, instead of 5 mg L^{-1}, 10 mg L^{-1} phosphorous was added in nutrient solution for toxicity in each treatments of 2.8 and 5.6 µg Fe mL^{-1}. Initial pH of the nutrient solution was 6.7 to 6.8, which was monitored and adjusted periodically Zn with 1.0 m M KOH or 2.0 m M H_2SO_4 to maintain a value of 7.2.

MSS-5, Arka and Neera genotypes when subjected to Fe deficiency stress (2.8 mg Fe mL^{-1} treatment), resulted in root exudation, which decrease the pH of the nutrient medium and showed the chlorosis of younger leaves whereas older one remains green. The root exudation and their ability to absorb and utilize iron in the ferrous form vary, with the genotypes of the crop plants. The efficient genotype of spearmint MSS-5 only turned green after the Fe deficiency visualizes characters, where as the severe chlorotic Arka and Neera did not turn green. Chlorosis in terms of total chlorophyll were estimated for the cultivar MSS-5 genotype which behaves as a Fe efficient genotype with more root exudation which was measured with the method of Arnon (1949).

The cultivar MSS-5 genotype behaves as a Fe efficient genotype with more root exudation of phenolic compounds, especially the caffeic acids. The phenolic compounds, the caffeic acids were estimated in root exudation by the method of Singh *et al.* (2001). The plan tissue Fe and Zn contents were estimated with 1 N HCl extracts on atomic absorption Pye Unichem, 2900 (Misra, 1992). The lignin was estimated by the Kalson method of Browning (1967), 5 g samples were digested with 72% H_2SO_4, then diluted with acid; to hydrolize and solubilize the polysachharides. The insoluble residue was dried and weighed as lignin on % basis. This partially solubilized as acid soluble lignin from spearmint, were further quantified on UV absorbance at 410 nm.

HPLC analysis of γ-Glu-cys peptides or phytochelatins (PCs): For the separation of PCs, HPLC analysis was performed in crude extracts of plant tissue following the method of Grill *et al.* (1991). Frozen plant tissue (1 g FW) was homogenized in 0.5 ml 1 N NaOH containing 1 mg mL^{-1} sodium borohydride. Alter centrifugation at 13,000 g at 4°C, the supernatants were acidified by adding 3.6 N HCl and precipitated protein was removed by centrifugation. The protein was estimated by the method of Lowry *et al.* (1951). Separation of PC peptides was done on a reverse phase C-18 column (µ Bondapack, RP 4 µm) with a linear gradient 0.1% Trifluoro acetic acids at a flow rate 0.5 ml min^{-1} using the applied biosystem HPLC (model No. 783A) at 220 nm. Experiments were done and HPLC in triplicates, and repeated thrice.

Pn and essential monoterpene oil(s): Initially, Pn of the third leaf was measured in a closed system using a poratable computerized photosynthesis model Li-6000(Licor, Lincoln, USA), as described in Singh *et al.*(1999) and total essential monoterpene oil(s) were extracted by 100 g fresh chopped leaves in Clevenger's apparatus (Clevenger, 1928).

All measurements were taken in triplicate and the results are given as means±SE. The data were analyzed statistically by two way ANOVA followed by 't-test' for comparing the means following Armitage (1971). The correlation coefficient among the characters were also analyzed further the values are statistically analysed by paired t-test.

Results

The efficient genotype of spearmint cv MSS-5 exudes more root exudates in the nutrient medium (0.54 mg g^{-1}) followed by more acidic medium than the Arka and Neera Chlorosis of younger leaves were more pronounced in MSS-5 with more and more root exudates the older leaves became green, then the Arka and Neera cultivars (Table 1). Results showed that the most Fe efficient genotype is MSS-5, where the iron uptake is 1440 µg g^{-1} in roots tissues of spearmint with further more the recovery of chlorotic younger leaves. Converting more Fe for Fe uptake in an efficient genotype by the help of maximum production of 0.82 mg g^{-1} phenolic compounds and root exudates -Fe^{+++} reductants chelation to produce more Fe^{++} availability to the plant. Table 2 indicates more toxic Zn uptake in tissue concentrations in MSS-5 cultivars (94 µg Zn g^{-1}), with maximum production of protein 1.49 mg g^{-1} and phytochelatins 45.79 m mol g^{-1} FW. The role of Zn excess in spearmint is to behave as an antioxidant, as a scavenger to the excess free radicals removal during the

Table 1. Effects of different genotypes of spearmint in deficient Fe nutrition in younger leaves (at 2.8 µg Fe ml^{-1}) in phosphorous excess

Genotype	Chlorophyll (mg g^{-1})		Phenolic compounds (mg g^{-1})	Caffeic acid (mg g^{-1})	Fe uptake (µg g^{-1})	Lignin (g g^{-1})	P value
	Deficient	Recovered					
Arka	2.01	2.07	0.65	0.41	1109	1.1	0.01
Neera	1.99	2.01	0.68	0.47	1163	1.2	NS
MSS-5	1.85	3.11	0.82	0.54	1448	1.4	0.01
Control	1.89	3.01	0.71	0.51	1437	1.1	0.01

Table 2. Effect of different genotype of spearmint in deficient Fe nutrition in younger leaves (at 2.8 µg Fe ml^{-1}) in phosphorous excess

Genotype	Height (cm)	Zn accumulation (mg g^{-1})	Pn (mg (CO_2) $m^{-2}s^{-1}$)	Protein (mg g^{-1})	Oil (%)	γ-Glu Cys peptide (m mol g^{-1} FW)	P value
Arka	31	70	124±7*	1.01	0.42	6.48±0.3	0.01
Neera	25	82	239±4	1.24	0.49	33.49±1.5	NS
MSS-5	42	94	249±2**	1.49	0.56	45.79±2.1	0.01
Control	32	90	241±3*	1.37	0.47	41.62±2.1	0.01

*, ** mean values significant at *P*=0.05 and *P*=0.01, respectively (Paired t test); NS - nonsignificant

Table 3. Correlation coefficients between different characters *viz.*, phenolic compounds, caffeic- acids, Fe uptake, Zn accumulation in *M. spicata*

Characters	Phenolic compound	Caffeic acid	Zn accumulation	Fe ptake	Lignin	Protein
γ-Glu.-Cys.-peptide	0.719**	0.712**	0.871**	0.641*	0.179	0.971**
Phenolic compound		0.699*	0.811**	0.821**	0.827**	0.642**
Caffeic acid			0.749**	0.714**	0.912**	0.617
Zn-accumulation				-0.497	0.679*	0.747**
Fe uptake					0.579	0.621*
Lignin						0.639*

*, ** values are significant P=0.05 or P=0.01, respectively

metabolism of essential monoterpene oil(s).

Table 3 indicated the γ-Glu Cys. peptidase peptide for significantly associated with toxic Zn-accumulation (r=0.871, $P \leq 0.01$). Zn accumulation and lignin (r=679, $P \leq 0.01$) and Zn accumulation with protein (r=747, $P \leq 0.01$), respectively.

Discussion

Results indicated the most efficient and inefficient genotype of all the existing cultivars *viz.*, Arka, Neera and MSS-5. The most Fe-efficient genotype is MSS-5. The root exudation of phenolic compounds (10.87 mg g^{-1}) and caffeic acid (0.54 mg g^{-1}), is more in MSS-5 cultivar where as Arka and Neera had lesser amount. Greening in the form of chlorophyll formation (3.11 mg g^{-1}) and Fe uptake (1448 µg g^{-1}) was found in MSS-5 genotype, whereas it was very less in Arka and Neera. Other workers also reported the same root exudation, and greening of the chlorosis of the genotypes, in different crops (Marschner, 1996; Brown and Jolly, 1986; Kannan, 1982).

Fe efficient genotype MSS-5 showed the Zn (94 µg Zn g^{-1}) and γ-Glu-cys peptide phytochelatin (45.79 n mol g F.wt.) then lesser concentrations of Zn and phtochelatins in Arka and Neera (Table 2). The heavy P fertilization produced Zn induced Fe-deficiency (Table 1). This Zn induced Fe deficiency was reported in *Mentha arvensis* (Misra and Ramani, 1991). Furthermore, the Zn toxicity facilitates the proteins metabolism due to the antioxidants usage of Zn (Table 2). Phytochelatins and Zn accumulation were more pronounced in spearmint MSS-5.

Plants tolerate high levels of toxic micronutrients – Zn, by a variety of mechanism, such as reduced uptake of Fe, active efflux and intracellular on extracllular sequestration of Zn. Predominant molecules in intracellular detoxification are γ-Glu-cys peptides or the phtochelatins. These phytochelatins are activated by sublethal metal concentrations and play a crucial role in cytosolic metal detoxification (Steffens, 1990; Zenk, 1996). These intracellular metal binding ligands are being used as a specific indicators of metal tolerance (Grill *et al.*, 1988). Zinc is potentially toxic metal when transferred from plants via a food chain to human and then to agriculture systems and vice-versa. Sometimes as an antioxidant dosages, in facilitating the protein synthesis in plants and also make them the Zn tolerant spearmint crops which thus leads to the phytoremediation process for excess Zn removal from the contaminated water and soil.

Moreover, the correlation coefficient in Table 3 indicated the Zn accumulation with phytochelatins (r=0.871, $P \leq 0.01$) and Zn accumulation with lignin (r=679, $P \leq 0.05$). The above significant association showed that the phytoremediation processes remove the toxic Zn from the heavily P fertilized fields by the Fe-efficient MSS-5 genotype. The lignin association with Zn accumulation further support for increased insect resistance in spearmint genotype MSS-5.

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Evaluation of composted biosolid waste as an amendment to a standard horticultural nursery mix for container grown *Callicarpa* and *Ilex* production

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Abstract

Growth of *Callicarpa dichotoma* (Lour.) 'Early Amethyst' and *Ilex glabra* (L.) 'Compacta' liners were evaluated in substrate containing 20, 40, 60, 80 and 100% composted biosolids as compared to a 3:2:1 (v:v:v) pine bark:peat:sand horticultural mix. Biosolid waste substrate amended with biosolids had higher pH, EC, nitrate, bulk density and container capacity compared to a standard horticultural nursery mix. Total porosity and air-filled capacity were greater for the control compared to substrate amended with biosolids. The effects of substrate amended with composted biosolids on growth varied for each species. *Callicarpa dichotoma* "Early Amethyst' liners grown in substrate amended with 20, 40 and 60% biosolid waste had greater shoot and root dry weight and a better visual evaluation compared to the control. *Ilex glabra* 'Compacta' liners grown in the control (standard nursery mix) had greater shoot and root dry weight and a better visual evaluation compared to any biosolid amended substrate. It was concluded that substrate amended with biosolid waste can be utilized for the container production of plants, however, its usage may be species specific.

Key words: Biosolids, sewage sludge, *Callicarpa, Ilex*, pH, electrical-conductivity, nitrate.

Introduction

Sphagnum peat is a major organic component of container substrate utilized in the nursery industry. Due to the physical and chemical qualities of peat, it is a valuable component in container substrate for the production of ornamental plants. Peat is extracted from peatlands, which are a vital part of a healthy environment. Peat vegetation rids water of heavy metals, pollutants, pathogens, excess nutrients, and stores atmospheric carbon dioxide. It also provides a natural habitat for several animal and bird species. The excavation of peat can be harmful to the biodiversity, hydrological cycle, and purification of water in the area of harvest (Grundling and Dada, 1999). Furthermore, the cost of peat has risen over the past several years (Wilson *et al.*, 2001a).

Given these factors, the nursery industry has increased its interest toward exploring alternative amendments to utilize in traditional potting media (Wilson *et al.*, 2002). Some of the amendments tested include coconut coir (Evans, 2002; Meerow, 1994), rubber tire chips (Jarvis *et al.*, 1996), recycled paper (Craig and Cole, 2000), poultry litter (Fulcher *et al.*, 2002), kenaf stem core (Wang, 1994), spent mushroom compost (Chong *et al.*, 1991) and recycled municipal waste (Kahtz and Gawel, 2004). 2.2 million tons of biosolids (sewage sludge) is incinerated or buried in landfills each year in the United States, while at the same time new environmental regulations are making space in landfills a scarcity (Rosen *et al.*, 1993). Biosolids are the treated solid organic matter comprised of private or community wastewater that can be beneficially utilized as a substrate amendment in the nursery industry (US EPA, 1999).

Plants have been shown to successfully grow in substrate that incorporates composted biosolids (Wootton *et al.*, 1981). The usage of biosolids separately and mixed with other components such as municipal leaf waste (Bugbee *et al.*, 1991) and yard waste (Wilson *et al.*, 2003; Wilson *et al.*, 2001b) has also been explored. The utilization of biosolids in this manner could aid in the conservation of peatlands, and reduce land needed for the disposal of biosolids while providing the nursery industry with an inexhaustible alternative substrate amendment.

Several potential benefits of biosolids as a substrate amendment are recognized. Biosolids are a source of nutrients (Rosen *et al.*, 1993; Falahi-Ardakani *et al.*, 1987), improve the qualities of substrate (EPA, 1999) and may be a substitute for moss peat (Rosen *et al.*, 1993). Composted biosolids decompose at a slow rate, therefore releasing nutrients at a steadier rate compared to non-composted biosolids (USDA, 1998). Root rot diseases are also suppressed in substrates that are amended with biosolids (Hoitink *et al.*, 1997). In addition, composted biosolids are potentially economically feasible (Bugbee and Frink, 1989; Vega-Sanchez *et al.*, 1987).

Potential barriers in beneficial usage of biosolids include public reluctance based upon potential health and environmental concerns (US EPA, 1999). These concerns are based upon disease causing pathogens which can be found in untreated wastewater and biosolids. In addition, odors are also a potential problem.

Biosolid stabilization is achieved typically by the addition of quicklime (CaO) or hydrated lime ($Ca[OH]_2$), which is added to either liquid biosolids before dewatering or mixed with dewatered biosolids (US EPA, 1999). These types of lime stabilization procedures meet 40 CFR Part 503 rules governing land application of biosolids.

The primary objectives of this study were to evaluate incorporation of biosolids upon 1) substrate electrical-conductivity (EC), pH and nitrate over a four-month period, 2) examine physical

characteristics, and carbon (C) and nitrogen (N) ratios of substrate amended with differing volumes of biosolids and 3) to examine the final shoot and root total dry weights of two different species grown in the substrate. These factors were monitored in order to develop a horticultural nursery mix amended with composted biosolids suitable for containerized shrub production.

Materials and methods

Biosolids produced by Waste Water Treatment Plant of Cookeville, Tennessee was utilized in this project. Biosolids utilized in this study underwent a lime-envessel pasteurization process and met the United States Environmental Protection Agency Federal Register Rules and Regulations Part 503; standards for the use or disposal of sewage sludge (US EPA, 1999). The biosolids were dewatered and quicklimed (CaO) to raise the pH to 12 or above, and pasteurized to produce class A biosolids. The biosolids were then allowed to compost under outdoor conditions for 75 days. A primary goal of composting biosolids is to produce a more stable, less-odorous source of organic matter (Rosen *et al.*, 1993).

Uniform *Callicarpa dichotoma* 'Early Amethyst' and *Ilex glabra* 'Compacta' liners (approximately 7.5 cm tall) were potted in 1-gallon (2.19 L) containers filled with 3:2:1 (v:v:v) pine bark: peat : sand mix with composted biosolids. For purposes of this study each species was evaluated as a separate experiment. The biosolids were screened to 3.0 cm and incorporated at rates of 0 (control), 20, 40, 60, 80 and 100% by volume. C and N values were determined before the addition of six grams of 14N-4.2P-11.6K (14-14-14) Osmocote with micronutrients, topdressed on all containers. Plants were grown under a 30 % shadecloth. Three-hundred milliliters of water was supplied twice daily via individual spray emitters. The study was conducted at the Tennessee State University Otis L. Floyd Nursery Crop Research Center in McMinnville, Tennessee.

The Virginia Tech pour-through method was used to collect leachate solution from the container substrate (Wright, 1986). EC and pH readings of leachate samples were taken 15, 30, 45, 60, 75 and 90 days after treatment (DAT). Leachate EC and pH were measured with a Myron Ultrameter™ Model 6P (Myron L Company, Carlsbad, California). Nitrate readings and a visual assessment of each plant were taken 30, 60 and 90 DAT. An Accumet AR 25 and electrode were used to record nitrate readings. The visual assessment was given a scale of 1-5 as follows: 1) plant died, 2) plant was near death or lost many leaves, 3) average looking plant, moderate growth, 4) good growth, few if any problems and, 5) Excellent growth, healthy leaves, no signs of chlorosis or nutrient problems. At the end of the project plants were harvested at the soil level. Container medium was washed from the roots. Dry shoot and root weights were recorded after drying at 70 ˚C for 72 h.

Three replications of each substrate treatment (0, 20, 40, 60, 80 and 100%) were evaluated for total bulk density, container capacity, total porosity, percent moisture and air-filled porosity. The North Carolina State University Porometer was utilized to determine the above mentioned substrate physical properties. Percent moisture was determined by drying a known amount of substrate at 105˚C for 24 h and weighing before and after. Container capacity was calculated by dividing the weight of the wet substrate by the volume of the pot. Standard procedures were utilized to determine bulk density, total porosity, and air-filled porosity (North Carolina State University and Fonteno and Bilderback, 1993). Total C and N concentrations were determined by a CNS analyzer (Carlo-Erba NA-1500;BICO, Burbank, California).

The experimental design was a randomized complete block design. Each treatment was replicated 6 times. All data within each experiment were subjected to an analysis of variance (ANOVA). Dunnett's test were utilized to compare treatments with the control (0% biosolids). The control was a standard horticultural nursery mix.

Results

Initial pH readings 15 DAT for the *C. dichotoma* 'Early Amethyst' liners revealed that as greater percentages of biosolids were added to the substrate the alkalinity level increased, with the 80 and 100% treatments being significantly greater than the control at 7.8 and 8.9, respectively (Table 1). pH levels generally decreased linearly throughout the duration of the project. pH results were virtually identical for the *I. glabra* 'Compacta' liners (Data not shown).

