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# Factors affecting *in vitro* propagation of *Dracaena sanderiana* Sander ex Mast. cultivars. I. Sterilization, explant browning, and shoot proliferation

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*Key words:* Dracaena, growth regulators, proliferation rate, propagation, sterilization.

**Abstract: *Dracaena sanderiana* Sander ex Mast. (lucky bomboo) is an ornamental plant belonging to the family Agavaceae. The factors affecting sterilization, explant browning, and shoot proliferation of two cultivars Green and Variegated of *D. sanderiana* were studied. Micropropagation of *D. sanderiana* is very important because of the limitations in its conventional propagation by classical vegetative propagation methods which give rise to several bacterial, fungal, viral and mycoplasma diseases. The *in vitro* condition can overcome these problems. Half-strength Murashige and Skoog's (MS) media supplemented with different concentrations of 6- benzyl-amino-purine (BA mg $l^{-1}$) and naphthalene acetic acid (NAA) (0.25 and 0.5 mg $l^{-1}$) were used for shoot proliferation and plant regeneration studies. The effect of explant positioning on the culture media, whether horizontal or vertical, was also assessed on proliferation and growth of shoots produced. Explants of the 'Green' cultivar, cultured horizontally on media, were successful in yielding proliferated shoots. The highest mean value (4.8) was recorded on the medium supplemented with 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA. Explants of the 'Variegated' cultivar, cultured horizontally on media, were also successful in yielding proliferated shoots. The highest mean value (3.66) was recorded on the medium supplemented with 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA.**

## 1. Introduction

*Dracaena sanderiana* Sander ex Mast. (Lucky bomboo) belongs to the family *Agavaceae*. It is distributed in tropical and subtropical open lands of Africa and India. Despite its medicinal and ornamental importance, not much work has been undertaken with regard to its *in vitro* propagation; conventional vegetative propagation is the most prevalent method (Junaid *et al*., 2008). Vegetatively propagated plants accumulate several bacterial, fungal, viral, and mycoplasma diseases however the *in vitro* condition can overcome these problems and offers rapid vegetative multiplication of plants (Predieri, 2001; Muthusamy *et al*., 2007).

Usually this plant is propagated by means of seeds or cuttings however micropropagation is a proposed technique to produce healthy plants. To cover the needs for such plants, it is necessary to study the different factors affecting productivity of the explants to standardize the technique used for enhancing the multiplication of this important plant for indoor and outdoor uses.

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Paek *et al*. (1985) found that *Cordyline terminalis* Electra and *Scindapsus aureum* Marble Queen shoot tips were successfully multiplied on a solid MS medium supplemented with Indole-3-acetic acid (IAA) at 1.0 mg $l^{-1}$, Kinetin at 3.0 mg $l^{-1}$ and adenine sulphate at 100 mg $l^{-1}$ and $Na_2H_2PO_4.H_2O$ at 150 mg $l^{-1}$. Atta-Alla *et al*. (1996) stated that the highest rate of shoot proliferation in *Dracaena marginata* Lam. 'Tricolor' was achieved on MS medium supplemented with BA at 4.0 mg $l^{-1}$ plus NAA at 0.05 mg $l^{-1}$. Furthermore, Tian-Lang *et al.* (1999) reported that using MS medium supplemented with BA (3-3.5 mg $l^{-1}$) and NAA (0.02 mg $l^{-1}$) was suitable for multiplication of *D. sanderiana* cv. Virescens. El-Sawy *et al*. (2000) produced the largest number of shoots per explant on MS medium supplemented with BA at 4.0 mg $l^{-1}$. Kobza and Vachunova (1989) reported that MS medium enriched with BA and IAA or BA and NAA was the best. Stem explants of *D. deremensis* Warneckii 'Lemon Lime' with a dormant bud provided the most suitable propagation material. Leffring *et al*. (1985) found that, as both growth regulators (IAA and ABA) increased over a few month culture period, the downward movement of IAA resulted in basal callus formation of *Cordyline* cultivars and prevented shoot development as it antagonized the effects of Kinetin in the culture medium. Kobza and Vachunova (1991) found that MS medium supplemented with 1.0 mg $l^{-1}$ Kinetin and 0.8 mg

$l^{-1}$ IAA was the best medium for *D. concina* Kunth shoot production.

The objective of the present investigation was to study different aspects of micropropagation of *D. sanderiana* (lucky bomboo) and factors affecting its sterilization, browning decrease in explants, and proliferation of shoots to propose a commercial protocol for propagating this plant.

## 2. Materials and Methods

This experiment was carried out at the Tissue Culture and Biotechnology Lab, Department of Horticultural Science, College of Agriculture, Shiraz University.

*Sterilization stage and effects of antioxidants*

Numerous shoot explants of *D. sanderiana* (both cultivars) measuring 3-4 cm long were removed from the mother plants and placed in a weak detergent solution (about 0.2%) for 20 min. To prevent the formation of polyphenols and the occurrence of browning, the explants were exposed to citric acid and ascorbic acid at 50 and 100 mg $l^{-1}$, respectively, and an integrated combination, for 30 min. They were then washed with running tap water for 20 min, followed by surface sterilization with 70% ethanol for 3 min plus 10% or 15% Clorox (a commercial bleach, containing 5.25% sodium hypochlorite) with 3-5 drops of Tween-20 for 10, 15, and 20 min. They were then rinsed three times with sterilized distilled water.

*Culture media*

Murashige and Skoog's (1962) (MS) medium was used for the establishment stage supplemented with 30 g $l^{-1}$ sucrose and 8 g $l^{-1}$ agar. The pH was adjusted to 5.8 before the addition of agar, then 40 ml of the culture medium were poured into 40 ml culture jars and autoclaved at 121ºC at a pressure of 1.5 kg $cm^{-2}$ for 20 min using the following media: full strength MS medium and 1/2 strength MS medium. All the chemicals were purchased from Sigma-Aldrich authorized distributer, Kimia Gostar Pooyesh Co., Ltd., Tehran, Iran.

*Shoot proliferation and plant regeneration*

Half strengths MS media supplemented with different concentrations of BA (1, 2, 3, 4 and 5 mg $l^{-1}$) and NAA (0.25 and 0.5 mg $l^{-1}$) were used for shoot proliferation and plant regeneration of both *Dracaena* cultivars. Also, the effect of the direction of explant positioning on media was studied to evaluate the proliferation and growth rate of the produced shoots.

*Data recording and analysis*

All the main experiments were conducted in a completely randomized design with eight replicates; the experiments were repeated at least two times. Data were statistically analyzed and the means were calculated using SPSS (version 15) software whereby means were compared using LSD test at 5% level.

## 3. Results and Discussion

*Sterilization stage*

Results indicated that the use of Clorox significantly affected explant contamination (Table 1). Increasing Clorox concentration increased the decontaminated explants in *D. sanderiana*. The highest mean value of explant decontamination (0%) was obtained when applying 15% (v $v^{-1}$) of Clorox for 20 min.

An optimum value of healthy explants, free of contamination, could be obtained by using Clorox at 15%, whereas the higher concentration had a negative effect on this characteristic. Our results are consistent with those obtained by Kunisaki (1975) on *Cordylin terminalis* (L.) Kunth, Chua *et al*. (1981) on *D. marginata* Link, Sagawa and Kunisaki (1990) on *Dracaena* sp., and Badawy *et al*. (2005) on *D. fragrans* (L.) Ker Gawl. cv. 'Massangeana'.

Table 1 - Effect of different concentrations of Clorox on surface sterilization of explants of *Dracaena sanderiana* Sander ex Mast.

| Clorox concentrations (%) | Time (min) | Contamination (%) |
|---|---|---|
| Control (0) | 0 | 100.00 a [z] |
| | 10 | 43.75 b |
| 10 | 15 | 37.50 bc |
| | 20 | 18.75 bcd |
| | 10 | 18.75bcd |
| 15 | 15 | 6.25 cd |
| | 20 | 0.00 d |

[z] Means followed by the same letter(s) are not significantly different using LSD test at 5% level.

*Effects of antioxidants*

Antioxidants were used to reduce browning in explants. After placing the explants in a weak detergent solution (about 0.2%), they were placed in a solution containing the concentrations of 0, 50 and 100 mg $l^{-1}$ ascorbic acid and citric acid separately and in combination for at least 30 min.

The data presented in Table 2 clearly show that both antioxidants significantly prevented browning in cultured explants of *D. sanderiana*. The highest mean of explants saved from browning was recorded when 100 mg $l^{-1}$ of both ascorbic acid and citric acid were mixed and used in combined form. The highest browning frequency and intensity belonged to the control. The use of 100 mg $l^{-1}$ ascorbic acid, significantly reduced the browning of explants compared with utilization of the same amount of citric acid. In a study conducted by Huang *et al*. (2002) it was observed that PPO (polyphenol oxidase) enzyme activity re-

Table 2 - Effect of different antioxidants on browning rate of *Dracaena sanderiana* Sander ex Mast.

| Antioxidants concentrations (mg $l^{-1}$) | Browning [z] |
|---|---|
| Control (0) | 8.60 a [y] |
| As50 | 5.20 bc |
| Ci50 | 5.80 b |
| As100 | 3.80 cd |
| Ci100 | 5.20 bc |
| As50+Ci50 | 3.60 d |
| As100+Ci100 | 2.20 e |

[z] 10 the highest rate of browning and 1 the lowest rate of browning.
[y] Means followed by the same letter(s) are not significantly different using LSD test at 5% level.
As= Ascorbic acid; Ci= Citric acid.

mains stable at pH 10 and strongly acts against browning; the use of ascorbic acid was to prevent the occurrence of browning. Elmore *et al*. (1990) also used ascorbic acid as an antioxidant and anti-browning substance in the culture medium for plant cells, tissues, and organs.

*Shoot proliferation rate in horizontal and vertical explants: Green cultivar*

Table 3 shows the difference between the proliferation rate of horizontal and vertical explants of *D. sanderiana* 'Green' after 60 days of deployment following treatment with concentrations of 1, 2, 3, 4 and 5 mg $l^{-1}$ BA, and 0.25 and 0.5 mg $l^{-1}$ NAA. According to the results, the direction of explant positioning in the culture medium, either horizontal or vertical, had significant effects on successful establishment and proliferation rates. The highest mean value (4.8) was recorded when using 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA. Generally, explants showed a good response to this BA concentration to the extent that some cultures produced up to 10 shoots. However, regardless of growth regulator concentration, proliferation failed to occur on vertical explants (Table 3, Fig. 1).

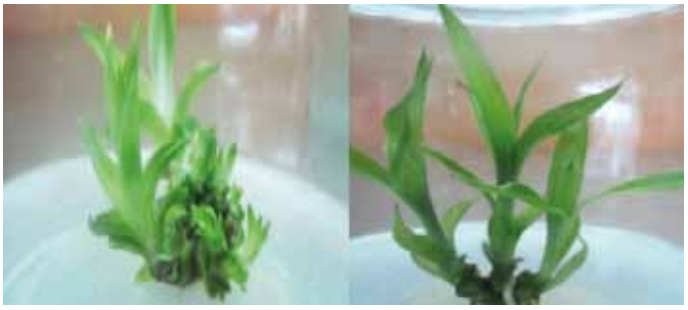

Fig. 1 - Rate of shoot proliferation in Dracaena 'Green' cultured horizontally on 1/2 MS medium with mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA- 40 days after culture (left) and 60 days after culture (right).