The *C. dichotoma* 'Early Amethyst' EC of substrate amended with composted biosolids was significantly higher for all treatments compared to the control beginning 15 DAT until conclusion of the project (Table 2). EC reading for the 100% treatment was four and half times greater than the control 15 DAT. However, the EC reading was never above recommended levels for any treatment of either species. The general trend, for all treatments except the control, was for the EC levels to decline or remain relatively constant from 15 DAT until the final reading 90 DAT. The control

Table 1. pH readings by days after treatment for *Callicarpa*. Values presented for the 20, 40, 60, 80 and 100% treatments are the relative increase or decrease as compared to the non-treated (0%) control

Treatment	pH reading by days after treatment					
(% by volume)	15	30	45	60	75	90
0	7.2[z]	7.1[z]	7.4[z]	7.1[z]	7.1[z]	7.0[z]
20	0.2	0.1	0.2**	0.1	0.1*	0.0
40	0.1	0.1	0.2**	0.2*	0.2**	0.2*
60	0.2	0.2	0.2**	0.2**	0.2**	0.2**
80	0.6**	0.2	0.2**	0.2**	0.2**	0.2**
100	1.7**	0.7**	0.4**	0.3**	0.3**	0.2**

[z]Actual pH reading of treatment.

*, ** Dunnett's test significant at P=0.05 or P=0.01, respectively when compared to 0 % treatment.

Table 2. EC readings by days after treatment for *Callicarpa*. Values presented for the 20, 40, 60, 80 and 100% treatments are the relative increase or decrease as compared to the non-treated (0%) control

Treatment	EC reading by days after treatment (dS m^{-1})					
(% by volume)	15	30	45	60	75	90
0	0.21[z]	0.23[z]	0.24[z]	0.24[z]	0.26[z]	0.27[z]
20	0.28	0.15**	0.25**	0.19**	0.12**	0.18**
40	0.44*	0.34**	0.39**	0.33**	0.20**	0.29**
60	0.52**	0.41**	0.51**	0.45**	0.40**	0.40**
80	0.47*	0.45**	0.47**	0.42**	0.36**	0.41**
100	0.75**	0.40**	0.41**	0.39**	0.43**	0.39**

[z]Actual EC reading of treatment.

*, ** Dunnett's test significant at P=0.05 or P=0.01, respectively when compared to 0 % treatment.

Table 3. Nitrate readings by days after treatment for *Callicarpa*. Values presented for the 20, 40, 60, 80 and 100% treatments are the relative increase or decrease as compared to the non-treated (0%) control

Treatment	Nitrate readings (ppm) by days after treatment		
(% by volume)	30	60	90
0	9.7[z]	11.9[z]	5.8[z]
20	0.5	4.3	1.3
40	9.2	15.1	2.9
60	18.2**	27.4**	4.7
80	11.0*	26.4**	10.5
100	13.6**	29.3**	21.0**

[z] Actual ppm of treatment.; ** Dunnett's test significant at P=0.05 or P=0.01, respectively when compared to 0 % treatment.

Table 4. Physical properties of composted substrate amended with biosolids. Values presented for the 20, 40, 60, 80 and 100% treatments are the relative increase or decrease as compared to the non-treated (0%) control

Treatment (% by volume)	Bulk density (g cm^3)	Container capacity (% by vol)	Total porosity (% by vol)	Air-filled Porosity (% by vol)	Moisture (%)
0	0.21[z]	56.0[z]	78.8[z]	21.1[z]	25.1[z]
20	0.08**	5.5*	-0.9	-4.6*	11.2*
40	0.16**	7.8**	-2.1	-10.0**	12.5*
60	0.27**	10.6**	-3.5	-14.2**	11.5*
80	0.37**	10.3**	-4.4	-15.1**	9.2
100	0.46**	12.4**	-5.4*	-15.9**	10.7*

[z] Actual measurement of treatment; *, ** Dunnett's test significant at P=0.05 or P=0.01, respectively when compared to 0 % treatment.

Table 5. Nitrogen and carbon concentrations of composted substrate amended with biosolids. Values presented for the 20, 40, 60, 80 and 100% treatments are the relative increase or decrease as compared to the non-treated (0%) control

Treatment (% by volume)	N(%)	C(%)	C/N ratio
0	0.65[z]	49.9[z]	76[z]
20	0.31*	-13.0*	-39*
40	0.47*	-19.9*	-50*
60	0.64*	-25.7*	-58*
80	0.77*	-29.4*	-62*
100	0.83*	-31.7*	-64*

[z] Actual measurement of treatment; *, ** Dunnett's test significant at P=0.05 or P=0.01, respectively when compared to 0 % treatment.

EC levels slowly increased from 15 DAT until 90 DAT. EC results were virtually identical for the *I. glabra* 'Compacta' liners (Data not shown). Nitrate levels for *C. dichotoma* 'Early Amethyst' were significantly greater for the 60, 80 and 100% treatments compared to the control, 30 and 60 DAT (Table 3). At 90 DAT the 100% treatment was significantly greater than the control. Nitrate levels were greatest with increased amounts of incorporated biosolids. Nitrate levels for *I. glabra* 'Compacta' were statistically similar (Data not shown).

Generally, as the percentage of incorporated biosolids increased the bulk density, container capacity and moisture significantly while the total porosity and air-filled porosity decreased (Table 4). Treatments that had greater amounts of incorporated biosolids significantly contained more N and less C resulting in lower C/N ratios (Table 5).

C. dichotoma 'Early Amethyst' grown in 40 and 60% biosolid amended substrate had the greatest shoot and root weight of any of the treatments, respectively (Table 6). The initial visual evaluation 30 DAT showed the control was the most marketable

Table 6. Growth characteristics of *Callicarpa* and *Ilex* liners grown for four months in composted substrate amended with biosolids. Values presented for the 20, 40, 60, 80 and 100% treatments are the relative increase or decrease as compared to the non-treated (0%) control

Treatment (% by volume)	Shoot dry wt (g)	Root dry wt (g)	Shoot: root ratio
Callicarpa			
0	22.85[z]	13.31[z]	1.73[z]
20	6.73	5.30*	-0.12
40	11.61**	5.60*	0.10
60	16.96**	5.73*	0.40
80	-7.43	-5.61*	0.32
100	-17.87**	-10.83**	0.23
Ilex			
0	8.70[z]	2.22[z]	3.87[z]
20	-4.22	-0.80	-0.72
40	-5.63**	-1.26**	-0.67
60	-5.05*	-0.95*	-1.00
80	-	-	-
100	-	-	-

[z] Actual weight of treatment; *, ** Dunnett's test significant at P=0.05 or P=0.01, respectively when compared to 0 % treatment.

Table 7. Visual evaluation ratings of *Callicarpa* and *Ilex* liners. Values presented for the 20, 40, 60, 80 and 100% treatments are the relative increase or decrease as compared to the non-treated (0%) control

Treatment	Visual evaluation by days after treatment		
(% by volume)	30	60	90
Callicarpa			
0	5.00[z]	4.16[z]	4.08[z]
20	-0.16	0.41	0.33
40	-0.66	0.50	0.58
60	-0.91*	0.25	0.58
80	-3.00**	-1.16**	-0.18
100	-3.90**	-2.06**	-0.48
Ilex			
0	4.25[z]	4.50[z]	4.58[z]
20	0.33	-0.33	-0.83*
40	-0.41	-1.00**	-1.16**
60	-0.41	-1.00**	-1.16**
80	-	-	-
100	-	-	-

[z] Actual measurement of treatment; *, ** Dunnett's test significant at P=0.05 or P=0.01, respectively when compared to 0% treatment.

of all treatments (Table 7). However, upon conclusion of the project, at 90 DAT, the 40 and 60% treatments had the best visual evaluation. In contrast, *I. glabra* 'Compacta' grown in the 0% (control) had the greatest shoot and root weight compared to the treatments that had incorporated biosolids (Table 6). The control also received the best visual evaluation 30, 60 and 90 DAT (Table 7). Five replications grown in each of the 80 and 100% biosolids died during the first 10 days of the project. Therefore, the 80 and 100% treatment that involved *I. glabra* 'Compacta' were excluded from the data analysis.

Discussion

The addition of quicklime during the dewatering process greatly contributed to the increased pH levels. Fitzpatrick *et al.* (1998) reported that stabilized biosolids typically had a high pH due to the chemical stabilizers, such as lime, utilized before composting. In addition, they state that the pH of most commercially produced

compost ranges from 6.7 to 7.7. The pH recommended for optimum nutrient uptake by plants ranges from 5.0 to 6.0 (Southern Nursery Assoc., 1997) to 5.2 to 6.2 (Ruter and Garber, 1993). However, depending upon the crop and method of fertilization, variations will exist. Therefore, the substrate with 80 and 100% biosolids volumes may have had a detrimental impact upon *C. dichotoma* 'Early Amethyst' root growth and subsequent foliar and overall plant development. Mortality in *I. glabra* 'Compacta' planted in 80 and 100% sewage sludge appears to be due to an excessively alkaline pH. *C. dichotoma* 'Early Amethsyt' appears to be more tolerant of alkaline substrate than *I. glabra* 'Compacta'. The pH values of the 80 and 100% treatments for both plant species were greater than the pH of most commercially produced compost (Fitzpatrick *et al.*, 1998). Furthermore, the control had the lowest pH of any of the treatments for both species.

High soluble salt content has been observed with many substrates that had incorporated biosolids (Chaney *et al.*, 1980). Results revealed that EC levels were higher with greater amounts of sewage sludge incorporated into the substrate. However, EC levels at no point during the project were above recommendations for the best management and growth of either species. This is in contrast to Chaney *et al.* (1980) reporting that soluble salt levels may be problematic in biosolid compost. Research has shown that compost comprised of biosolids and yard waste resulted in the greatest plant growth compared to other composted materials (Fitzpatrick and Verkade, 1991).

Increased growth of *C. dichotoma* 'Early Amethyst' may be attributed to increased NO_3-N, which is presumably derived from nitrification of NH_4-N. Decreased plant growth may be attributable to excessive amounts of NH_4-N and excessively alkaline substrate (Bugbee and Frink, 1989). Excessive levels of NH_4-N and pH may become more favorable to plant growth by further modifying the composting process. The addition of sawdust, for example, during the composting process may help to reduce substrate pH.

Greater bulk densities and container capacities of the biosolid amended substrate compared to the standard nursery mix are attributed to increased amounts of incorporated biosolids. Reduced total porosity and air-filled capacity are also attributed to increased amounts of biosolids. Due to the physical density of biosolid material it tends to reduce air-filled porosity, thus reducing drainage.

Composts with a C/N ratio less than 20 are considered to be optimal for the growth and development of plants (Davidson *et al.*, 1994). Compost with C/N ratios higher than 30 may be immature or lacking stability, which may promote plant phytotoxicity and mineral immobilization (Zucconi *et al*., 1981). Hue and Sobieszczyk (1999) reported that N immobilization was minimal if C/N ratios are maintained between 15 to 20. Given these results, the composted substrate utilized in the study was considered stable and beneficial for the release of N.

Depending upon the plant species, a standard nursery mix amended with biosolids up to 60% or less (by volume) provided an adequate substitute for moss peat for containerized production of *C. dichotoma* 'Early Amethyst'. However, the growth of *I. glabra* 'Compacta' in substrate amended with biosolids was detrimental to the plants' growth and development with the standard nursery mix yielding the best plant growth and development. All *C. dichotoma* 'Early Amethyst' treatments were visually considered marketable. In contrast, only the control and 20% biosolid treated *I. glabra* 'Compacta' plants were visually considered marketable. However, the results demonstrate that there is a potential usage for biosolids in the container production of plants. These results reveal that the use of dewatered biosolids as a substrate amendment for increased plant production is species specific.

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Evaluation of seasonal nutrient status in the leaves of different olive varieties grown on calcareous soils

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Abstract

The study was conducted for two successive years at a private farm in El-Saf, Giza, Egypt on 19 years old trees of olive cultivars, Picual, Aggizi and Manzanillo, grown in calcareous soils. Leaf nutrients were measured bi-monthly during the 2001-2002 growing season. The study revealed that most of nutrients in the soil were at inadequate level. Nutrient concentrations in the leaves of the three cultivars were nearly the same. Results revealed that leaf N ranged between low to satisfactory. P contents were adequate in spring while inadequate in summer. K leaf contents were adequate. Peaks of Mg were found to be the highest during winter. Ca peaks were observed during March-June. Fe and Zn were inadequate while Mn was adequate. The concentrations of Fe, Mn and Zn peaked during June, which could be due the repeated foliar application of these nutrients during this period. The seasonal nutrient changes (N, P, K, Ca, Mg, Fe, Mn, Zn and Cu) of the olive leaves are supposed to be used as a guide for proper fertilization. Nutrients should be added as acidic fertilizer to the soil, which is useful in calcareous and high pH conditions.

Key words: Olive, *Olea europaea* L., leaf nutrients, seasonal variations.

Introduction

During recent years Egypt, calcareous soil under cultivation in was estimated to reach about one million feddans (1 feddan= 0.42 ha). The programs of agricultural development in Egypt aim to increase the cultivated areas with olive trees in these soils. Soils in El-Saf area are highly calcareous. These soils contain about 3% to more than 20% $CaCO_3$ with pH values in the range of 8.0 to 9.0. Their inherited fertility is low. Olive is extensively grown in these calcareous soils. Proper nutrient management is required to grow olive successfully on such soils. Olive trees do much better with changes to their nutritional status through good nutrition. Controlled nutrient supply during different seasons can produce higher yield.

Few data are available concerning seasonal nutrient status. Soil and leaf analysis have been developed over the years to help growers to diagnose tree nutrient status and soil nutrient availability and make adjustments on fertilization programs accordingly. The purpose of the present investigation was to detect seasonal changes in the nutrient status of some olive varieties grown in calcareous soils, to be used as a tool to optimize fertilizers use.

Materials and methods

The study was conducted at an olive orchard established on calcareous soil located in El-Saf, (Giza, Egypt) during two successive years (2001-2002). The orchard contained nineteen years old olive trees (*Olea europae*a L.) of three different varieties namely, Picual, Manzanillo (dual purpose) and Aggizi (table olives), the three most common olive varieties grown in this area. Trees were uniform in growth and cultivated at 6 x 6 m distance (278 tree/ha). The trees were in good physical condition, free from insect damage and diseases and were subjected to the same management treatments. The trees received the following agricultural practices during the period of the study:

Fertilization: (1) 28 m^3 cattle manure ha^{-1}, in January; (2) 278 kg N ha^{-1}, as ammonium nitrate (33.5% N), added in 4 equal doses in March, May, July, and August. (3) 74 kg P_2O_5 ha^{-1}, as single super phosphate (15.5% P_2O_5) in 1 dose in February; (4) 228 kg K_2O ha^{-1}. as potassium sulfate (48% K_2O) in 3 equal splits in March, May and July; (5) concerning micro nutrient fertilizers, the trees were sprayed three times in March, May and June using a compound containing 6.5% Zn-EDTA, 4.5% Mn-EDTA and 2.25% Fe-EDTA in concentrations of 1.5 g L^{-1} in the spray solution.

The trees were drip irrigated from deep well of water containing 838 ppm total soluble solids with pH 8.24 for 8 hours every 2 days from the beginning of June till the end of September. Then the irrigation period extended to 4 days during the rest eight months. Total amount of water used was 5450 m^3/year/ha. Plant protection treatments were applied when required. The average yield/tree was 8.33 kg in non-bearing year (2001), and 35.57 kg, in bearing year (2002).

Sampling

Soil samples: Samples were taken from the root tip zone of the trees in May. Soil samples were air dried then sieved to pass a 2 mm sieve.

Leaf samples: Leaves from the selected trees were collected from the fully mature leaves of spring flush in the first week of every month. The 2nd and the 3rd leaves from the fruit bearing branches of about 20 trees, 5-10 leaves from each, were randomly taken around the tree. During the two years, the number of samples were 36 for each variety. The samples were washed with tap water, 0.01 N HCL and distilled water, respectively, then dried at 70 °C and ground in a stainless steel mill.

Analysis: Soil samples were analyzed for texture with a hydrometer (Kilmer and Alexander, 1949), for pH and electric

conductivity (EC) using water extract (1:2.5) method, (Jackson, 1973), for total calcium carbonate ($CaCO_3$%) by calcimeter method as described by Alison and Moodle (1965) and organic matter (OM%) content according to Walkley and Black (1934) using potassium dichromate (Chapman and Pratt, 1978). Nitrogen calculated from soil organic matter due to its quick changes, depending on environmental factors. Phosphorus was extracted using sodium bicarbonate (Olsen *et al.*, 1954). Potassium (K^+) and Magnesium (Mg^{+2}) were extracted using ammonium acetate. Iron (Fe^{+2}), Manganese (Mn^{+2}), Zinc (Zn^{+2}) and Copper (Cu^{+2}) were extracted using DPTA (Lindsay and Norvell, 1978).

The plant material was digested using an acid mixture consisting of nitric, perchloric and sulfuric acids in the ratio of 8:1:1 (v/v), respectively (Chapman and Pratt, 1978). Nitrogen (N) was determined in the dry plant material using the boric acid modification described by Ma and Zuazaga (1942), and distillation was done using a Buechi 320-N_2-distillation unit. Phosphorus was photometrically determined using the molybdate vanadate method according to Jackson (1973). Potassium was determined using flame photometer Eppendorf. Mg^{2+}, Fe^{+2}, Mn^{+2}, Zn^{+2} and Cu^{+2} were determined using the atomic absorption spectrophotometer PMQ3. The soil data were evaluated using the criteria published by Ankerman and Large (1974) and Lindsay and Norvell (1978), whereas the leaf analysis data were evaluated according to the criteria suggested by Bouat (1964), Hartmann *et al.* (1966) and Recalde and Chaves (1975).

Results and discussion

Soil properties and nutrient status of soils: From Table 1, it can be noticed that soil pH had high value (8.33). Under such high alkaline conditions, availability of some nutrients is expected to be low. Electric conductivity (EC) was high. The soil samples were found to be high in $CaCO_3$; contained greater than 10% $CaCO_3$ which is expected to have an adverese effect on the nutrient availability (Ankerman and Large, 1974). The soil of the orchard was very poor in organic matter content (0.31%).

Table1. Soil characteristics

Characteristic		Nutrient content	
Sand %	93	N (mg/100g)	15.50 VL
Silt %	04	P (mg/100g)	4.55 H
Clay %	03	K (mg/100g)	8.26 VL
Texture	Sandy	Mg (mg/100g)	20.00 L
pH	8.33H	Ca (mg/100g)	270.00 H
E.C dS/m	0.68H	Na (mg/100g)	37.20 H
$CaCO_3$ %	10.8H	Fe (mg/kg)	15.57 M
OM%	0.31VL	Mn (mg/kg)	3.33 VL
		Zn (mg/kg)	0.80 L
		Cu (mg/kg)	0.83 L

VL= very low L= low M= medium H= high

Table 1 also depicts the average values of the major nutrient concentration in the soil samples, total nitrogen was very low and available P-content was 4.55mg/100 g soil. According to (Ankerman and Large, 1974), this P-concentration is considered to be high. However, under the conditions of such soil (high $CaCO_3$ and high pH), the availability of P is expected to be reduced, and plants might suffer from P- deficiency. Extracted potassium levels seem to be very low. Therefore, potassium fertilizers were added to compensate the K deficiency in the soil, especially those soils of high sand content as the soil under investigation. The extractable Ca was high, while the extractable Mg was low. In this context, Mengel and Kirkby (1987) mentioned that Mg^{+2} uptake by plants can be restricted by the high levels of Ca^{+2} in the root medium, which might lead to Mg deficiency in plants, in spite of its high levels in the soil.

Calcareous soils may contain medium levels of total Fe, but in unavailable forms to plants. The extractable Fe^{2+} level of the present soil samples was in the medium range and Mn^{+2} was very low, and it tends to form Mn – organic matter complexes under such high pH conditions, which makes it less available to trees. Available Zn^{+2} can be reduced due to formation of Zn-carbonates. The values of Zn in this study were low. Also, Cu was low in the soil, as it's known in most calcareous soils, which contain inadequate levels of available copper. In addition, the uptake of micronutrients is depressed by increasing P contents in the soil.

Nutrient status of olive leaves: It is well known that olive trees are very efficient in absorption of nutrients from the soil. Nutrient concentration in the leaves followed the same behaviour in the cultivars under study. Nitrogen concentration in the leaves ranged between 1.0-2.6% (on dry matter basis) (Fig. 1), which tends to be low. This may be due to high leaching of ammonium nitrate in such soil with 93% sand. It is recommended to add nitrogen as ammonium sulphate, which is less leached and more efficient in calcareous and high pH conditions. Johnston (2004) reported that when ammonium sulphate is applied one pH unit can be decreased and this pH change is important for P supply and also for micronutrient supply. P-concentrations in the mature spring leaves ranged between 0.10-0.20% (Fig. 1) which are adequate, while in summer, they were less than 0.10%.

Potassium concentrations in the leaves ranged between 1.0-2.2% (Fig.1), which, is in the adequate range. Some researchers mentioned that low concentrations of K in olive leaves are not uncommon in olive groves planted on calcareous soils. Higher magnesium concentration peaks were found during winter (November-January), while calcium concentrations were high during March-June.

Fig. 2 illustrates the micronutrient concentrations in the leaves of olive trees throughout the year. Fe concentrations can be higher than, equal to, or lower than those in normal trees. Thus, this disorder on calcareous is not always attributable to Fe deficiency; a high concentration of calcium in the soil is likely to make iron deficiency more severe, a condition known as lime-induced Fe chlorosis. The severity of the disorder increases at high pH. In general, Fe-concentrations were inadequate and peaked in May-June to reach about 200-300 ppm. Mn^{2+} concentrations followed the same trend in the three cultivars under study. Concentrations of Mn were adequate and peaked in June to reach the range between 70-80 ppm. Olive trees are known to be very sensitive to Zn deficiency. Marschner (1993) found that in soils with very high pH and $CaCO_3$ and very low in organic matter, availability of Zn to plant roots is extremely low. As a result of factors described above most Zn concentrations in this study were low. Zn concentration was less than 25 ppm except in June when it reached to about 60 ppm. The concentrations of Fe, Mn, and Zn (peaked during June), were affected by the repeated foliar application of these nutrients

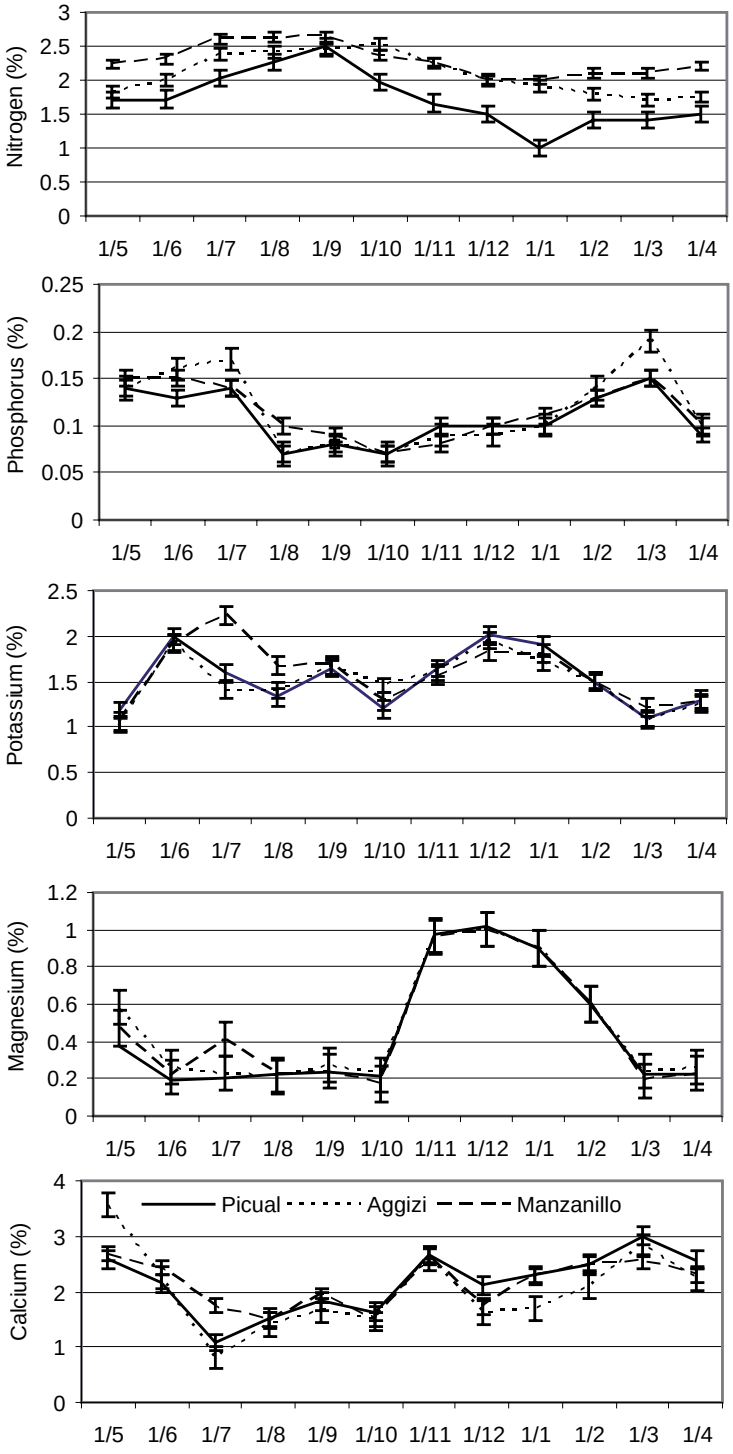

Fig. 1. Monthly concentrations of macronutrients in the leaves of different olive cultivars (Each value is the mean ± SE of three replicates)

to trees after fruit setting during this period.

The peak of Fe, Mn and Zn in June, could be due to foliar application of these nutrients, which improved its concentrations in leaves. Boaretto *et al.* (2002) and Sanchez and Righetti (2002) found that when severe Zn deficiency symptoms appear, early spring foliar sprays could increase the micronutrient concentration in the targeted organs. Also, Swietlik (2002) mentioned that it could stimulate vegetative growth. Similar results were found by Shaaban and El-Fouly (2005).

It is well known in calcareous soil that Cu-availability and

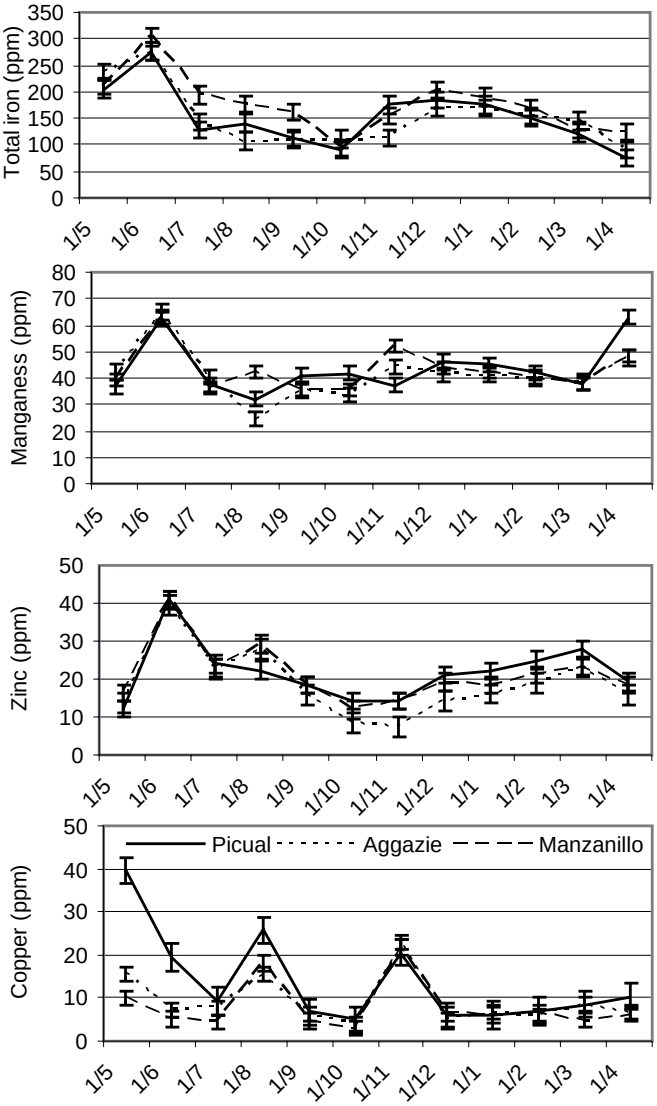

Fig. 2. Monthly concentrations of micronutrients in the leaves of different olive cultivars (Each value is the mean ±SE of three replicates)

consequently its concentration in leaves of grown plant are expected to be low due to high soil pH. In this study Cu-concentration was found to be less than the adequate range in most samples. In some samples Cu-concentration was found to be high. This might be due to use of Cu-containing pesticides.

It could be concluded that nutrient concentration in olive leaves is greatly affected by soil characteristics as well as farm management. Soil characterstics and plant nutrient requirement should be considered when preparing a fertilizer recommendations.

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Evaluation of zinnia cultivars for field grown cut flower production

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Abstract

The objective of the study was to evaluate the effects of cultivar and planting date on zinnia (*Zinnia elegans*) cut flower production. Parameters evaluated were the number of days to harvest, duration of harvest period for each planting date, number of stems per plant, stem length and diameter. Plants from the May planting date produced stems over a longer period of time compared to plants from the June and July plantings with the exception of 'Scarlet Splendor' from the July planting. Within each of the three planting dates, there were no statistically significant differences in the number of stems produced per plant due to the cultivar effect for 10 of the 13 cultivars evaluated. A trend of increasing stem and bloom size from the May planting date to the July planting was observed. The median number of stems produced by the zinnia cultivars in this study from the May, June, and July planting dates were respectively 21.6, 10.8 and 14.5 stems per plant for plants spaced one foot apart in the row. The potential stem yield for a single 100 ft row of the zinnia cultivars included in this trial was 2160, 1080 and 1450 stems for the production life of May, June, and July plantings, or 4690 stems for the three plantings combined. The cut flower zinnias evaluated in this study were very productive during the summer growing season.

Key words: *Zinnia elegans*, zinnia, cut flower, field production

Introduction

Floriculture and ornamental crop production has grown to be one of the largest segments of U.S. agriculture, and in 2003 with a farm value of $14.4 billion, trailed only corn and vegetables in value among crops (Jerardo, 2004). Cut flower production, a segment of the floriculture industry, in the U.S. in 2006 had an estimated value of $385 million. This was about half the value of imported cut flowers in 2006, $750 million (Jerardo, 2006). These production figures are for 'mainstream' florist flowers that are produced in ideal microclimates around the world and then transported to distant markets.

In US, there is growing interest in producing specialty cut flowers that are not considered 'traditional' cut flowers but are still in demand by florists, designers, and consumers (Armitage, 1993). Research efforts have identified floral crops that can be produced in Mississippi that meet the quality criteria of local florists who have indicated a willingness to purchase these crops if supply and quality were available (Sloan and Harkness, 2002; Sloan *et al.*, 2003). Other studies indicated that zinnia has excellent field production potential (Starman *et al.*, 1995). The Association of Specialty Cut Flower Growers conducted on-farm trials to identify superior cut flower cultivars, including zinnia, for field production (Dole, 2005). The 'Benary's Giant' series of zinnia was awarded the Association of Specialty Cut Flower Growers Cut Flower of the Year in 1999 (ASCFG, 1999). The 'Benary's Giant' series has been an industry standard for cut flower zinnia since then. An inclination by consumers to purchase locally produced flowers has been identified. Hudson and Griffin (2004) reported that consumers responding to survey indicated a willingness to pay a premium price for flowers grown in Mississippi compared to those flowers grown outside of Mississippi. The objective of this study was to evaluate the effects of cultivar and planting date on zinnia cut flower production.

Materials and methods

Thirteen zinnia cultivars were seeded into 1204 cells containing Metro Mix 366 media on three dates: April 25, May 27, and June 30, 2003. The seedlings were fertilized with 100 ppm (mg L^{-1}) N using Peter's Peat Lite Special 20-10-20 water soluble fertilizer (20N-4.3P-16.7K; The Scotts Company, Marysville, OH) until the first leaf emerged after which they were fertilized with 250 ppm (mg L^{-1}) N from Peter's 20-10-20. The seedlings were drenched with Banrot (etridiazole + thiophanate methyl) at a rate of 59.15 mL / 3.78 L prior to transplanting to the plant beds. The seedlings were transplanted to raised field beds on a Savannah sandy clay loam soil at the North Mississippi Research & Extension Center in Verona, Miss. (lat. 34.2° N, long. 88.8° W) on three dates; May 19, June 20, and July 27, 2003. Raised beds were formed with a three-point hitch bed shaper. The beds were 0.76 m across the top and were spaced 1.52 m center to center. A single drip tape was placed in the center of the bed and buried 2.54 cm below the bed surface. The beds were fertilized before planting with 8-8-8 (8N-3.5P-6.6K IMC Rainbow Agribusiness, Florence, AL) at a rate of 0.45 kg 9.29 m^{-2} of bed. Beds were fertigated weekly at the rate of 0.25 kg of Peter's 20-20-20 (20N-8.8P-16.5K) per 92.90 m^2 during the growing season. Irrigation was supplied as needed through the drip tape to provide 1892.70 L/92.90 m^2 of row per irrigation. The experimental design was a split plot with the planting date being the whole plot factor, and the cultivar being the sub-plot factor with four replications. The experimental unit consisted of two plants of each cultivar that were planted in

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pairs, one plant on each of two parallel rows that were spaced 30.5 cm apart; the plants within each row were spaced 30.5 cm apart. Zinnia stems were harvested as soon as the blooms were completely opened. Stems were not harvested unless they were at least 45.7 cm long. The data collected during the trial were analyzed by SAS PROC MIXED (SAS Institute Inc, Cary, NC). Means separation were conducted with Fisher's Protected LSD at P=0.05. The data recorded in this experiment were date of harvest, stem length, stem diameter, bloom diameter, and number of stems per plant.

Results and discussion

There was an interaction between planting date and cultivar in the analysis of days to first harvest. Within the May planting date, 'Envy', 'Sun Red', 'Benary's Giant Crimson', 'Benary's Giant Carmine Rose', 'Yoga', and 'Cactus Jewels Mix' needed more time to produce the first mature stems, 44.7–42.2 days, compared to 'Benary's Giant Scarlet', 'Scarlet Splendor', and 'State Fair Mix', 38.0 days (Table 1). 'Scarlet Splendor' took longer to grow to maturity, 45.1 days, than the other cultivars in the June planting except 'Envy' and 'Benary's Giant White'. 'Benary's Giant Carmine Rose' required longer, 51.0 days, than the other cultivars in the July planting to grow to maturity while 'Scarlet Splendor' and 'Sun Red' required the least amount of time, 27.0 days. There were no statistical differences between the May and July planting dates in the time required to produce mature stems for 'Benary's Giant Crimson', 'Benary's Giant Deep Red', 'Benary's Giant Mix', 'Benary's Giant White', 'Cactus Jewels Mix', 'Envy', 'Ruffles Scarlet', 'State Fair Mix', and 'Yoga'. These findings disagree with those of Young *et al.* (2003) where zinnias planted in June took longer to grow to harvest compared to those planted in May. Within the 'Benary's Giant' series, there were biologically inconsequential, but statistically significant, differences in the time required to grow to maturity due to planting date for 5 of the 6 cultivars, but 'Benary's Giant Carmine Rose' was the notable exception.