The highest average length of shoots in horizontally cultured explants of 'Green' was observed when BA and NAA were utilized at concentrations of 1 mg $l^{-1}$ and 0.5 mg $l^{-1}$, respectively. This resulted in the production of shoots measuring 3.25 cm long. Furthermore, shoots were successfully produced measuring 3.05 cm long when BA and NAA were used at 3 mg $l^{-1}$ and 0.5 mg $l^{-1}$, respectively. The greatest average length of shoots achieved in vertically cultured explants of 'Green' was 4.12 cm when 2 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ NAA were used (Table 4).

Table 3 - The interaction effect of different concentrations of BA and NAA, and exposure position of explants on the proliferation rate of 'Green'

| Treatments (mg $l^{-1}$) | | Average number of shoots | | Mean |
|---|---|---|---|---|
| | | Exposure position of explant | | |
| BA | NAA | Horizontal | Vertical | |
| 0 | 0 | 1.00 c [z] | 1.00 c | 1.00 B |
| 1 | 0.25 | 1.20 bc | 1.00 c | 1.11 B |
| | 0.50 | 1.40 bc | 1.00 c | 1.22 B |
| 2 | 0.25 | 4.80 a | 1.00 c | 3.11 A |
| | 0.50 | 1.60 bc | 1.00 c | 1.33 B |
| 3 | 0.25 | 2.20 b | 1.00 c | 1.66 B |
| | 0.50 | 1.60 bc | 1.00 c | 1.33 B |
| 4 | 0.25 | 1.00 c | 1.00 c | 1.00 B |
| | 0.50 | 1.40 bc | 1.00 c | 1.22 B |
| 5 | 0.25 | 1.20 bc | 1.00 c | 1.11 B |
| | 0.50 | 1.60 bc | 1.00 c | 1.33 B |
| Mean | | 1.72 A | 1.00 B | |

[z] Data followed by the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level.

Table 4 - The interaction effect of different concentrations of BA and NAA, and exposure position of explants on the length of shoots in 'Green'

| Treatments (mg $l^{-1}$) | | Average length of shoots (cm) | | Mean |
|---|---|---|---|---|
| | | Exposure position of explant | | |
| BA | NAA | Horizontal | Vertical | |
| 0 | 0 | 2.40 b [z] | 1.88 b | 2.16 AB |
| 1 | 0.25 | 1.95 b | 2.30 ab | 1.91 B |
| | 0.50 | 3.25 ab | 2.62 b | 2.40 AB |
| 2 | 0.25 | 2.30 b | 2.25 b | 2.80 AB |
| | 0.50 | 2.35 b | 4.12 a | 3.14 A |
| 3 | 0.25 | 2.96 ab | 2.88 ab | 2.92 AB |
| | 0.50 | 3.05 ab | 2.00 b | 2.27 AB |
| 4 | 0.25 | 2.70 b | 2.12 b | 2.40 AB |
| | 0.50 | 2.70 b | 3.00 ab | 2.83 AB |
| 5 | 0.25 | 2.50 b | 2.25 b | 2.7 AB |
| | 0.50 | 2.40 b | 2.62 b | 2.50 AB |
| Mean | | 2.59 A | 2.51 A | |

[z] Data followed by the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level.

Average leaf number in horizontally cultured explants of 'Green' showed no significant difference in any of the treatments used, but in vertically cultured explants the greatest number of leaves (5.75) was obtained with 2 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ NAA (Table 5).

Table 5 - The interaction effect of different concentrations of BA and NAA, and exposure position of explants on the average leaf number in 'Green'

| Treatments (mg $l^{-1}$) | | Average number of leaves (cm) | | Mean |
|---|---|---|---|---|
| | | Exposure position of explant | | |
| BA | NAA | Horizontal | Vertical | |
| 0 | 0 | 3.80 b [z] | 4.00 b | 3.88 AB |
| 1 | 0.25 | 3.70 b | 4.75 ab | 4.16 AB |
| | 0.50 | 4.30 ab | 3.50 b | 3.94 AB |
| 2 | 0.25 | 4.45 a | 4.25 ab | 5.02 A |
| | 0.50 | 3.90 b | 5.75 a | 4.05 AB |
| 3 | 0.25 | 4.46 ab | 4.75 ab | 4.60 AB |
| | 0.50 | 4.30 ab | 3.25 b | 3.83 AB |
| 4 | 0.25 | 4.00 b | 3.50 b | 3.77 B |
| | 0.50 | 4.50 ab | 3.75 b | 4.16 AB |
| 5 | 0.25 | 4.20 b | 4.00 b | 4.11 AB |
| | 0.50 | 3.80 b | 4.25 ab | 4.00 AB |
| Mean | | 4.12 A | 4.15 A | |

[z] Data followed by the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level.

With increasing concentration of growth regulators, deformity was observed in most of the shoots produced. Shoots created from horizontally cultured explants of 'Green', at a concentration of 4 mg $l^{-1}$ BA, showed the greatest deformity and deviated from their natural state. The least deformity was observed in the control and 2 mg $l^{-1}$ BA treatments. The level of deformity in shoots obtained from vertically cultured explants of 'Green' was also higher at 5 mg $l^{-1}$ BA. Deformity was not detected at low growth regulator concentrations and in the control explants (Table 6).