Multiple stems are generally harvested from zinnia plants over a period of time. Cultivars that keep producing stems over long periods would be desirable for cut flower growers. There was an interaction between planting date and cultivar for the duration of the harvest period. 'Benary's Giant Deep Red' produced stems over a longer period of time than the other cultivars in this trial for plants from the May and June plantings, 85.0 and 55.0 days, respectively (Table 2). The June planting date of 'State Fair Mix' had a shorter harvest period, 41.7 days, than 'Benary's Giant Deep Red', but longer than the other cultivars. 'Scarlet Splendor' and 'Sun Red' produced stems over a longer period, 56.0 and 52.0 days, compared to the other cultivars in the July planting. The duration of harvest period was shorter for plants from the June and July plantings compared to the May planting for all cultivars except 'Scarlet Splendor' in July. This suggests that the plants from the May planting were stronger compared to those from the other planting dates.

Plants of 'Benary's Giant Deep Red' from the May planting date produced more stems per plant (34.6) during the life of the plant than the other cultivars in the May planting. 'Benary's Giant Deep Red' followed only 'Sun Red' in production in the June planting, and was in the top statistical grouping for cultivars in the July planting for number of stems per plant (Table 3). Comparisons within each cultivar across planting dates showed that plants from the May planting produced more stems for 10 of the 13 cultivars compared to the June planting. However, comparisons between the May and July planting dates with regard to the number of stems produced per plant show that 9 of the 13 cultivars showed no significant difference due to planting date. Within each of the

Table 1. Effects of cultivar and planting date on the number of days required to grow stems to first harvest maturity for zinnia cultivars[z]

Cultivar	Days required to first harvest		
	Planting date		
	May 19	June 20	July 27
Benary's Giant Carmine Rose	43.7 b A[x]	39.0 c B	51.0 a A
Benary's Giant Crimson	44.0 a A	38.5 b B	44.0 a B
Benary's Giant Deep Red	41.0 ab AB	39.0 b B	44.0 a B
Benary's Giant Mix	41.0 a AB	39.0 a B	40.0 a B
Benary's Giant Scarlet	38.0 b B	39.0 b B	43.9 a B
Benary's Giant White	40.7 a AB	41.2 a AB	40.0 a B
Cactus Jewels Mix	42.2 ab AB	39.0 b B	44.0 a B
Envy	44.7 a A	42.7 a AB	44.0 a B
Ruffles Scarlet	40.7 ab AB	39.0 b B	44.0 a B
Scarlet Splendor	38.0 b B	45.1 a A	27.0 c C
State Fair Mix	38.0 b B	39.7 ab B	43.0 a B
Sun Red	44.7 a A	40.0 a B	27.0 c C
Yoga	42.5 a A	39.7 a B	40.5 a B

LSD (cultivar) = 4.4299[w], LSD (date) = 4.7578[y]

[z]There was an interaction between cultivar x planting date (P<0.0001) in the analysis of the number of days required to first harvest of cultivars. Two comparisons; one within the cultivar and across planting dates, and the other within planting date and across cultivars; were needed for the analysis.

[y]LSD within cultivar and across planting dates

[x]Means compared by Fisher's Protected LSD at P=0.05. Means with the same-upper case letter in a column do not differ at the 5% significance level. Means with the same lower-case letter in a row do not differ at the 5% significance level.

[w]LSD within planting date and across cultivars

Table 2. Effects of cultivar and planting date on the duration of the harvest period of zinnia cultivars[z]

Cultivar	Duration of harvest period		
	Planting date		
	May 19	June 20	July 27
Benary's Giant Carmine Rose	54.2 a C[x]	25.2 b C	32.0 b CD
Benary's Giant Crimson	54.0 a C	23.5 c CD	39.0 b BC
Benary's Giant Deep Red	85.0 a A	55.0 b A	28.0 c D
Benary's Giant Mix	57.0 a BC	15.2 c DE	43.0 b B
Benary's Giant Scarlet	63.0 a B	25.2 c C	38.9 b BC
Benary's Giant White	55.5 a BC	19.0 c CD	41.2 b B
Cactus Jewels Mix	55.7 a BC	21.9 c CD	39.0 b BC
Envy	53.2 a C	17.5 c C-E	38.5 b BC
Ruffles Scarlet	57.2 a BC	21.9 c CD	35.5 b B-D
Scarlet Splendor	54.7 a BC	9.2 b E	56.0 a A
State Fair Mix	63.0 a B	41.7 b B	40.0 b BC
Sun Red	56.2 a BC	24.2 b C	52.0 a A
Yoga	57.7 a BC	22.5 c CD	42.5 b B

LSD (cultivar) = 8.5975[w], LSD (date) = 9.2875[y]

[z]There was an interaction between cultivar x planting date (P<0.0001) in the analysis of the number of days required to first harvest of cultivars. Two comparisons; one within the cultivar and across planting dates, and the other within planting date and across cultivars; were needed for the analysis.

[y]LSD within cultivar and across planting dates

[x]Means compared by Fisher's Protected LSD at P=0.05. Means with the same-upper case letter in a column do not differ at the 5% significance level. Means with the same lower-case letter in a row do not differ at the 5% significance level.

[w]LSD within planting date and across cultivars

Table 3. Effects of cultivar and planting date on the number of stems produced per plant by zinnia cultivars[z]

Cultivar	Stems produced per plant		
	Planting date		
	May 19	June 20	July 27
Benary's Giant Carmine Rose	20.1 a B-E[x]	8.7 b C	13.4 ab C-E
Benary's Giant Crimson	21.7 a B-D	11.9 b C	21.5 a AB
Benary's Giant Deep Red	34.6 a A	22.2 b B	25.7 b A
Benary's Giant Mix	17.0 a C-E	8.7 b C	14.1 a C-E
Benary's Giant Scarlet	21.6 a B-E	13.6 b C	15.3 ab B-E
Benary's Giant White	16.2 a DE	9.9 a C	10.8 a E
Cactus Jewels Mix	26.1 a B	8.8 b C	14.0 b C-E
Envy	18.4 a C-E	9.1 b C	14.5 ab B-E
Sun Red	22.4 b B-D	29.7 a A	26.0 ab A
Ruffles Scarlet	21.9 a B-D	10.8 b C	11.9 b DE
Scarlet Splendor	14.6 a E	15.5 a BC	20.1 a A-C
State Fair Mix	19.4 a B-E	8.9 b C	12.0 b DE
Yoga	23.2 a BC	12.1 b C	17.9 ab B-D
LSD (cultivar) = 7.0753[w] , LSD (date) = 7.300[y]			

[z]There was an interaction between cultivar x planting date (P=0.05) in the analysis of the number of days required to first harvest of cultivars. Two comparisons; one within the cultivar and across planting dates, and the other within planting date and across cultivars; were needed for the analysis.

[x]LSD within cultivar and across planting dates

[y]Means compared by Fisher's Protected LSD at P=0.05. Means with the same-upper case letter in a column did not differ at the 5% significance level. Means with the same lower-case letter in a row did not differ at the 5% significance level.

[w]LSD within planting date and across cultivars

Table 4. Effects of cultivar and planting date on zinnia stem length

Cultivar	Stem length (cm)
Benary's Giant Carmine Rose	49.05 ab[y]
Benary's Giant Crimson	48.68 abcd
Benary's Giant Deep Red	48.18 d
Benary's Giant Mix	48.89 abc
Benary's Giant Scarlet	48.39 cd
Benary's Giant White	49.16 ab
Cactus Jewels Mix	49.05 abc
Envy	48.5 bcd
Sun Red	48.96 abc
Ruffles Scarlet	47.56 e
Scarlet Splendor	48.51 bcd
State Fair Mix	49.23 a
Yoga	49.12 ab
LSD	0.7025
Planting Date	**Stem length (cm)**
May 19	48.95 a
June 20	48.97 a
July 27	48.22 b
LSD	0.4313

[y]Means compared by Fisher's Protected LSD at P = 0.05. Means within a column with the same letter did not differ at the 5% significance level.

three planting dates, there were no statistical differences in the number of stems produced per plant for 10 of the 13 cultivars.

For stem length there was no planting date x cultivar interaction (Table 4). The stem length ranged from 49.2-47.6 cm. 'Ruffles Scarlet' produced the shortest stem 47.6 cm. The decision of not to harvest and record stems that were less than 45.7 cm in length reduced the possibility for differences to be observed in stem length. Differences in productivity were reflected in the number of stems harvested per plant. The May and June planting dates produced statistically longer stems compared to the July planting date.

Table 5. Effects of cultivar and planting date on zinnia stem diameter

Cultivar	Stem diameter (cm)
Benary's Giant Carmine Rose	0.83 cd[y]
Benary's Giant Crimson	0.82 de
Benary's Giant Deep Red	0.93 ab
Benary's Giant Mix	0.81 de
Benary's Giant Scarlet	0.88 bcd
Benary's Giant White	0.79 e
Cactus Jewels Mix	0.81 e
Envy	0.67 f
Sun Red	0.79 e
Ruffles Scarlet	0.67 f
Scarlet Splendor	0.90 bc
State Fair Mix	0.99 a
Yoga	0.83 de
LSD	0.0719
Planting Date	**Stem diameter (cm)**
May 19	0.79 b
June 20	0.85 a
July 27	0.83 a
LSD	0.0497

[y]Means compared by Fisher's Protected LSD at P = 0.05. Means within a column with the same lower-case letter do not differ at the 5% significance level.

Table 6. Effect of cultivar and planting date on bloom diameter of zinnia cultivars[z]

Cultivar	Bloom diameter (cm)[y]		
	Plant date		
	May 19	June 20	July 27
Benary's Giant Carmine Rose	8.01 ab BC[w]	7.79 b C-E	8.50 a C-E
Benary's Giant Crimson	7.77 b B-D	8.15 ab A-C	8.64 a C-E
Benary's Giant Deep Red	7.27 b D	7.28 b EF	8.12 a E
Benary's Giant Mix	7.55 b CD	7.71 b C-E	8.94 a CD
Benary's Giant Scarlet	7.83 a B-D	7.10 b FG	8.40 a DE
Benary's Giant White	7.52 b CD	7.24 b EF	8.32 a E
Cactus Jewels Mix	8.07 b BC	8.49 b AB	9.59 a B
Envy	6.74 a E	6.56 a G	6.68 a F
Sun Red	7.27 b DE	7.48 b D-F	8.30 a E
Ruffles Scarlet	5.54 b E	5.56 b H	6.34 a F
Scarlet Splendor	8.22 b B	8.00 b B-D	9.02 a BC
State Fair Mix	9.01 b A	8.66 b A	10.09 a A
Yoga	7.51 b CD	6.91 c FG	8.32 a E
LSD (cultivar) = 0.5837[v], LSD (date) = 0.5915[x]			

[w]There was an interaction between cultivar x planting date (P=0.0297) in the analysis of bloom diameter. Two comparisons; one within cultivar across planting dates and the other within planting date across cultivars; were needed for the analysis.

[v]LSD within cultivar and across planting dates

[w]Means compared by Fisher's Protected LSD at P=0.05. Means with the same upper-case letter in a column do not differ at the 5% significance level. Means with the same lower-case letter in a row do not differ at the 5% significance level.

[x]LSD within planting date across cultivars

'State Fair Mix' produced larger diameter stems (0.99 cm) than the other cultivars in this trial except 'Benary's Giant Deep Red' while 'Envy' and 'Ruffles Scarlet' produced the smallest diameter stems (0.67 cm) (Table 5). The June and July planting dates produced larger diameter zinnia stems on an average than the May planting date.

The bloom diameter of 'State Fair Mix' was larger within each of the three planting dates compared to the other cultivars except for 'Cactus Jewels Mix' and 'Benary's Giant Crimson' from the June planting date (Table 6). 'Envy' and 'Ruffles Scarlet' produced smaller blooms than the other cultivars except 'Sun Red' in May. The trend in bloom diameter across planting dates

was the same for stem diameter. Nine of the 13 cultivars produced larger diameter blooms in the June and July plantings compared to the May planting.

The May planting of zinnia cultivars in this trial produced plants that yielded stems for a longer period of time. Many of the zinnia cultivars within the June planting date had a shorter harvest period, probably due to stressful, hot weather conditions at transplanting. While, 'Benary's Giant Deep Red' produced flower stems over the longest period of time in the May and June plantings, it was at the bottom of the list for duration of stem production for the July planting. Within each planting date, there were no statistical differences in harvest duration for 8 of the 13 cultivars, which could indicate that there was little difference between the cultivars for this response. While there was little difference in stem length due to planting date or cultivar, there was a trend for increased stem and bloom diameter from May to the July planting date. 'Benary's Giant Deep Red' and 'Sun Red' were consistently the top producers of flower stems across the 3 planting dates. For each planting date, there was relatively little difference between the other cultivars regarding the number of stems produced per plant. However, the median number of stems produced by all cultivars from the May, June, and July planting dates were respectively 21.6, 10.8, and 14.5 stems per plant that were planted 30.5 cm apart in the row. This would result in a potential stem yield for a single 30.4m row of zinnia cultivars included in this trial of 2160, 1080 and 1450 stems for the production life of May, June, and July plantings, or 4690 stems for the three plantings combined.

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Growth and yield of grape as influenced by soil-site parameters in Nasik district of Maharashtra

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Abstract

Six grape growing typical pedons in Nasik district, Maharashtra were characterised and soil-site parameters were correlated with yield and yield attributes of the crop. These soils were very shallow (Darana), moderately deep (Mahiravani, Kothure), shallow (Shivdi), deep (Talegaon) and very deep (Andersool) and characterised by well drained (Darana, Mahiravani, Shivdi) and moderately well drained (Talegaon, Kothure, Andersool). The height, stem girth, spread volume, bunch per plant, berries per bunch were very much related with soil depth, drainage, pH, available water content and DTPA extractable micronutrient cations.

Key words: Grape, soil characterstics, growth, yield, drainage, depth, available water content

Introduction

The grape (*Vitis vinifera* L.) is one of the important commercial export oriented horticultural crop mostly grown in the state of Maharashtra, Andhra Pradesh, Karnataka, Tamilnadu, Haryana and Punjab in India under varied pedo-edaphic environments of sub-tropical to hot tropical regions. The hot tropical region is major viticulture region accounting for 70 per cent of the area under grape in the country. It occupies 44.3 thousand hectares of area associated with production of 1137.8 thousand MT and productivity of 25.7 MT/ha. About 85 per cent of the total production, irrespective of the variety, is consumed fresh. In Maharashtra, grape cultivation is mostly confined to Nasik, Ahemadnagar, Pune, Satara, Sangli and Osmanabad districts which contribute major share in grape production of the country., although a significant variation in productivity was observed among districts. Nasik district emerged as a grape district in Maharashtra with large area and high productivity. However, there is a wide variation in productivity of grape, owing to varied landscape, soil characteristics (Jagdish Prasad *et al.,* 1995) and agro-managements.

Through present study, an attempt has been made to characterize the typical grape growing soils of Nasik district to understand their potential and constraints in grape production. The relationship between plant characterstics and soil parameters like soil depth, drainage, pH, available water content and DTPA extractable micronutrient cations have been worked out.

Materials and methods

Nasik district falls under sub region AER 6.2 associated with dry sub-humid to semi-arid ecosystem in the grape growing areas. The mean minimum and maximum temperatures are 25.9 and 30.9°C, respectively and represent *Ustic* moisture and *Isohyperthermic* temperature regimes. To ascertain the variability in grape yield *vis-à-vis* the soil-site characteristics, six typical soils in the villages of *viz.* Darana (P_1), Talegaon (P_2), Mahiravani (P_3), Shivdi (P_4), Kothure (P_5), and Andersool (P_6) under grape orchards of 5-7 yrs old were investigated (Soil Survey Division Staff, 1995) from Satana, Dindori, Nasik, Niphad and Yeola tehsils of Nasik district.

The growth and growth parameters *viz.,* height, stem girth (15 cm above ground), canopy (spread volume), average number of bunches/plant and berries/bunch were recorded (mean value of 5 plants). The horizon-wise soil samples were analysed for soil properties (coarse fragments, sand, silt, clay, bulk density, water retention, pH, EC, organic carbon, $CaCO_3$, exchangeable Ca, Mg, Na, K, micronutrient cations and CEC) following the standard procedures and soils were taxonomically classified (Soil Survey Staff, 1998).

Results and discussion

The climate, landscape and soil characteristics and their range reported by different research workers against resources available (yield also) in present study (Table 1) were considered

Table 1. Growth and yield parameters in selected vineyards

Pedon/ Location	P_1 Darana village (Satana)	P_2 Talegaon village (Dindori)	P_3 Shivdi village (Niphad)	P_4 Mahiravani village (Nasik)	P_5 Kothure village (Niphad)	P_6 Andersool village (Yeola)
Height (m)	1.52	1.64	1.49	1.74	1.40	1.40
Stem girth (cm)	18.20	18.00	20.00	14.40	20.30	18.50
Spread (m^2)	2.26	3.33	3.14	2.65	3.93	4.00
Average bunches $plant^{-1}$	35.00	52.00	46.00	45.00	43.00	40.00
Average berries $bunch^{-1}$	68.00	115.00	68.00	86.00	122.00	85.00
Yield $plant^{-1}$ (kg)	7.14	17.90	9.30	11.60	15.70	10.20
Yield (t ha^{-1})	12.50	36.80	14.89	23.80	21.50	23.60

Table. 2. Physical properties of soils

Horizon	Depth (cm)	Coarse fragments (%)	Particle-size distribution (%)			Bulk density (mg m^{-3})	Water retention		AWC (%)	AWC (mm)
		v/v	Sand	Silt	Clay		33 kPa	1500 kPa		
Pedon 1:Clayey, smectitic (calcareous) Typic Ustorthents										
Ap	0-10	9.44	25.9	21.6	52.5	1.62	31.56	29.4	12.16	197.0
Pedon 2: Fine, smectitic (calcareous) Typic Haplustepts										
Ap	0-20	6.3	20.0	25.5	54.5	1.53	38.08	15.54	22.50	377.2
Bw1	20-49	4.7	24.4	23.6	52.0	1.65	34.30	17.75	16.50	272.2
Bw2	49-91	12.8	21.3	25.2	53.5	1.70	34.70	16.01	18.69	217.7
BC	91-150+	10.7	48.4	20.1	31.5	1.72	22.46	9.81	12.65	217.5
Pedon 3: Loamy over sandy, mixed (calcareous) Typic Ustorthents										
Ap	0-15	4.3	41.4	18.7	39.9	1.23	24.34	11.28	13.06	160.6
AC	15-30	9.8	45.0	26.5	28.5	1.26	19.56	10.64	8.92	112.3
Pedon 4: Clayey, smectitic (calcareous) Typic Haplustepts										
Ap	0-11	8.3	22.6	24.9	52.5	1.36	31.98	21.35	10.63	144.5
Bw	11-29	8.4	19.3	26.7	54.0	1.39	39.95	28.94	13.10	182.0
Pedon 5: Very- fine smectitic (calcareous) Leptic Haplusterts										
Ap	0-16	9.8	12.2	21.8	66.0	1.59	46.19	30.94	15.25	242.4
Bw	16-36	11.7	10.2	25.3	64.5	1.69	39.6	29.36	10.21	172.5
Bss	36-69	3.2	9.8	26.2	64.0	1.72	40.6	29.93	10.67	183.5
Pedon 6: Very- fine smectitic (calcareous) Typic Haplusterts										
Ap	0-14	2.7	19.6	22.9	58.5	1.57	40.47	25.09	15.38	241.4
Bw	14-43	3.2	10.2	28.3	61.5	1.64	38.77	25.71	13.06	241.1
Bss1	43-83	1.2	10.4	28.6	61.0	1.69	45.98	22.47	22.51	397.3
Bss2	83-125	1.4	8.4	27.1	64.5	1.73	51.23	32.60	18.83	325.7
Bss3	125-155	-	7.7	25.3	67.0	1.73	50.22	21.22	29.00	501.7

for grouping the soils/sites into different suitability classes for growing grape in these sites.

These soil pedons had their development over basalt or basaltic alluvium (P_3) and occur at an elevation of 550 to 700 m above MSL but within a similar climatic zone. These soil pedons were very shallow (P_1), shallow (P_4), moderately deep (P_3 & P_5), deep (P_2) and very deep (P_6) and were endowed with well drained (P_1, P_3 and P_4) and moderately well drained (P_2, P_5 & P_6) environment. These soil pedons exhibited dark grayish brown (10YR) matrix colour barring two pedons (P_1 & P_4) that had brown (7.5YR) matrix colour.

Soil properties and grape yield: Pedon 1 soil being very shallow, strongly alkaline (pH 8.7) associated with ESP 5.0 had vine height of 1.52 m, stem girth 18.2 cm, number of bunches 35 per plant and number of berries 68 per bunch. It seems that soil constraints particularly of depth and ESP are managed by manure/agro management which has been reflected in high organic carbon content of the soil (Table 3) also. The height, stem girth, spread volume, bunches per plant, berries per bunch were 1.64 m, 18cm, 3.33 m^3, 52 and 115, respectively in vineyard of pedon 2 which clearly demonstrates the effect of soil solum, favourable DTPA -extractable micronutrients, pH than the soil of pedon 1. Shallow solum, sandy substratum, low AWC are the factors, which caused low yield in pedon 3, but girth was more than the pedon 1 and 2. Although pedon 4 had lower values with respect to stem girth and spread volume per plant, yield was better than pedon 1 and 3 owing to well drained soil, neutral to slightly alkaline pH favouring availability of nutrients and more particularly of DTPA-extractable micronutrients.

The very-fine (more than 60 % clay) Vertisols (pedons 5 & 6) associated with moderately well drained drainage and sodicity impairing the hydraulic conductivity (Kadu *et al.,* 2003) and $CaCO_3$ (pedon 6) seems to be the factors (Table 2 and 3) responsible for lower yield of grape expressed through other growth factors.

The correlation study indicated that the plant height had significant negative relationship with pH and ESP. Negative correlations were also observed between stem girth and AWC, however, spread volume and berries/bunch had significant negative correlation with DTPA- extractable Cu but positive with DTPA-Mn. $CaCO_3$ content adversely affected bunch per plant owing to its adverse effect on nutrient availability (Kadao *et al.,* 2002). The multiple regression analysis related with plant parameters had the following relationship with different soil parameters.

Plant height (m) = 60.54 + (-9.523 x depth) + (-0.546 x pH) + (-0.31 x EC) + (0.209 x $CaCO_3$ + (-120 x C4) + (-0.009 x Mn) + (0.017 x CEC) + (0.306 x ESP) + (-0.014 x clay) R^2 = 86

Stem girth (cm) = -42.181 + (-0.013 x depth) + (8.030 x pH) + (13.642 x EC) + (-0.052 x Mn) + (-0.192 x Cu) + (-1.421 x ESP) + (0.124 x clay) R^2= 0.69

Spread (m^2) = 12.097 + (0.004 x depth) + (-1.055 x pH) + (3.763 x EC) + (-0.018 x $CaCO_3$) + (-0.036 x Cu) + (0.066 x Mn) + (0.054 x CEC) + (0.017 x ESP) + (-0.075 x clay) R^2 = 0.68

Bunches per plant = -139.902 + (0.165 x depth) + (-11.36 x pH) + (56.24 x EC) + (1.629 x $CaCO_3$) + (-0.68 x Cu) + (-0.913 x Mn) + (0.892 x EC) + (-2.325 x ESP) + (-9.556 x clay) R^2 = 0.80

Table 3. Chemical properties of soils

Horizon	Depth (cm)	pH (1:2.5)	EC (1:2.5) dSm^{-1}	Organic carbon g kg^{-1}	$CaCO_3$ g kg^{-1}	DTPA-extractable (mg kg^{-1})				Exchangeable cations cmol (p^+) kg^{-1}				CEC cmol (p^+) kg^{-1}	Base saturation (%)	ESP
						Cu	Fe	Zn	Mn	Ca^{++}	Mg^{++}	Na^+	K^+			
Pedon 1: Clayey, smectitic (calcareous) Typic Ustorthents																
Ap	0-10	8.7	0.20	9.0	74.9	7.9	5.20	1.3	5.94	36.5	6.00	2.87	0.72	49	94.0	5.8
Pedon 2: Fine, smectitic (calcareous) Typic Haplustepts																
Ap	0-20	8.2	0.26	6.6	101.9	11.64	5.22	1.70	11.84	42.5	5.16	0.54	0.82	51	96.1	1.0
Bw 1	20-49	8.3	0.25	5.5	177.1	2.50	2.52	0.80	10.80	37.0	4.50	0.48	0.35	45	94.5	1.0
Bw 2	49-91	8.4	0.18	2.4	217.1	1.94	3.82	0.18	7.36	25.5	2.66	0.46	0.28	32	90.3	1.4
BC	91-150	8.5	0.15	2.3	227.1	1.16	4.50	0.16	4.33	19.2	2.16	0.76	0.22	25	89.3	3.0
Pedon 3 : Loamy over sandy, mixed (calcareous) Typic Ustorthents																
Ap	0-15	8.3	0.17	7.3	174.3	22.0	5.5	1.58	12.84	21.1	4.33	0.55	1.33	32	85.3	1.7
AC	15-30	8.4	0.13	5.6	169.4	2.84	6.18	0.16	18.4	15.3	3.33	0.65	0.66	23	86.0	2.8
Pedon 4: Clayey, smectitic (calcareous) Typic Haplustepts																
Ap	0-11	7.4	0.11	3.5	28.0	19.04	7.82	3.06	5.78	22.2	6.83	0.36	0.92	33	87.9	1.0
Bw	11-29	7.2	0.08	1.2	41.0	5.92	9.92	0.68	3.44	14.8	8.0	0.39	0.49	29	74.7	1.3
Pedon 5: Very- fine smectitic (calcareous) Leptic Haplusterts																
Ap	0-16	8.4	0.17	9.0	41.0	2.12	6.40	0.40	32.1	44.1	8.16	0.90	0.88	59	91.5	1.5
Bw	16-36	8.5	0.20	5.0	40.7	1.52	8.74	0.06	16.3	43.9	7.83	1.36	0.66	54	98.1	2.5
Bss	36-69	8.0	0.23	3.1	73.5	1.50	8.16	0.16	15.7	38.5	6.83	1.34	0.65	52	91.0	2.5
Pedon 6: Very- fine smectitic (calcareous) Typic Haplusterts																
Ap	0-14	8.2	0.38	7.6	162.2	11.52	3.10	1.66	24.2	24.6	8.0	1.08	2.91	38	94.3	2.8
Bw	14-43	8.4	0.37	6.5	215.3	2.46	3.94	0.30	16.9	18.4	13.5	2.31	0.62	38	91.6	6.0
Bss1	43-83	8.6	0.40	2.5	217.0	2.92	3.92	0.20	25.5	16.3	17.83	4.59	0.46	43	91.1	10.7
Bss2	83-125	8.8	0.53	3.6	216.3	2.10	4.92	0.16	8.66	14.9	20.0	3.28	0.51	42	92.1	7.8
Bss3	125-150+	8.9	1.22	1.1	227.0	1.74	4.62	0.06	7.16	13.7	17.83	3.07	0.48	40	87.7	7.6

Berries per bunch = 446.915 + (0.165 x depth) + (-46.729 x pH) + (23.284 x EC) + (0.216 x $CaCO_3$) + (-1.323 x Cu) + (1.577 x Mn) + (2.864 x CEC) + (-2.232 x ESP) + (-1.962 x clay) $R^2 = 0.74$

Yield (kg/plant) = 136.804 + (0.057 x depth) + (-15.941 x pH) + (6.672 x EC) + (-0.134 x $CaCO_3$) + (-0.258 x Cu) + (0.567 x Mn) +(0.945 x CEC) + (-0.140 x ESP) + (-0.707 x clay) $R^2 = 0.69$

Multivariate regression analysis of the different plant parameters with soil characteristics indicated regression coefficient (R^2) of plant height 0.86, stem girth 0.69, spread 0.68, bunches per plant 0.80, berries per bunch 0.74 and yield 0.69. This shows that the soil parameters such as depth, pH, EC, Mn, Cu, ESP and clay combinedly express the per cent variation in plant parameters such as height, stem girth, spread, bunches per plant, berries per bunch and yield (kg $plant^{-1}$) by 86, 69, 68, 80, 74 and 69 per plant, respectively.

The suitabilities arrived are permanently not suitable for P_1 due to limitations of soil characteristics *viz*. pH 8.7 that limit the nutrient availability and depth (<10 cm) that limit the availability of foothold. However the yield reported at this site was 12.5 t/ha, which might be due to intensive agro-managements rather than landscape and soil characteristics. Pedons 3 and 4 have moderate limitation of pH, calcium carbonate and texture therefore they could be rated as moderately suitable. Pedons 2 and 5 have slight limitation of pH, $CaCO_3$, hence they are ranked as highly suitable. Pedon 6 is presently not suitable due to limitations in nutrient availability.

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In vitro propagation schedule of *Picrorhiza kurroa*: An endangered medicinal plant of Central Himalaya

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Abstract

Picrorhiza kurroa Royle ex Benth (Kutki) has traditionally been used to treat disorders of the liver and upper respiratory tract, fever, and to treat dyspepsia, chronic diarrhoea and scorpion sting in Ayurveda medicine owing to the presence of active principles in root and rhizomes. The plant is self-regenerating but unregulated over-harvesting has caused it to be threatened to near extinction. The current research describes a protocol of micro propagation of this important medicinal plant from establishment to hardening in field conditions. Multiple shoots were induced in apical and axillary meristems derived from mature explants on Murashige and Skoogs (1962) medium supplemented with 0.25 mg L^{-1} 6-benzylaminopurine (BA), 0.25 mg L^{-1} kinetin (KN), 0.5 mg L^{-1} ascorbic acid and 3% (w/v) sucrose. Optimal rooting (86.6%) and growth of microshoots were observed on a medium containing 0.25 mg L^{-1} indole-3-butyric acid (IBA) with 2 % (w/v) sucrose. Micropropagated plantlets were acclimatized and successfully grown in soil.

Key words: *Picrorhiza kurroa,* axillary bud, *In vitro* multiplication, micropropagation

Introduction

Picrorhiza kurroa Royle ex Benth (Scrophulariaceae) commonly known as kutki is an important medicinal plant which occurs at higher altitude, between 3000-4500m, in Himalayas. It is a perennial herb with elongated stout and creeping stolon (Semwal *et al.,* 1983). It secretes a large quantity of glucosidal bitter principle named kutkosides and picrosides which are the constituents of kutkin (Rastogi *et al.*, 1949). The drug Picrorhiza is obtained from dried stolon and roots. Its herbal preparation is antiperiodic, stomachic, laxative in small doses and cathartic in large doses and useful in dropsy (Kirtikar and Basu, 1933). However there are many problems that restrict its multiplication on a large scale.

Propagation through seed is unreliable because of disease and pest problems, short viability and heavy rains during the seeding season in the natural habitat. Pharmaceutical companies largely depend upon materials procured from naturally occurring stands raising concern about possible extinction and providing justification for development of *in vitro* techniques for mass propagation of *P. kurroa*. Conservation of genetic stability in germplasm collections and micropropagation of elite plants is of the utmost importance and propagation of plants through apical or axillary meristem culture allows recovery of genetically stable and true to type progeny (Hu and Wang, 1983). The present study describes an optimized protocol upto hardening and acclimatization in field conditions in its natural habitat for mass propagation of homogenous and elite material of *P. kurroa* for increased production and improving the socio economic status of hill farmer community.

Materials and methods

Plant material and explant source: Actively growing young plants of *P. kurroa* were collected from greenhouse as well as field grown plants from Herbal Research and Development Institue, Mandal, Gopeshwar in the month of July, 2003. These plants were established in earthen pots at controlled environment containment facility, College of Basic Sciences and Humanities, GBPUA&T, Pantnagar. After establishment, explants (axillary and apical shoot tips 4-5cm in length) were collected at weekly intervals from two elite survived plants. These explants (4-5) were washed with 2% (v/v) detergent 'Tween 20' and rinsed several times with running tap water. The explants were surface sterilized in 0.1 % (w/v) aqueous mercuric chloride solution for 15 min followed by four washings with sterile distilled water. The apical and axillary meristems were inoculated on media (25-30 mL) containing different sets of phytohormones with single explant in each culture vessel (Jam Bottle of 500 mL capacity) for *in vitro* establishment.

Culture medium and growth conditions: The meristems (apical and axillary) were placed on semi-solid basal MS (Murashige and Skoog, 1962) medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BA: 0.0, 0.25, 0.5 and 1.0 mg L^{-1}), kinetin (KN: 0.0, 0.25, 0.5 and 1.0 mg L^{-1}), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0.0, 0.10, 0.25 and 0.5 mg L^{-1}) for shoot proliferation and multiplication. The pH of the media was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl before autoclaving at 121^0C and 1.06 kg cm^{-2} pressure for 20 min for sterilization. The cultures were maintained at 25 ± 2^0C under 14h photoperiod (55 mmol $m^{-2}s^{-1}$) from cool, white fluorescent tube lights. The cultures were maintained by regular subcultures at 4-week intervals on fresh medium with the same compositions. To avoid blackening, the medium was supplemented with 0.5 mg L^{-1} ascorbic acid.

Induction of rooting and acclimatization: For root induction, excised microshoots (1-2 cm length) were transferred to MS basal medium supplemented with different concentrations of IAA or IBA (0.0, 0.1, 0.25 and 0.5 mg L^{-1}) and 2% (w/v) sucrose. One

excised shoot was placed in each culture vessel having 25-30 mL of the culture media. All the cultures were incubated at 25±2^0 C under 16h photoperiod with cool, white fluorescent light. Rooted micro-propagules were thoroughly washed to remove the adhering gel and planted in 2.5 cm plastic pots (250mL) containing a sterile mixture of sand, soil and cow-dung manure in the ratio of 1: 1: 1 (v/v) and kept in the greenhouse for acclimatization.

Results and discussion

Meristem proliferation and multiplication: Meristem proliferation and multiplication was initiated from apical and axillary explants of *P. kurroa* within 8-10 days of inoculation onto MS basal medium supplemented with BA, KN and 0.5 mg L^{-1} ascorbic acid. Of the different cytokinins tested, BA + KN was the most effective for shoot proliferation and multiplication. The maximum shoot proliferation and multiplication was observed both in apical and axillary meristems cultured on MS medium supplemented with 0.25 mg L^{-1} BA, 0.25 mg L^{-1} KN and 0.5 mg L^{-1} ascorbic acid within 4 weeks of culture under 14h photoperiod (Table 1). The apical and axillary shoots proliferated and elongated to 1.0-1.5 cm within 4 weeks of culture. There was no sign of shoot proliferation when explants were cultured in media devoid of cytokinin. At higher concentrations of BA or kinetin, the rate of shoot proliferation declined. Inclusion of either IAA or IBA in the culture medium did not help in proliferation and multiplication of shoot. In most of the cases, the growth was inhibited and only 1-2 shoots elongated; some produced compact callus at the base of the explants. Prolonged culture on the proliferation and multiplication media resulted in the blackening of the basal ends of the developing shoots. There were differences among the treatments for both the percentage of cultures with multiple shoots and the mean number of shoots/culture. The axillary meristems produced more number of shoots (4.57) than the apical meristems (3.25). The highest percentage of cultures with multiple shoots was observed on media containing 0.25 mg L^{-1} BA, 0.25 mg L^{-1} KN and 0.5 mg L^{-1} ascorbic acid when the cultures were incubated in the continuous light for 4 weeks (Table 1). The frequency of multiple shoots per culture varied from 1.24 to 4.42 in case of the 14h photoperiod. The rate of multiplication was high and stable upto 5th subculture and declined in subsequent subcultures.