*Shoot proliferation rate in horizontal and vertical explants: variegated cultivar*

Table 7 shows the difference between the proliferation rate of horizontally and vertically cultured explants of *D. sanderiana* 'Variegated' after 60 days of deployment with the concentrations of 1, 2, 3, 4 and 5 mg $l^{-1}$ BA, and 0.25 and 0.5 mg $l^{-1}$ NAA. According to the results, the direction of explant position in culture medium had a significant effect on proliferation rate: horizontally cultured explants showed a greater shoot proliferation rate. The highest mean value (3.66) was recorded when using 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA (Table 7, Fig. 2).

Table 6 - The interaction effect of different concentrations of BA and NAA, and exposure position of explants on deformity in 'Green'

| Treatments (mg $l^{-1}$) | | Deformity [z] | | Mean |
|---|---|---|---|---|
| | | Exposure position of explant | | |
| BA | NAA | Horizontal | Vertical | |
| 0 | 0 | 0 g [y] | 0 g | 0 E |
| 1 | 0.25 | 1.00 d-g | 0 g | 0.55 DE |
| | 0.50 | 1.00d-g | 0.50 fg | 0.77 DE |
| 2 | 0.25 | 1.20 d-g | 0.75efg | 1.00 CD |
| | 0.50 | 0.80 efg | 0.62 fg | 0.72 DE |
| 3 | 0.25 | 1.60 def | 1.50 def | 1.55 C |
| | 0.50 | 3.00 abc | 1.87cde | 2.50 B |
| 4 | 0.25 | 2.20 bcd | 2.75abc | 2.40 B |
| | 0.50 | 3.70 a | 3.40 a | 3.61 A |
| 5 | 0.25 | 3.20 ab | 3.50 a | 3.33 A |
| | 0.50 | 3.50 a | 3.50 a | 3.50 A |
| Mean | | 1.96 A | 1.67 B | |

[z] 5= Highest deformity and 1= Lowest deformity.

[y] Data followed by the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level.

Table 7 - The interaction effect of different concentrations of BA and NAA, and exposure position of explants on the shoot proliferation rate in 'Variegated'

| Treatments (mg $l^{-1}$) | | Average number of shoots | | Mean |
|---|---|---|---|---|
| | | Exposure position of explant | | |
| BA | NAA | Horizontal | Vertical | |
| 0 | 0 | 1.00 c [z] | 1.00 c | 1.00 B |
| 1 | 0.25 | 1.00 c | 1.00 c | 1.00 B |
| | 0.50 | 1.00 c | 1.00 c | 1.00 B |
| 2 | 0.25 | 3.66 a | 1.00 c | 2.33 A |
| | 0.50 | 1.00 c | 1.00 c | 1.00 B |
| 3 | 0.25 | 2.00 b | 1.00 c | 1.5 AB |
| | 0.50 | 2.00 b | 1.00 c | 1.5 AB |
| 4 | 0.25 | 1.00 c | 1.00 c | 1.00 B |
| | 0.50 | 1.00 c | 1.00 c | 1.00 B |
| 5 | 0.25 | 1.00 c | 1.00 c | 1.00 B |
| | 0.50 | 1.00 c | 1.00 c | 1.00 B |
| Mean | | 1.42 A | 1.00 B | |

[z] Data followed by the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level.

When explants were treated with 1 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ NAA, the average length of shoots in vertically cultured explants of 'Variegated' measured 3.00 cm more than other treatments.

The average length of shoots in vertically cultured explants of 'Variegated' was the highest with 2 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ NAA. The average length was 2.83 cm (Table 8).

Five leaves were obtained on average with the use of 1 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ NAA. This average was higher

Fig. 2 - Rate of shoot proliferation in Dracaena 'Variegated' cultured horizontally on 1/2 MS medium with 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA- 55 days after culture (left) and on 1/2 MS medium with 3 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA 70 days after culture (right).

Table 8 - The interaction effect of different concentrations of BA and NAA, and exposure position of explants on the length of shoots in 'Variegated'

| Treatments (mg $l^{-1}$) | | Average lenght of shoots (cm) | | Mean |
|---|---|---|---|---|
| | | Exposure position of explant | | |
| BA | NAA | Horizontal | Vertical | |
| 0 | 0 | 1.00 f [z] | 1.33 def | 1.16 CD |
| 1 | 0.25 | 1.00 f | 1.00 f | 1.00 D |
| | 0.50 | 3.00 a | 1.33 def | 2.16 AB |
| 2 | 0.25 | 1.50 c-f | 2.16 a-e | 1.83 A-D |
| | 0.50 | 2.33 a-d | 2.83 ab | 2.58 A |
| 3 | 0.25 | 1.63 c-f | 2.33 a-d | 1.98 AB |
| | 0.50 | 2.00 a-f | 1.33 def | 1.66 BCD |
| 4 | 0.25 | 1.16 ef | 2.00 a-f | 1.58 BCD |
| | 0.50 | 1.83 b-f | 1.16 ef | 1.50 BCD |
| 5 | 0.25 | 1.33 def | 1.66 c-f | 1.50 BCD |
| | 0.50 | 1.16 ef | 2.50 abc | 1.83 A-D |
| Mean | | 1.63 A | 1.70 A | |

[z] Data followed by the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level.

than other treatments. The average leaf number in vertically cultured explants of 'Variegated' was 5.66 when 3 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA were used (Table 9).