Induction of root from microshoots: Elongated shoots (1-2 cm long) were rooted on MS basal medium supplemented with various concentrations of either IAA or IBA. The rooting in the microshoots was inhibited in the medium devoid of growth regulator. Root initiation took place within 10-12 days of transfer to MS basal medium supplemented with 0.1-0.5 mg L^{-1} IAA or IBA. However, optimal rooting (70.6%) and growth of microshoots was observed on medium containing 0.25 mg L^{-1} IBA with 2% (w/v) sucrose (Table 2). The rooting ability was reduced with the increase in the concentration of IAA or IBA in the culture medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of IAA or IBA.

Acclimatization and field establishment: About 96% of the rooted plantlets established in the greenhouse within 2-3 weeks of transfer. The plant grew well and attained 6-8 cm height within 4 weeks of transfer. The whole plant regeneration and acclimatization has been depicted in Fig. 1.

Table 1. Effect of BA, kinetin and 2 mg L^{-1} ascorbic acid on shoot growth from apical (A) and axillary (B) meristems of *Picrorhiza kurroa* after 4 weeks of culture under 14h photoperiod

MS + Growth regulators (mg L^{-1})		Percent of cultures with multiple shoots (Mean ± S.E.)*		Number of shoots/ explant (Mean ± S.E.)*	
Kinetin	BA	A	B	A	B
0.0	0.0	0.0	0.0	0.0	0.0
0.0	0.25	38.7 ± 0.3	46.7 ± 0.3	2.72 ± 0.5	2.31 ± 0.2
0.0	0.50	40.2 ± 0.5	43.3 ± 0.7	1.25 ± 0.4	1.73 ± 0.5
0.0	1.0	17.7 ± 0.4	21.3 ± 0.6	1.87 ± 0.3	1.34 ± 0.6
0.25	0.0.	31.6 ± 0.4	41.4 ± 0.3	2.40 ± 0.2	2.93 ± 0.5
0.25	0.25	61.8 ± 0.5	73.7 ± 0.3	3.25 ± 0.2	4.57 ± 0.4
0.25	0.50	50.7 ± 0.4	57.6 ± 0.5	3.15 ± 0.4	3.20± 0.7
0.50	0.0	41.2 ± 0.5	43.8 ± 0.6	2.19 ± 0.4	2.30 ± 0.4
0.50	0.25	51.3 ± 0.6	56.4 ± 0.3	2.20 ± 0.7	2.17 ± 0.6
0.50	0.50	40.4 ± 0.4	41.8 ± 0.5	1.80 ± 0.6	1.88 ± 0.5
0.5	1.0	20.6 ± 0.4	23.4 ± 0.5	1.17± 0.5	1.13 ± 0.3
1.0	0.0	28.6 ± 0.7	23.5 ± 0.4	1.12 ± 0.5	1.30 ± 0.6
1.0	0.5	22.7 ± 0.5	25.5 ± 0.4	1.17 ± 0.6	1.27 ± 0.6

* Mean of 20 cultures per treatment, repeated thrice

Table 2. Effect of IAA and IBA on rooting from excised shoots of *P. kurroa* cultured on MS basal medium supplemented with 2% (w/v) sucrose

MS + Growth regulators (mg L^{-1})		Percentage of rooted shoot (Mean ± S.E)*	Days to rooting
IAA	IBA		
0 .10	0.0	30.6±0.20	10-11
0.25	0.0	45.4±0.6	10-13
0.50	0.0	50.2±0.4	11-12
0.0	0.10	57.20±0.70	10
0.0	0.25	70.60±0.30	12
0.0	0.50	54.83±0.50	11

*Data represent mean of 20 cultures/treatment, repeated thrice

Present study showed that it was possible to explore the morphogenetic potential of *P. kurroa* by modification of growth regulators and light conditions. The use of different combinations of cytokinins for the induction and multiplication of shoots derived from apical and axillary meristems and the regulatory action of cytokinin and apical dominance in shoot induction and multiplication *in vitro* is well documented (Wickson *et al.*, 1958). The higher shoot induction and multiplication was observed both in apical and axillary meristems cultured on MS medium supplemented with 0.25 mg L^{-1} BA, 0.25 mg L^{-1} kinetin and 0.5 mg L^{-1} ascorbic acid within 4 weeks of culture under 14h photoperiod. At higher concentrations of BA or kinetin, the rate of shoot proliferation declined. The axillary meristems produced more number of shoots than the apical meristems. The results demonstrated that inclusion of either IAA or IBA in the culture medium did not help in shoot multiplication. Prolonged culture on the proliferation and multiplication media containing IAA or IBA resulted in the blackening of the basal ends of the developing shoots. The results are consistent with earlier reports indicating cytokinins and auxins effect on shoot multiplication in other plants using shoot tip or axillary bud explants (Rout *et al.,* 1999). With the increase in the concentration of either BA or KN, the percentage of shoot multiplication declined. The results also implies that there were differences among the treatments for

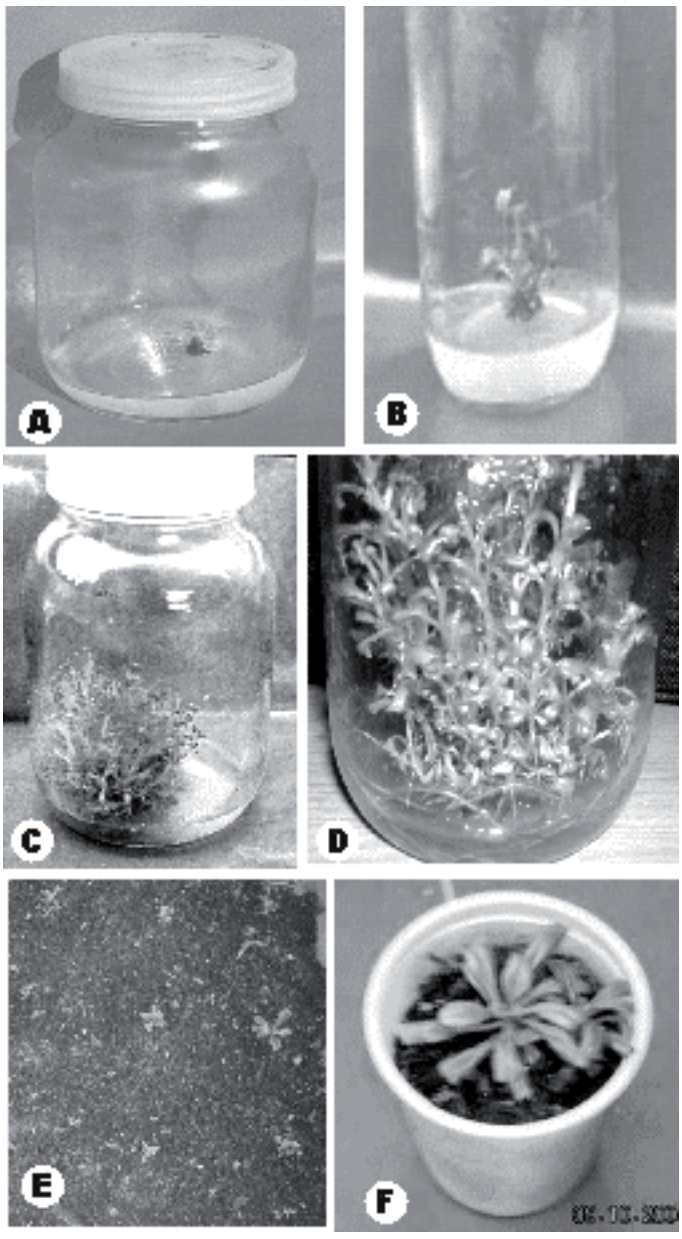

Fig. 1. Micropropagation of kutki (*P. kurroa*) (A) Establishment stage, (B & C) Shoot proliferation, (D) Rooting. Hardening and acclimatization (E) in pot (F) in field.

both the percentage of culture developing multiple shoots and the mean numbers of shoots per culture. The rate of multiplication was high and stable upto 5th subculture and declined in subsequent subcultures. This might be due to the balancing of the endogenous and exogenous growth regulators and the ionic concentration of nutrient salts as reported earlier in other plants (Zimmermann, 1985). Elongated shoots rooted better in MS basal medium supplemented with 0.25 mg L^{-1} IBA and 2% sucrose. The rooting ability was reduced with the increase in the concentration of IAA or IBA in the medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of IAA or IBA. The rooted plantlets were established in the field and grew normally.

In conclusion, an attempt was made to develop an *in vitro* protocol for mass multiplication of *P. kurroa* by manipulating the nutrient salts, growth regulators and culture conditions. This investigation may be useful for conservation of this economically important medicinal plant species.

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A comparison of three mathematical models of response to applied nitrogen using lettuce

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Abstract

Modern fertilization recommendation must optimize crop yield and quality and minimize chances of negative environmental effects due to over fertilization. Data from fertilizer studies can be fitted to several mathematical models to determine optimum fertilizer rates, but resulting recommendations can vary depending on the model chosen. In this research, lettuce (*Lactuca sativa* L.) was used as a case study vegetable crop to compare models for estimating fertilizer N requirements. Field studies were conducted to measure yield response to applied N. The area was located at 25˚21´ E longitude and 51˚38´ N latitude in the North of Varamin city, (Tehran province, Iran) in the alluvial plain of Varamin. Soil family was fine, mixed, active, thermic, typic haplocambids based on Soil Taxonomic system (USDA, 1999). Plants were grown in Central Research Station of Varamin and received five rates of N (0, 150, 200, 250 and 300 kg ha^{-1}) as a urea in split applications. Data for plant fresh mass and N uptake were recorded. Logistic, linear-plateau and quadratic models were compared for the field data. The logistic model described the data for cultivar quite well, with correlation coefficients of 0.90 and above. Coefficients for the linear-plateau model were derived from the logistic model. All three models for lettuce production were compared graphically and analytically. The model coefficients were used to make improved estimates of fertilizer recommendations for field production of lettuce.

Key words: *Lactuca sativa*, logistic equation, nitrogen

Introduction

Recommendations for fertilization of crops are derived from field studies in which crop yield and quality responses to a range of fertilizer rates are measured. Responses are often modeled to determine optimum fertilizer rate. Today, the relationship of nutrient management to environmental pollution is also an important aspect of any fertilization recommendation. There are many mathematical models for fitting crop response data. The research seeks to find a model that describes the data well and aids in defining reasonable fertilization recommendations that result in optimum crop yield and quality without the risk of over fertilization.

Quadratic models have been very popular for describing crop response to fertilization but tend to overestimate response if the maximum point on the curve is taken as the best fertilization rate. Often, fertilization rates less than the function maximizing rate are statistically similar to the single function maximizing rate (Cerrato and Blackmer, 1990; Hochmuth *et al.*, 1993a). Models other than quadratic functions have been used to describe crop response to fertilizer. Plateau models, such as linear-plateau (Dahnke and Olson, 1990; Nelson and Anderson, 1977), have been used with agronomic crops (Bullock and Bullock, 1994; Cerrato and Blackmer, 1990; Fageria *et al.*, 1997) and vegetables (Abdul-Baki *et al.*, 1997; Hochmoth *et al.*, 1993a, 1993b; Sanchez *et al.*, 1991) and logistic models with agronomic crop (Overman, 1995; Overman *et al.*, 1990, 1993). More research with vegetable crops to test functions such as the logistics model is need. Vegetables such as lettuce that require fertilization for optimum yield and quality is ideal crop for such research.

Lettuce is an important vegetable crop that is grown widely throughout the Iran, with much of the commercial production in Varamin, Tehran, Gilan and Mazandaran regions. Most of Varamin·s lettuce is produced on Aridisols soils of northern Varamin. Varamin is a major supplier of lettuce for Tehran.

Because of the high proportion of leaf tissue in lettuce, yields are greatly impacted by N fertilization. Research in Varamin with lettuce grown on loamy soils showed that N fertilization requirements were from 150 to 200 kg ha^{-1}. Sources of N fertilizer did not differ in their effects on lettuce yield or head quality (Gardner and Pew, 1979). Low levels of N result in small head size and poor yields. Even short periods of N deficiency can have a long-lasting negative effect on lettuce yield (Burns, 1988). Current N recommendation is 200 kg ha^{-1} for lettuce grown on loamy soils in Varamin. Yield and N uptake tend to increase linearly with N application rate. At high levels of N, plant yields and N uptake asymptotically approach maximum values. Decisions concerning optimum rates of fertilization usually involve fitting some type of model to yield data in response to several rates of fertilizer application. Regression analyses have been conducted on numerous data sets for response of agronomic forage crops to applied nutrients (Overman and Evers, 1992; Overman and Wilkinson, 1992; Overman *et al.*, 1990, 1991, 1992, 1993, 1994a, 1994b, 1995). In all these studies, the logistic equation accurately described data for dry-matter yields of forages and corn. In several studies, the extended logistic model also described plant N uptake as well as yield (Overman and Evers, 1992; Overman *et al.*, 1994a, 1994b, 1995). In the latter case, a common N response coefficient, c, existed between yield and plant N uptake. As a consequence, yield could be expressed as

a hyperbolic function of plant N uptake. Willcutts *et al.* (1998) studied models of response to applied nitrogen using lettuce. They found the logistic model offers a useful tool for evaluation of lettuce response to applied N.

The objective of this study was to demonstrate the utility of the logistic model to describe response of lettuce to applied N. A comparison was made with the linear-plateau and quadratic models for data obtained in field. Coefficients of the linear-plateau model were obtained as approximations from the logistic model. Both the linear-plateau and quadratic model predicted negative yields at very low N levels, whereas the logistic equation shows asymptotic approach to zero. The general characteristic and a rational basis for the logistic equation have been given by Overman (1995). Output (yield or plant N uptake) remains positive for all applied N, which must be true of the system by definition. Linear-plateau and quadratic models do not meet this constraint.

Materials and methods

Field experiments: Field experiments were conducted in spring with lettuce (*L. sativa* L.) on Aridisols soils and soils family with fine, mixed, active, thermic, typic haplocambids based on Soil taxonomic system (USDA. 1999). The area was located between 25° 21′ E longitude and 51° 38′ N latitude in the North of Varamin city (Tehran province, Iran) in the alluvial plain of Varamin. After soil was prepared by ploughing and disking, plots were formed. Irrigation method was furrow irrigation. Lettuce seeds were planted on 9 March. Plots were 15 m long and 5 m wide and consisted of five rows on 40 cm spacing × 20 cm between plants, for a total of 93 plants per plot (62000 plant ha^{-1}). Plants were grown in field of Central Research Station (Varamin Agricultural Research Center) and received five rates of N (0, 150, 200, 250 and 300 kg ha^{-1}) as urea in split applications. Treatments were replicated three times, with irrigation and pest control following recommended cultural practices (Hochmuth and Maynard, 1996). Lettuce heads were harvested on 4 June and fresh mass of marketable lettuce was recorded. N uptake with plant was measured in laboratory (Bremner and Mulvaney. 1982).

Model description: Data were analyzed using several models for comparison. The logistic models for yield and plant uptake are given by equations [1] and [2].

$$Y=A/[1+\exp(b-cN)] \quad [1]$$
$$Nu=A'/[1+\exp(b'-cN)] \quad [2]$$

Where Y= yield in fresh, mass, kg $plant^{-1}$; Nu=nitrogen uptake by lettuce, g $plant^{-1}$; N=nitrogen applied, g $plant^{-1}$ or kg ha^{-1}; A=maximum yield in fresh mass, kg $plant^{-1}$; b= intercept parameter for yield; b′= intercept parameter for nitrogen uptake; c= N response coefficient, plant g^{-1} or ha kg^{-1}. Following Overman *et al.* (1994a), Eqs. [1] and [2] can be combined to give the hyperbolic phase relation between yield and plant uptake,

$$Y=Y_m N_u/(K'+N_u) \quad [3]$$

Where, parameters Y_m and K′are defined in terms of the logistic parameters by,

$$Y_m=A/[1-\exp(b-b')] \quad [4]$$
$$K'=A'/[\exp(b'-b)-1] \quad [5]$$

Note that Y_m represents maximum potential yield and that $N_u=K'$ produces $Y=Y_m/2$, or one- half of maximum potential yield. Calculus techniques show that maximum incremental response to applied N occurs at an application rate $N_{1/2}= b/c$, where Y=A/2. This is the point of maximum slope T vs N. Similarly, maximum incremental response of plant N to applied N occurs at $N'_{/2} =b'/c$, with $N_u=A'/2$. The N response coefficient can be redefined as characteristic N given by N′=1/c, which converts units to more familiar g $plant^{-1}$ or kg ha^{-1}.

The linear-plateau model is given by,

$$Y_{lp}=B_{lp}+C_{lp}N \quad \text{for } N<N_x \quad [6]$$
$$Y_{lp}=A_{lp} \quad \text{for } N>N_x \quad [7]$$

Where for Y_{lp}= linear-plateau estimate of yield in fresh mass, kg $plant^{-1}$; A_{lp}= plateau or maximum freshyield, kg $plant^{-1}$; B_{lp}= intercept parameter, kg $plant^{-1}$; C_{lp}= slope parameter, ha $plant^{-1}$; and N_x= N application rate for intersection between Eqs. [6] and [7]. The linear-plateau parameters can be approximated from the logistic parameters as,

$$A_{lp}=A \quad [8]$$
$$B_{lp}=A/2(1-b/2) \quad [9]$$
$$=A/4N'(2N'-N_{1/2})$$
$$C_{lp}= Ac/4= A/4N' \quad [10]$$

This occurs because the logistic model approximates a straight line in the midrange of response. It follows that the intersection of the linear and plateau portions occurs at,

$$N_x= (A_{lp}-B_{lp})/C_{lp} \quad [11]$$
$$=(b+2)/c= N_{1/2}+2N'$$

The quadratic model can be written as,

$$Y_q= A_q+b_qN+C_qN^2 \quad [12]$$

Where, Y_q= quadratic estimate of yield in fresh mass, kg $plant^{-1}$; A_q= intercept parameter, kg $plant^{-1}$; B_q= linear response coefficient, ha $plant^{-1}$; and C_q= quadratic response coefficient, ha^2 kg^{-1} per plant. Peak production can be estimated from the maximum where the derivative, $dY_q/dn=0$, which occurs at,

$$N_{peak}= B_q/2c_q \quad [13]$$

And gives peak production of,

$$Y_{peak}= A_q+B_q{}^2/4C_q \quad [14]$$
$$=A_q+B_q/2N_{peak}$$

Fertilization rates of N_{peak} may be optimal for production because of diminishing returns obtained as N approaches N_{peak}. Therefore, optimum applied N rates would tend to be below N_{peak} (*i.e.*, $N_{opt}<N_{peak}$).