With increasing concentration of growth regulators, deformity was observed in most shoots produced. Shoots created from horizontally cultured explants of 'Variegated', resulting from 5 mg $l^{-1}$ BA with 0.25 and 0.5 mg $l^{-1}$ NAA, showed the greatest deformity and were deviated from their natural state. Deformity in shoots obtained from vertical explants of 'Variegated' was also higher at concentrations of 5 mg $l^{-1}$ BA and 0.25 or 0.5 mg $l^{-1}$ NAA; deformity was absent in low growth regulator concentrations and in control explants (Table 10).

Successful growth of explants under controlled conditions is a function of the proper combination of nutrients, especially compounds controlling growth. Because of the small size of the explants used, synthesis and production of these compounds is greatly needed for organ growth and development. Therefore, in order to achieve maximum growth rate, it is necessary to use plant hormones in appro-

Table 9 - The interaction effect of different concentrations of BA and NAA, and exposure position of explants on the average leaf number in 'Variegated'

| Treatments (mg $l^{-1}$) | | Average number of leaves | | Mean |
|---|---|---|---|---|
| | | Exposure position of explant | | |
| BA | NAA | Horizontal | Vertical | |
| 0 | 0 | 3.25 c [z] | 3.00 c | 3.12 B |
| 1 | 0.25 | 3.66 bc | 3.00 c | 3.33 B |
| | 0.50 | 5.00 ab | 3.00 c | 4.00 AB |
| 2 | 0.25 | 3.25 c | 3.50 bc | 3.45 AB |
| | 0.50 | 3.00 c | 3.66 bc | 3.33 B |
| 3 | 0.25 | 3.41 bc | 5.66 a | 4.53A |
| | 0.50 | 3.66 bc | 3.00 c | 3.33 B |
| 4 | 0.25 | 3.33 bc | 3.50 bc | 3.50 AB |
| | 0.50 | 3.33 bc | 3.00 c | 3.16 B |
| 5 | 0.25 | 3.33 bc | 3.00 bc | 3.33 B |
| | 0.50 | 3.66 bc | 4.00 bc | 3.83 AB |
| Mean | | 3.53 A | 3.42 A | |

[z] Data followed by the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level.

Table 10 - The interaction effect of different concentrations of BA and NAA, and exposure position of explants on deformity in 'Variegated'

| Treatments (mg $l^{-1}$) | | Deformity [z] | | Mean |
|---|---|---|---|---|
| | | Exposure position of explant | | |
| BA | NAA | Horizontal | Vertical | |
| 0 | 0 | 0 e [Y] | 0 e | 0.00 E |
| 1 | 0.25 | 0.33 de | 1.00 cde | 0.66 DE |
| | 0.50 | 1.00 cde | 1.25 cd | 1.16 CD |
| 2 | 0.25 | 1.33 cd | 1.50 bc | 1.41 BCD |
| | 0.50 | 1.33 cd | 1.50 bc | 1.41 BCD |
| 3 | 0.25 | 1.40 cd | 1.66 bc | 1.53 BCD |
| | 0.50 | 1.33 cd | 1.66 bc | 1.50 BCD |
| 4 | 0.25 | 2.00 bc | 1.80 bc | 1.90 BC |
| | 0.50 | 1.66 bc | 2.50 abc | 2.08 BC |
| 5 | 0.25 | 3.33 a | 3.25 a | 3.29 A |
| | 0.50 | 3.40 a | 3.66 a | 3.53 A |
| Mean | | 1.52 B | 1.90 A | |

[z] 5 Highest deformity and 1 Lowest deformity.
[Y] Data followed by the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level

priate concentrations. Having this goal necessitates the use of different hormones responsible for growth stimulation (Hu and Wang, 1983). The increase in shoot proliferation rate is partly due to the increase in BA concentration. This can be interpreted through the rate of cytokinins role in stimulating cell division and growth of lateral buds. Furthermore, shoot proliferation culture media that is rich in cytokinins often results in lateral buds that have been released from terminal bud dominance (Taji *et al*., 1997). Explants that were taken from young stems of *D. sanderiana*, as compared to older explants, showed greater shoot production in the media used. The results of this experiment were in agreement with those of Tian Lang *et al*. (1999) on *D. sanderiana* cv. Virescens. They found that the use of 3 to 3.5 mg $l^{-1}$ BA with 0.02 mg $l^{-1}$ NAA yields the highest shoot proliferation rate in this species. Atta-Alla *et al*. (1996) and El-Sawy *et al*. (2000) on *D. marginata* cv. Tricolor showed the highest shoot proliferation rate on MS medium containing 4 mg $l^{-1}$ BA and 0.05 mg $l^{-1}$ NAA. Moreover, our results were more or less the same as those of Maheran *et al*. (1996) on *D. fragrans* 'Massangeana' and Ying *et al*. (2008) on *D. cambodiana* Pierre ex Gagnep.

## 4. Conclusions

Explants of the 'Green' cultivar, cultured horizontally on media, were successful in yielding proliferated shoots. The highest mean value (4.8) was recorded on the medium supplemented with 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA. Explants of the 'Variegated' cultivar, cultured horizontally on media, were also successful in yielding proliferated shoots. The highest mean value (3.66) was recorded on the medium supplemented with 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA.

## References


ATTA-ALLA H., ZAGHLOUL M., WALY A.K., KHATTAB S.H., 1996 - *Micropropagation of some ornamental plants. 3.* In vitro *culture and establishment of* Dracaena marginata *var. 'Tricolor'*. - J. Agric. Sci., 34: 1153-1162.

BADAWY E.M., HABIB A.M.A., BANA A.E., YOSRY G.M., 2005 - *Propagation of* Dracaena fragrans *plants by tissue culture technique*. - Arab J. Biotech., 8: 329-342.

CHUA B.U., KUNISAKI J.T., SAGAWA Y., 1981 - In vitro *propagation of* Dracaena marginata *'Tricolory'*. - Hort. Sci., 16: 494-498.

ELMORE H., SAMPLES B., SHARMA S., HARRISON M., 1990 - *Influence of cultural and physiochemical factors on ascorbate stability in plant tissue culture media*. - Plant Cell Tissue Organ Culture, 20: 131-135.

EL-SAWY A., BEKHEE S.A., HOSSNY Y.A., 2000 - *A protocol for micropropagtion of* Dracaena marginata. - Egypt. J. Hort., 27: 29-40.

HU C.Y., WANG P.J., 1983 - *Meristem shoot tip and bud culture*, pp. 177-227. - In: EVANS D.A., W.R. SHARP, P.V. AMMIRATO, and Y. YAMADA (eds.) *Handbook of plant cell culture*. - Macmillan Publishing Company, NY, USA, pp. 833.

HUANG L.C., LEE Y.L., HUANG B.L., KOU C.I., SHAW J.F., 2002 - *High polyphenol oxidase activity and low titratable acidity in browning bamboo tissue culture*. - In Vitro Cell Dev. Biol. - Plant, 38: 358-365.

JUNAID A., MUJIB A., SHARMA M.P., 2008 - *Effect of growth regulators and ethylmethane sulphonate on growth, and cholorophyll, sugar and proline contents in* Dracaena sanderiana *cultured* in vitro. - J. Plant Biol., 52: 569-572.

KOBZA F., VACHUNOVA J., 1989 - *Propagation of Dracaena* in vitro. *I. Propagation of* Dracaena deremensis. - Acta University Agric. Faculty of Hort., 4: 33-37.

KOBZA F., VACHUNOVA J., 1991 - *Propagation of Dracaena* in vitro. *II. Propagation of* Dracaena concina *Kunth*. - Acta University Agric. Faculty of Hort., 6: 51-55.

KUNISAKI J.T., 1975 - In vitro *propagation of* Cordyline terminalis *L. Kunth*. - Hort. Sci., 10: 601-602.

LEFFRING L., VONKK C.R., RIBOT S.A., 1985 - *Hormonal effects on shoot formation of Cordyline cultivars*. - Acta botanica Neerlandica, 34: 238-245.

MAHERAN A.A., OOI H.L., RASHID A.A., 1996 - In vitro *responses of* Dracaena fragrans *massangeana to growth regulators*. - Pertanika J. Trop. Agric. Sci., 19: 123-127

MURASHIGE T., SKOOG F., 1962 - *A revised medium for rapid growth and bioassays with tobacco tissue cultures.* - Physiol. Plant, 15: 473-497.

MUTHUSAMY A., VASANTH K., SIVASANKARI D., CHANDRASEKAR B.R., JAYABALAN N., 2007 - *Effects of mutagens on somatic embryogenesis and plant regeneration in groundnut*. - J. Plant Biol., 51: 430-435.

PAEK KY., OH M.O., CHOI J.K., 1985 - *Mass propagation of Cordyline and Scindapsus* in vitro. - Korean J. Hortic. Sci., 26: 83-92.

PREDIERI S., 2001 - *Mutation induction and tissue culture in improving fruits*. - Plant Cell Tissue Organ Culture, 64: 185-210.

SAGAWA Y., KUNISAKI J.T., 1990 - *Micropropagation of floricultural crops*, pp. 25-34. - In: EVANS D.A., W.R. SHARP, P.V. AMMIRATO, and Y. YAMADA (eds.) *Handbook of plant cell culture*. - Macmillan Publishing Company, NY, USA, pp. 833.

TAJI A.M., DODD W.A., WILLIMS R.R., 1997 - *Plant tissue culture practices*. - UNE Press, Australia, 3rd Edition, pp. 71.

TIAN-LANG T.H., ZHANG-LIN T.L., TAN H.Y., ZHANG L., 1999 - *Stem segment culture and tube propagation of* Dracaena sanderiana *cv. Virscens*. - Acta Hort. Sin., 26: 133-134.

YING X., BAO-LING H., CHENG-QUN L., YIN-PING Z., CHUANMING Z., YING L., 2008 - *Rapid propagation of* Dracaena cambodiana in vitro. - J. Anhui Agric. Sci., 32: 112-120.

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# Factors affecting *in vitro* propagation of *Dracaena sanderiana* Sander ex Mast. cultivars. II. MS salt strengths, subculturing times, rooting and acclimatization

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*Key words:* acclimatization, *Dracaena*, MS salts strength, rooting, subculture.

**Abstract: The interaction effect of MS salt strengths and subculturing times were studied in *Dracaena sanderiana* Sander ex Mast. (lucky bomboo). Stem pieces, each bearing a single node, were cultured horizontally on full, 3/4 and 1/2 strength MS media supplemented with 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA. Proliferation rate, shoot length, and number of leaves increased when 1/2 strength MS medium was used. The highest shoot length was recorded after the third subculture. Moreover, the greatest number of leaves was obtained at this stage. The highest mean value for parameters such as rooting percentage, the number of roots, and root length were observed on media supplemented with 2 mg $l^{-1}$ Indole-3-butyric acid (IBA). Plantlets were subsequently planted in a mixture of perlite and vermiculite (1:1) under 95% relative humidity and were then transferred to greenhouse conditions. The proposed protocol can be used in commercial mass production of *D. sanderiana*.**

## 1. Introduction

Dracaenas rank second in Europe (Vonk Noordegraaf, 1998) and third in the United States (U.S. Department of Agriculture, 1999) as popular foliage plants used for interiorscaping. Dracaenas are also rich in steroidal sapogenins and saponins (Mimaki *et al*., 1998, 1999; Yokoduk *et al*., 2000), some of which have cytotoxic activities against cultural tumor cells (Mimaki *et al*., 1999), making them an important group of plants for pharmacognosy research.