Results and discussion

Field experiments: Response of field lettuce to applied N is shown in Fig. 1. Logistic, linear-plateau, and quadratic models were fitted to the data with parameters listed in Table 1. The logistic model provides a reasonable basis for the linear-plateau model (Fig. 1). The intersection point can be calculated from Eq. [11], and peak N values for the quadratic model were calculated from Eq. [13]. A summary of critical values of model parameters is listed in Table 2. At $N=N_{1/2}$yield is 505 of the plateau, whereas

Table 1. Model parameters for field lettuce at Varamin, Iran

Model		Parameters	Value
Logistic		A, kg plant^{-1}	0.48
$Y=A/[1+\exp(b-cN)]$		b	0.64
$Nu=A'/[1+\exp(b'-cN)]$		c, ha kg^{-1}	0.028
		A´	490.00
		b´	30.00
Linear-plateau		A_{lp}, kg plant^{-1}	0.48
$Y_{lp}=B_{lp}+C_{lp}N$	for $N<N_x$	B_{lp}, kg plant^{-1}	0.163
$Y_{lp}=A_{lp}$	for $N>N_x$	C_{lp}, ha plant^{-1}	0.00336
Quadratic		A_q, kg plant^{-1}	0.1984
$Y_q=A_q+b_qN+C_qN^2$		B_q, ha plant^{-1}	0.0021
		C_q, ha^2 kg^{-1} plant^{-1}	0.000004

Table 2. Critical N value (kg ha^{-1}) for the models for field-grown lettuce in Varamin, Iran.

$N_{1/2}$	N_x	N_{peak}
23	94	202

at $N=N_x$, yield is 88% of plateau. For $N=N_{peak}$ yields are well out on the plateau, beyond the region of significant response to applied N. Fig. 2 show dependence of fresh mass on plant N uptake for field lettuce.

From these results, the logistic model apparently provides an adequate description field results for response of lettuce to applied N. From analysis of the field data, N_x appears to give the most reasonable level for a nitrogen fertilizer recommendation, *viz.*, 150 kg ha^{-1} for these conditions. This is considerably below the current Varamin recommendation of 200 kg ha^{-1}.

The logistic model offers a useful tool for evaluation of lettuce response to applied N. Parameters A, b, and c in Eq. [1] can be estimated from data by nonlinear regression. One can also use the following simple alternative procedure. Parameter A (the plateau) can be estimated by visual inspection of the data for yield *vs*. applied N (such as Fig. 1). Then parameter b follows from,

$$B=\ln(A/Y\circ-1) \qquad [15]$$

Where Y◦= estimated intercept yield at N=0. Finally, parameter c is calculated from,

$$C=b/N_{1/2} \qquad [16]$$

Where $N_{1/2}$ is estimated as the value of N corresponding to y=A/2 (50% of the plateau) on the graph of yield response to applied N. With parameters b and c in hand, N_x can then be estimated from Eq. [11]. Estimates of yield at given applied N levels are easily made with Eq. [1] using a calculator with an equation writer.

Present study revealed that the logistic model contains the right characteristics to describe field data and is relatively simple to use in practice.

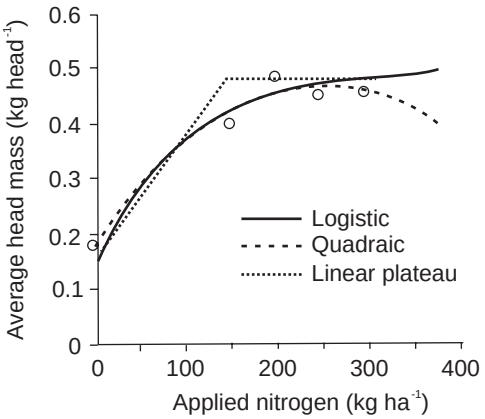

Fig. 1. Comparison of logistic, linear plateau and quadratic models for response of field grown lettuce to N application at Varamin. Model values calculated with parameters from Table 1.

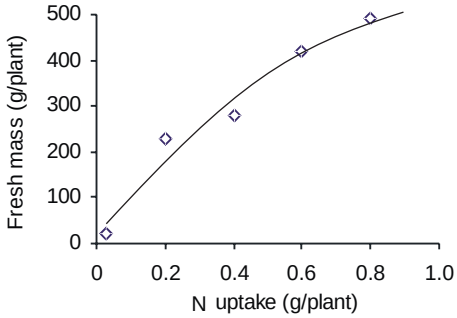

Fig. 2. Dependence of fresh mass on plant N uptake for field grown lettuce at Varamin. Curve drawn from Eq. 3 with parameters calculated by Eqs. 4 and 5 using logistic parameters from Table 1.

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Comparison of conventional fertilization and vermicompost use for basil cultivation

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Abstract

The effect of conventional fertilization was compared with a vermicompost that was mixed with substrate for sweet basil (*Ocimum basilicum* L.) in a greenhouse experiment. The study was conducted in a completely randomized block design with 4 replications. Eight treatments were compared: a control treatment of a substrate mixture (T0: with no vermicompost added), five treatments with increasing percentages of vermicompost added to the substrate mixture (H1 to H5), and two treatments using two application rates of a chemical fertilizer (F1 and F2). Both fertilizer and vermicompost presented very low levels of heavy metals, which assured agronomical suitability. Vermicompost from SS-MSW (Source-Separated Municipal Solid Waste) and slaughterhouse sludge, presented significant value as soil conditioner and biofertilizer and produced increased levels of C and N ($P<0.05$). The phosphorus addition by vermicompost was high, with a decrease of zinc absorption by plants and potential contamination risk. Mixtures including more than 50% of the vermicompost and the highest rate of fertilizer showed statistically significant differences for dry weight, leaf length, plant survival and P-Zn antagonism ($P<0.05$).

Key words: Fertilization, vermicompost, *Ocimum basilicum* L., basil

Introduction

There is a controversy concerning the use of inorganic and organic fertilizers. Inorganic fertilizers are easy to manage and hygienic, but organic ones present the advantage of lower costs and environmental benefits (Ghosh, 2004). Environmental problems related with heavy metal contamination should be considered, as organic manures are generated by urban and industrial developments (Marbán *et al.*, 1999). Organic wastes of a different nature and environmental risks are generated as consequence of such activities, and their recycling can offer an ecological solution for waste treatment and disposal, and could be an efficient and economic alternative for conventional waste disposal procedures (Govil, 2001).

Composting is a biological process that produces a stable organic matter, free of pathogens and toxins: "*compost*" (Polo, 1997). Usually, in agriculture, the most important restrictive factors to use composts as soil amendments have been human pathogenic content, the presence of heavy metals, nutrient excesses, high salt concentrations, organic pollutants and immaturity (Madrid *et al.*, 2000). Vermicomposts, which are produced by the fragmentation of organic wastes by earthworms, have a fine particulate structure and contain nutrients in forms that are readily available for plant uptake. In greenhouse trials, they have shown to enhance growth of different seedlings and cultivations (Atiyeh *et al.* 2000). After the thermophilic stage of composting, the material is inoculated with earthworms and a commercial biofertilizer (vermicompost) is obtained as the final result. The organic matter of this biofertilizer contains a high percentage of humic and fulvic acids and a beneficial microbial load. Vermicompost incorporation increases soil aggregation, structure, water retention, cation exchange capacity, and releases nutrients required by plants, in a balanced way. It adsorbs pollutants such as heavy metals due to its high adsorption capacity and protects soil from erosion (Movahedi and Cook, 2000). It also contains phytohormones such as indolacetic and gibberellic acid, together with other biologically active substances (Ruiz *et al.*, 1999) and as a consequence of the high microbial load contributes to the protection of roots from bacterial and parasitic nematode attacks. Enough evidence exists to ensure that human pathogens do not survive the vermicomposting process (Eastman *et al.*, 2001), but special attention should be paid to heavy metal contents, since they could be dangerous in the food chain.

Sweet basil (*Ocimum basilicum* L.) is grown commercially as a cultivated herb plant in many parts of the world, and used both as a fresh and a dried food spice, for the commercial production of essential oil and in traditional medicine. Research has also shown that it may be a good indicator of the adverse effect of various environmental signals to plants, including high concentrations of trace metals in composts (Zheljazkov and Warman, 2003).

In the present study, soil and plant characterstics were evaluated with biofertilizer (vermicompost) and inorganic fertilization for basil cultivation.

Materials and methods

The urban solid wastes from Chivilcoy town were classified, by manual separation for the inert materials (glass, plastic, cardboard and metals), from the organic ones. These urban organic residues, also denominated SS-MSW: Source-Separated Municipal Solid Waste (Zheljazkov and Warman, 2004), were mixed with reduced fat slaughterhouse sludge prior to being composted. When the first thermophilic stage was finished, the material was inoculated with earthworms (*Eisenia andrei*).

Sweet basil was sown in conventional 60 x 30 cm trays. After

twenty days, 3-4 cm plants were transplanted to 1 kg pots. Substrate (peat) was sterilized with methyl bromide and mixed with different quantities of vermicompost. Three plants per pot were left in each pot, and the study was conducted in greenhouse conditions, with pots moisture maintained near field capacity.

The experiment was conducted in a completely random block design with 4 replications for each treatment. In the experiment, eight treatments were compared: a control treatment of a substrate mixture (T0: with no vermicompost added), five treatments with increasing percentages of vermicompost added to the substrate mixture (H1 to H5), and two treatments using two application rates of a chemical fertilizer (F1 and F2). Treatments were: T0: substrate mixture (peat without vermicompost), H1: 95 % mixture + 5% vermicompost, H2: 90 % mixture + 10% vermicompost, H3: 80 % mixture + 20% vermicompost, H4: 50 % mixture + 50% vermicompost, H5: 100 % vermicompost, F1: substrate + chemical fertilizer: 3 g KEMIRA NPK 12-5-11 per pot, F2: substrate + chemical fertilizer: 30 g of KEMIRA NPK 12-5-11 per pot.

Agronomic variables that were evaluated in substrate mixture were: pH: water extraction 1: 2.5, total organic carbon (%C): Walkley –Black technique (Nelson and Sommers, 1982), total nitrogen (%N): microKjeldahl procedure; available phosphorus (P, mg kg^{-1}): Bray 1 method (Bray and Kurtz, 1945); electrical conductivity (EC, dS m^{-1}): saturation extract. (Rhoades, 1996).

Heavy metals, cadmium (Cd), lead (Pb), zinc (Zn), nickel (Ni), copper (Cu), chromium (Cr) and mercury (Hg) were quantified in both the fertilizer and vermicompost with an aqua regia extraction and quantified by ICP Baird 2070 (Page, 1982).

The greenhouse study was concluded when the basil reached the commercial pre-bloom stage. Plant variables considered were dry matter weight and leaf size. Phosphorus (%P) and zinc (Zn mg kg^{-1}) concentrations were measured in leaves. Data were statistically analysed for ANOVA using Statistix 4.0 software.

Results and discussion

The composition of the vermicompost was: 1.2 %N, 184 mg kg^{-1} P-Bray, and K 1.1 $cmol_c$ kg^{-1}, and the fertilizer contained: 12.8% N, 4.5% P_2O_5 and 11.3 % K. Toxic metals, Cd, Pb, Zn, Ni, Cu, Cr and Hg, were evaluated in chemical fertilizer and biofertilizer; both compounds had lower levels of inorganic toxics than the maximum acceptable concentrations for biosolids of high quality according to the United States Environmental Protection Agency (US-EPA), and did not present limitations for European Union (EU). Fig. 1 shows the comparison between levels of metals and soil quality criteria for Argentine Law 24051, to remark the agricultural suitability of the materials.

Total organic carbon was associated to the highest rate of vermicomposts (50 and 100%), with a statistically significant difference from the control ($P < 0.05$). The inorganic fertilizers did not show any variation in the total organic carbon content. The total nitrogen levels also increased (Fig. 2) and H4, H5, F1 and F2 differed significantly from the control ($P < 0.05$). The C/N relationship was nearly 10/1 in all treatments. This relationship indicated that the vermicompost could be considered mature for use, without causing any nitrogen problems in the crop (Madrid *et al.*, 2000). Stability is an important property of compost as its application as soil conditioner or part of a growing medium requires a stable product characterized by odour, water content, pH and other parameters (Eggen, 2001). The results agree with those presented by Smith *et al.* (1999), about beneficial effect of composted materials in increasing soil carbon and nitrogen content.

The evolution of Bray-available phosphorus with higher rates of vermicompost showed a great increase, with particularly large values in H5 (800 mg kg^{-1}), such levels could cause contamination and nutritional imbalance in plants (Fig. 3).

The pH values were nearly neutral for the control and all doses of vermicompost, while those for inorganic fertilizers F1 and F2 were 5.6 and 5.3, respectively. Electrical conductivity values were higher in vermicompost (H4 and H5) and maximum in fertilizer (F2), with statistically significant differences ($P<0.05$) compared with control (T0).The elevated electric conductivity values indicated importance of contents of soluble salts, which could increase the osmotic pressure in the system and produce nutritional problems that could be associated with damage and death of plants. Zheljazkov and Warman (2003) also recorded the same tendency of increasing pH and EC of the growth medium with an increased addition of vermicompost.

Results of plant analysis are shown in Table 1. Dry matter yield weights of basil plants showed a significant decrease ($P < 0.05$) between the control (T0) and the higher rates of the vermicompost and fertilizer (H4, H5, F2), and growth of plants was similar for the rest of the treatments (H1, H2, H3 and F1) , with non significant differences ($P < 0.05$).

The size of the basil leaf is very important for commercial use. The treatment H2 (10 % vermicompost) produced the greatest leaf length (10.74 cm) followed by H1 and H3. These three treatments behaved as a statistically homogeneous group. The treatments producing lower leaf sizes were H4 and H5 (50 and 100 % vermicompost added).

The percentage survival of plants was in general 100%, but amending the soil with 100% of vermicompost and the highest rate of the fertilizer produced significant plant mortality.

The treatment that had adverse effects on growth, measured as reduction in dry weight and reduced development of the leaf and plant mortality, occurred when the vermicompost was used more than 50% and with highest rate of fertilizer, that could have a phytotoxic effect.

Table 1. Effect of different treatments on plant characterstics

Treatment	Dry weight (g)	Leaf length (cm)	Survival (%)	P (%)	Zn (mg kg^{-1})
T0	3.16b	8.43b	100b	0.38a	152.2b
H1	2.94b	9.55c	100b	0.44a	138.2b
H2	3.90b	10.74c	100b	0.50b	153.6b
H3	3.86b	8.96c	100b	0.51b	119.6ab
H4	1.55a	6.19a	100b	0.53b	72.2ab
H5	0.38a	4.20a	65a	0.55b	44.0a
F1	3.40b	8.49 b	100b	0.38a	159.2b
F2	1.41a	8.41b	65a	0.38a	277.0b

Column values followed by the same letter are not significantly different at $P<0.05$

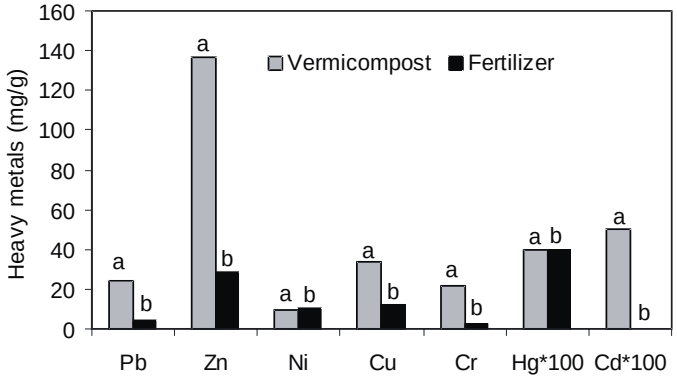

Fig. 1. Heavy metals in fertilizer and vermicompost compared to argentinian soil quality criteria (SQ criteria). Different characters designate statistical significant differences ($P \leq 0.05$)

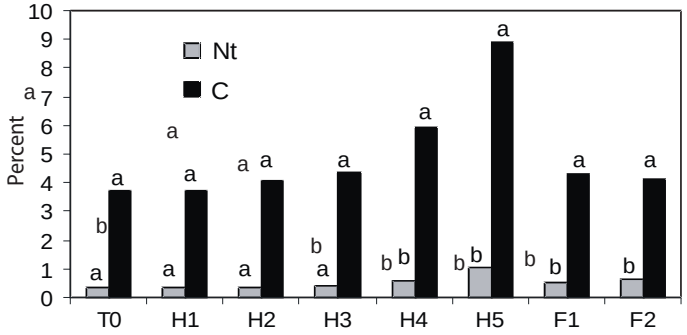

Fig. 2. Evaluation of C and N for all treatments. Different characters designate statistical significant differences ($P \leq 0.05$)

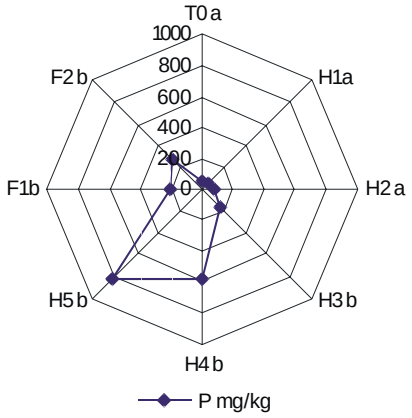

Fig. 3. P evaluation for all treatments. Different characters designate statistical significant differences ($P<0.05$)

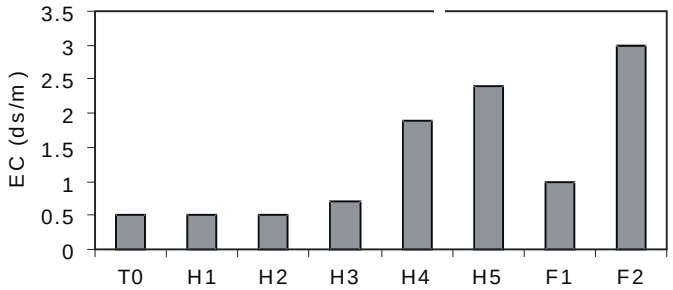

Fig. 4. Evaluation of electrical conductivity for all treatments. Different characters designate statistical significant differences ($P<0.05$)

The P content of basil leaf varied from 0.386 % P in T0 to 0.550 % P in H5. The inverse situation was observed with the Zn content, which decreased with the rate of vermicompost application. Zn levels varied between 152.2 mg kg^{-1} of Zn in T0 to 44.0 mg kg^{-1} of Zn in H5 (Table 1). The antagonism between P and Zn is a well-known interaction. As the content of P increased in the media, a decrease in the absorption and transport of the Zn from the roots could take place, and in some cases, it could cause nutritional problems and yield decrease (Malavolta, 1994). An antagonism P-Zn was manifested in treatments H3, H4 and H5.