Dracaenas as ornamental plants are propagated through stem cuttings, which are predominantly imported from Central America, but imported cuttings may carry and spread pathogens and pests (Palm and Rossman, 2003; Prado *et al*., 2008). For example, an invasive pathotype of *Ralstonia solanacearum* race 1 was identified from eye cuttings of golden pothos imported from Costa Rica to Florida (Norman and Yuen, 1998). Childers and Rodrigues (2005) reported some pest mite species on ornamental plants imported to the US from Central America. Mass propagation through seeds has many limitations like seed dormancy, low rate of germination, and progeny variation in other plant species (Venkataramaiah *et al*., 1980; Chand and Singh, 2004). To overcome these problems and fulfill the required demand has necessitated restoring the productivity of plants through the use of plant tissue culture techniques (Bhattacharjee, 2006) such as *in vitro* micropropagation which includes the rapid vegetative multiplication of valuable plant material for agriculture and forestry. In addition, the *in vitro* technique is also widely used in the commercial field for the micropropagation of ornamental plants in large numbers; however, the process is regulated by the biochemical reserve localized in specific organs (Thorpe, 1990; Bhattacharjee, 2006).

The objective of the present investigation was to study different aspects of micropropagation of *D. sanderiana* (lucky bomboo) and factors affecting its multiplication and rooting, and subsequent acclimatization to propose a commercial protocol for propagation of this plant.

## 2. Materials and Methods

The experiment was carried out at the Tissue Culture and Biotechnology Lab, Department of Horticultural Science, College of Agriculture, Shiraz University.

*Effects of MS salt strength and number of subcultures*

Green cultivar stem segments, bearing a node, were cultured horizontally on full MS, 3/4 MS and 1/2 MS media supplemented with 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA. The case of subculture times was also assessed. Leaf and shoot numbers and the average length of shoots (cm) were recorded.

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*Rooting*

The experiment was conducted to study the effects of different concentrations of supplementary IBA (0, 1, 2, 3 mg $l^{-1}$) in MS medium on the rooting of both cultivars of *D. sanderiana*. All treatments contained 30 g $l^{-1}$ sucrose and 8.0 g $l^{-1}$ agar in the media used. The pH was adjusted to 5.8 before the addition of agar, then the culture media were poured into 40 ml culture jars and autoclaved at 121ºC at a pressure of 1.5 kg $cm^{-2}$ for 20 min. Shoots were incubated at 25±2ºC under 16/8 (light/dark) photoperiod. The light intensity was 30 µm $m^{-2}$ $s^{-1}$ provided by cool white fluorescent lamps.

*Transfer and adaptation*

Plantlets were removed from the culture media; roots were washed with distilled water. They were then cultured in a sterilized mixture of 1:1 perlite and vermiculite (v $v^{-1}$) inside 5 cm pots covered with plastic bags and maintained at a relative humidity of about 95% to later initiate compatibility with field conditions. Plastic bags were gradually removed to allow steady acclimatization with lower humidity levels. After two months, they were transferred to the greenhouse where more compatibility was expected to be observed. For better growth, plants were fed with Crystalon fertilizer (0.5 mg $l^{-1}$, once every 15 days).

## 3. Results and Discussion

*Effects of MS salt strength, number of subcultures, and number of shoots*

From the data obtained for the number of shoots (Table 1), the greatest mean value (2.00) was recorded when using 1/2 compared to 3/4 and full strength MS media, which resulted in 1.6 and 1.26 shoots, respectively. Proliferation rates decreased in subsequent subcultures: 1.8 and 1.2 shoots after the second and third subcultures, respectively. Furthermore, the comparison of subcultures derived from full strength MS medium revealed that the first and second subcultures yielded 1.4 shoots, whereas the third subculture yielded one shoot, respectively. Probably, the amount of internal tissue hormones have altered with time.

*Shoot length*

The data presented in Table 1 show the effect of MS salt strength, i.e. full, 3/4 and 1/2 strength, on shoot length of cultured *D. sanderiana*. The longest shoot (3.33 cm) was measured in explants cultured on 1/2 MS; full MS medium gave 2.6 cm and 3/4 MS medium gave 2.5 cm shoot length. With regard to the effect of the interaction between concentrations of MS salts and the number of subcultures on shoot length, it was found that the highest value for shoot length (4.10 cm) was obtained by using 1/2 MS medium after the third subculture, as compared with that obtained from using full MS medium after the first subculture, which gave the lowest value (2.20 cm shoot length). The results reveal that as the salt concentration of MS medium decreases the proliferation and shoot growth rates increase.

Moreover, as the number of subcultures increased, more positive values for shoot length were obtained which may be attributed to the explants' freshness and adequate nutrient supply. The presence of vitamins and growth regulators were partly responsible for growth enhancement.

*Number of leaves*

The data obtained for the number of leaves (Table 1) shows that the greatest mean value (4.86) was recorded when using 1/2 MS; 3/4 and full strength MS media produced 3.93 and 4.06 leaves, respectively.