Our results showed that vermicompost has agronomic value if used as an amendment, and also for leaf growth with commercial application, but possible negative effects must be kept in mind that could result from large doses, as well as in the case of fertilizers. Vermiculture to process organic waste and generate fertilizer can be a useful tool to promote sustainable development on a local scale in Latin-American cities (Spiaggi *et al.*, 2001). As it was proposed by EPA (2001), we followed key factors contributing to vermicompost project success: comparison with other scientific research; comparative tests with different application rates of vermicompost, fertilizer and control plots; and a good trial design with statistically comprable results.

Zheljazkov and Warman (2004) reported that mature composts could be safely used as soil conditioners for agricultural crops, as addition of compost reduced bioavailability and transfer factors for micronutrients as Cu and Zn. It had also been demonstrated that soil amendment with compost is an effective non-chemical, environment-friendly means to prevent or to reduce the damage caused by *Fusarium oxysporum* f. sp. *basilici* that causes wilt in sweet basil plants (Zheljazkov and Warman, 2003).

A relevant agronomic application could be the exploitation of integrated nutrition. Jeyabal and Kuppuswamy (2001) showed that the integrated application of vermicompost, fertilizer N, *Azospirillum* and phosphobacteria increased rice yield by 15.9% over application with N fertilizer alone.

Fortuna *et al.* (2003) also suggested that it could play a role in optimizing nutrient availability and potential carbon sequestration in an agroecosystem. They reported a comparison of compost and chemical fertilizer, for corn–corn–soybean–wheat rotation compared to continuous corn. Compost applications over 6 years increased the resistant pool of C by 30% and the slow pool of C by 10%. The compost treatment contained 14% greater soil organic C than the fertilizer management. Proper management of nutrients from compost, cover crops and rotations can maintain soil fertility and increase C sequestration.

Vermicompost was as useful as fertilizer to obtain a product with commercial value. Both materials presented low toxic metals content, and the maturity of vermicompost was appropriated (C/N relationship was near 10). The restrictive factors for large rates of both materials were soluble salts content, which increased electrical conductivity, and produced plant mortality and P-Zn antagonism in plant. Vermicompost acted as organic amendment which presented a significant increase in C and N levels. It should be taken into account that the application of vermicompost could hardly increase the amounts of available phosphorus, with a risk of nutritional imbalance and potential environmental problems. Mixture including more than 50% of the vermicompost and the highest rate of fertilizer showed statistically significant differences for dry weight, leaf length, plant survival and P-Zn antagonism ($P<0.05$).

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Chilling requirement studies on flower buds in some male pistachio genotypes (*Pistacia vera* L.)

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Abstract

Effects of different chilling periods were evaluated on growth and development of floral buds of male seedling trees **(***Pistacia.vera* L.**)** for chilling requirements of male genotypes helpful in predicting overlapping of flowering with female trees and escape from spring cold damage. The chilling requirement and responses of male genotypes to chilling treatment were determined by applying eight levels of chilling to shoots (*i.e.* 600-1300 h) at 3±1 °C. Based on the effect of chilling hours on bud break on four male pistachio genotypes were grouped to early (P_1 and P_6) and late flowering (P_7 and P_{10}) types. Percentage and rate of bud break, duration of flowering, growth and development of bud (length and width) were evaluated. The results indicated that genotypes had different chilling requirement. Among the male pistachio genotypes, the adequate chilling hours (bud break >80%) for P_1, P_6, P_7 and P_{10} genotypes were 800, 700, 1100, and 1300 hours, respectively. P_1 and P_6 had low chilling requirement (700 hours) for 50% bud break compared to P_7 and P_{10} (900 and 800 hours). Increased chilling led to decreased heat unit requirements for sprouting, resulting in greater overall growth and development. Chilling was a determining factor in floral bud break for all the genotypes, increasing chilling also produced greater bud break percentages. All genotypes required fewer heat units for bud break as chilling increased. Increasing the chilling hours also increased the length and width of flower buds and reduced duration of flowering.

Key words: Dormancy, bud break, cold storage, chilling requirement, bud development

Introduction

Dormancy in plants has been described as a state in which visible growth is temporarily suspended (Samish, 1954), a phase in plant development allowing it to survive under winter conditions (Saure, 1985) and a state in which deciduous plants are without leaf or are lacking visible growth (Westwood, 1993). Endodormancy released from within plant parts, as controlled by chilling temperatures, is a major factor in determining a plant's performance in a given climate or hardiness zone (Westwood, 1993). Temperate zone plants must be exposed to a certain period of chilling temperatures above freezing (Westwood, 1993) or a minimum number of hours below 7°C (45°F) (Saure, 1985) for dormancy break. This exposure period is referred as the chilling requirement. Dormancy requirements of landscape trees are of particular interest to the arborist and urban forester. Trees noted to perform well in northern climates, such as flowering cherries, spruce, or beech, may perform poorly or flower not at all in southern climates. In other cases, trees noted to perform well in the south may leaf out too early in the north, resulting in cold and frost damage (Lechowicz, 1984).

Much work has been reported on fruit species with respect to dormancy and chilling requirements. In a study with peaches, once chilling requirement was satisfied, prolonged chilling induced enhanced leafing over blooming (Citadin *et al.,* 2001). There are also cultivar differences in heat requirement for bloom. In a study with several fruit tree species, once chilling requirement was satisfied, prolonged chilling led to a decreased need for heat units for bud break (Couvillon and Erez, 1985).

The chilling optimum of temperate latitude forest trees varies between 0 and 2,000 h of below 5°C (Jensen and Gatherum, 1965; Steinhhoff and Hoff 1972; Van den Driessche, 1975; Burr *et al.,* 1989). Differences in chilling optimal within species may be caused by genetic variability, perhaps related to the different elevations and geographic regions in which the seed source was found (Rehfeldt, 1990). This genetic variation could in turn lead to the differences in chilling requirements between and within species. Most pistachios have a chilling requirement of 600-1200 hours. Not all buds of a plant have equal chilling requirements. Generally flower buds require less chilling than lateral buds. This is because flower buds often appear several days earlier than vegetative buds. Similarly, terminal buds have a lower chilling requirement than lateral buds. Therefore, in moderate climates (without severe cold in winters), terminal buds can begin to grow soon enough to establish apical dominance over laterals. In areas of severe winters, by the time the growing season begins both the apical and axillary's buds may have all of the chilling requirements met. Therefore when spring finally arrives, the plant will begin to grow from both lateral and terminal buds simultaneously.

Samish and Lavee (1962) indicated about the lack of standardized method to evaluate the depth of dormancy. To properly evaluate the depth of dormancy in the entire plant, it should be exposed to temperature for growth as in a greenhouse, but this is difficult with large plants. In a series of experiments, Erez and Lavee (1979) used rooted cutting of peach to study the effect of alternating temperature in breaking bud dormancy. One might ask whether the presence of roots in close proximity to the buds might affect response; however, Couvillon and Erez (1985) had previously demonstrated that bud break on rooted cutting paralleled that

of mature trees. Therefore, cuttings bearing many buds will be the better choice for researchers wishing to predict the field response. The larger cutting has better expected response. The source of cutting is also important, especially for theoretical studies; previous year's shoots are normally used, but their vigor could affect response. Therefore, selected shoots should be similar in length and taken from similar position on the plant (Dennis, 2003).

Investigators often speak of the 'end of rest' when evaluating bud dormancy. This is usually defined as the time when 50% of the buds on excised shoots are capable of growth within a given period of time when held at an appropriate temperature with their bases in water. Greening of the bud scales is some times taken as evidence of bud break. The bases of cutting must be cut frequently to prevent vessel occlusion. Another problem is the danger of desiccation unless they are kept under high humidity, as in a mist bed. Growing cutting and/or bubs *in vitro* can prevent this (Dennis, 2003). Not much information is available about the chilling requirements of pistachio during the winter to ensure adequate bloom and pollination in the following spring (Crane and Iwakiri, 1981; Crane and Takeda, 1979).

The aim of this study was to evaluate the chilling requirement of male genotypes by exposing shoots to varying degree of chilling hours. The study will be helpful in the understaning of overlapped flowering with female trees and resistance to spring cold damage.

Materials and methods

The experiment was carried out during 2005-2006 using four male pistachio genotypes (P_1, P_6, P_7, and P_{10}) growing in Pistachio Research Institute at Rafsanjan, Iran. Their chilling requirements were calculated according to the chill unit. Temperatures between 0-7°C in winter of past year was 800-900 hours, latitude and longitude; 30° 25´N, 55° 45´E, respectively. To determine chilling requirement of mentioned genotypes, after leaf fall in the early November, 96 shoots of 30 -35 cm length from each genotype were picked up. After treating with Benomyl (2%) to protect from fungi, the shoots were warped in humid cloth and plastic then placed at temperature 3±1°C in referegerator.

The shoots were taken out from referegerator after chilled for desirable time (600 to 1300 h). These shoots were placed in buckets with half Hoagland medium. At 100-hours interval bud sticks were removed from the referegerator and placed in nutrient solution in the growth chamber of Horticulture Department of Tehran University. The growth chamber at the laboratory, programmed to simulate a typical day in mid April (9 hour night with 11°C and15 hour light with 19°C), these parameters were based on the average of the past seven years. Fresh cuts were made in the shoot bases and the water was changed every 4 days. The experiment had a factorial design with two factors including chilling hours at 8 levels and four genotypes (early flowering (P_1, P_6) and late flowering (P_7, P_{10}) in base of randomized complete block. The flower bud breaking percentage was determined in each treatment and the data were analyzed by SAS software.

Results and discussion

There were significant differences among genotypes for chilling requirements (Table 1). The adequate chilling hours for P_1, P_6, P_7, P_{10} genotypes were 800, 700, 1100, and 1300, respectively. P_1, P_6 and P_{10}, had minimum chilling hour requirement (700, 700, 800 hours) to initiate 50% bud break than P_7 (800 h). None of the genotypes responded to 600 hours treatment, except P7. (Table 1).

Compared to early flowering genotypes, late flowering types required less chilling hours. In all genotypes, increasing the level of chilling accelerated the rate of flower bud break (Table 1). Differences in the number of heat units required to reach bud break at every chilling level were determined for each genotype. The level of chilling exposure required for flower bud break was inversely related to heat unit accumulation. The higher chilling treatments also generally exhibited the highest mean percentage bud break over the course of the experiment (Fig. 2). These observations were similar to those recorded by Ashby *et al.*(1991) and Couvillon and Erez (1985).

Increasing the chill hours also increased the length and width of flower buds (Fig. 3) which is in accordance to the findings of Ferguson *et al.* (2003). Duration of flowering decreased from 26 days (in 700 hour treatments) to 11 days (in 1300 hour treatments) (Fig. 4).

Information from the present and future studies may be used to facilitate the development of models for regional planting recommendations based on the amount of chilling received

Table1. Effect of chilling on mean flower bud opening percentage and its rate in different genotypes

Chill hours	P_1		P_6		P_7		P_{10}	
	Flower bud opened (%)	Rate of flower bud opening	Flower bud opened (%)	Rate of flower bud opening	Flower bud opened (%)	Rate of flower bud opening	Flower bud opened (%)	Rate of flower bud opening
600	0.00	0.00	0.00	0.00	57.9cde	1.73i	0.00	0.00
700	69.33bcd	2.47hig	85.913ab	4.92d	56.44cde	2.25hig	87.05ab	2.375ghi
800	86.235ab	3.35efg	85.53ab	3.32efg	39.27e	3.03efgh	71.48bc	2.09ih
900	88.77ab	3.61ef	51.083de	3.01efgh	49.25e	1.77i	85.02ab	5.34d
1000	84.49ab	2.83fgh	76.3abc	3.05efgh	42.51e	2.78fgh	80.93ab	3.4e
1100	81.493ab	3.95e	89.76ab	5.282d	89.92de	5.01d	73.02bc	5.31d
1200	83.46ab	8.37b	88.65ab	3.65ef	74.1bc	2.982efgh	75.42bc	3.3efg
1300	80.2ab	8.05b	97.72a	9.77a	44.9e	3.94e	83.77ab	6.4c

*Means followed by different letters are significantly different by Duncan's Multiple Range Test at P=0.01

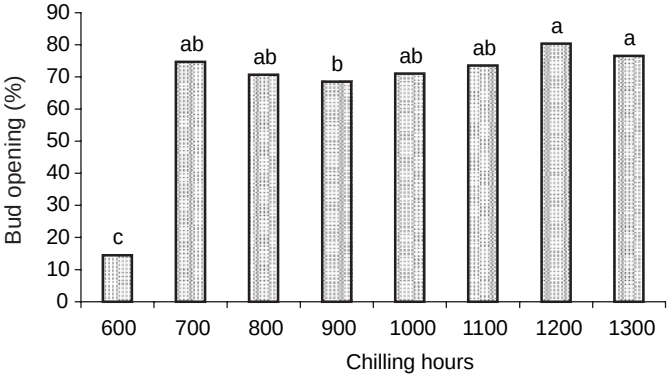

Fig.1. Influence of chilling hours on bud opening

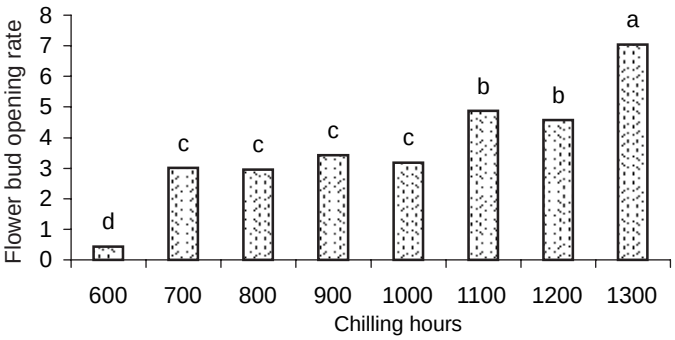

Fig. 2. Effect of chilling hours on the flower bud opening rate

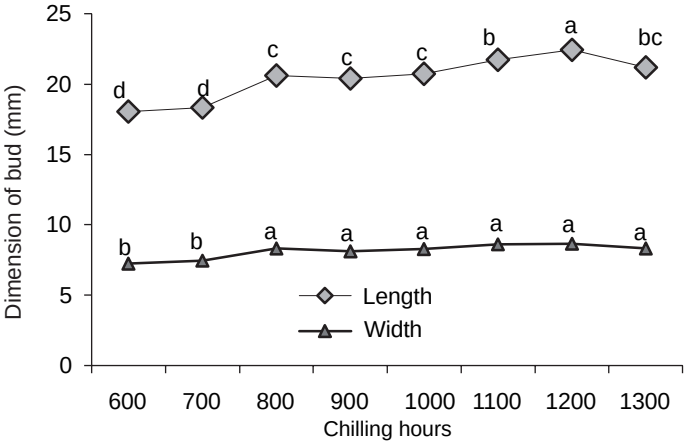

Fig. 3. Chilling effect on dimension of flowering buds of pistachio male genotypes in growth chamber.

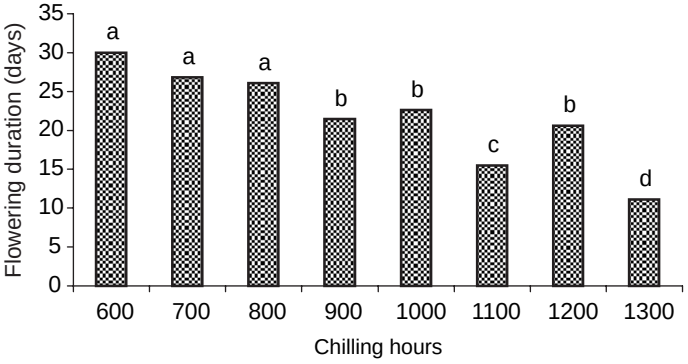

Fig. 4. Effect of chilling hours on the flowering duration

Fig. 5. Shoot bud showing anthesis under growth chamber conditions

at a given location. More research will be needed to develop regional planting models for adequate pollination. The processes that lead to dormancy and bud break within a plant consist of many interacting factors (temperature, light, physiological and chronological age of plant, apical dominance, provenance, hormonal balances, environmental conditions, drought, fertility, etc.). These factors are related to chilling and heat unit accumulation and must be studied to present a accurate picture of specific chilling requirements in individual cultivars. Finally, one of the most critical concerns yet to be addressed is a determination of the optimal temperatures for break dormancy.

The present study assumed ambient temperatures below 7°C and constant at 3°C as adequate to accomplish chilling requirement and maintaining the greenhouse environment above 22°C was ideal for flushing. Perhaps lower or higher temperatures could be considered more effective for breaking dormancy. Also, differences between constant versus fluctuating temperatures in a natural or simulated environment are the areas of additional study.

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