The interaction between MS salt strength and the number of subcultures significantly affected the number of leaves produced; i.e. the highest mean value (6.20) was obtained using 1/2 MS after three subcultures, as compared to 3/4 MS medium, regardless of the subculture stages, which gave 3.80 leaves after the first, and 4.00 leaves after the second and third subcultures. These results revealed that the MS salts at full strength were not effective in enhancing the initiation and formation of leaf primordia as compared to the lower concentrations. However, a positive impact was observed when subculturing was done again and again; in other words, the third subculture yielded more new leaves (Fig. 1).

*Rooting*

The data presented in Table 2 and figure 2 clearly show the effects of IBA concentration on the number of roots

Table 1 - The interaction effect of MS salt strength and subculture number on shoot length, number of leaves, and number of shoots during multiplication stage of *Dracaena sanderiana* Sander ex Mast.

| MS strength | Number of shoots | | | | Shoot length (cm) | | | | Number of leaves | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sub. 1 | Sub. 2 | Sub. 3 | Mean | Sub. 1 | Sub. 2 | Sub. 3 | Mean | Sub. 1 | Sub. 2 | Sub. 3 | Mean |
| Full MS | 1.40 b [(z)] | 1.40 b | 1.00b | 1.26 B | 2.20 c | 2.50 bc | 3.10 b | 2.60 B | 3.60 c | 4.00 bc | 4.60 b | 4.06 B |
| ¾ MS | 1.80 b | 1.50 b | 1.50b | 1.60 AB | 2.00 c | 2.50 bc | 3.00 b | 2.50 B | 3.80 bc | 4.00 bc | 4.00 bc | 3.93 B |
| ½ MS | 3.00 a | 1.80 b | 1.20b | 2.00 B | 2.70 bc | 3.20 b | 4.10 a | 3.33 A | 4.00 bc | 4.40 bc | 6.20 a | 4.86 A |
| Mean | 2.06 A | 1.56 B | 1.23 B | | 2.30 B | 2.63 B | 3.40 A | | 3.80 B | 4.13 B | 4.93 A | |

[(z)] Data followed with the same letter(s) (lower letters for interactions and capital letters for factor means) are not significantly different using LSD test at 5% level.

A. First subculture B. Second subculture C. Third subculture

Fig. 1 - Proliferation rate of *D. sanderiana* 'green' explants on 1/2 MS medium at different subcultures.

and root length of cultured *D. sanderiana*. It was found that using IBA at different concentrations significantly affected percentage of rooting. The highest percentage of rooting was observed at a concentration of 2 mg $l^{-1}$ IBA (81.25%). The highest mean value for root length (3.36 cm) was achieved when using 2 mg $l^{-1}$ IBA as compared with that of untreated explants (1.00 cm).The highest mean value for the number of roots (5.00) was obtained with 2 mg $l^{-1}$ IBA as compared with the control explants which gave the lowest mean number of roots (1.75).

These results are in agreement with those obtained by Paek *et al*. (1985) on *Cordyline*. They concluded that using 2.0 or 3.0 mg $l^{-1}$ IBA resulted in more success in the rooting stage. Debergh (1975, 1976) succeeded in achieving a 100% rooting percentage in *D. deremensis* Engl. and *D. fragrans* when using 2 mg $l^{-1}$ IBA. Badawy *et al*. (2005) obtained the greatest root numbers and length in *D. fragrans* 'Massangeana' with 1/2 MS medium supplemented with 0.5 mg $l^{-1}$ IBA.

*Transfer and adaptation of 'Green' plants*

A month after deployment in the rooting medium, the elongated and well-rooted plantlets were transferred to an equal ratio of sterilized perlite and vermiculite (v $v^{-1}$). The greatest average length of shoots after transferring to the pots and adaptability to environmental conditions was observed in treatments of 3 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA and 2 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ NAA, i.e. 6.75 and 5.80 cm, respectively.

Concentrations of 1 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA produced the highest number of leaves (8.80) compared to other treatments. Average stem diameter was greatest (5.17 mm) at the concentration of 2 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ NAA. Chlorophyll content in the treatments with 2 mg $l^{-1}$ BA and

Table 2 - Effect of different concentrations of IBA on rooting of *Dracaena sanderiana*

| IBA (mg $l^{-1}$) | Rooting percentage | Average root length (cm) | Number of roots |
|---|---|---|---|
| Control | 15.00 d [(z)] | 1.00 c | 1.75 c |
| 1 | 31.25 c | 1.58 bc | 2.25 c |
| 2 | 81.25 a | 3.36 a | 5.00 a |
| 3 | 57.50 b | 2.85 ab | 3.25 b |

[(z)] In each column, means followed by the same letter(s) are not significantly different using LSD test at 5% level.

Fig. 2 - Shoots rooted on ½ MS medium containing 2 mg $l^{-1}$ IBA, 30 days after culture.

0.25 and 0.5 mg $l^{-1}$ NAA was 43.94 and 52.16 mg $g^{-1}$ FW, respectively, which was higher than other treatments (Table 3, Fig. 3).

*Transfer and adaptation of 'Variegated' plants*

In transferred plantlets, the greatest shoot length (3.83 cm) was observed in the treatment with 2 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA, which was not significantly different from many other treatments. The highest average number of leaves was obtained in the treatment with 1 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ NAA. The stem diameter in transferred plantlets after treatment with 4 mg $l^{-1}$ BA and 0.25 mg $l^{-1}$ NAA was higher (3.56) than the other treatments. Chlorophyll content was the highest with 3 mg $l^{-1}$ BA and 0.25 and 0.5 mg $l^{-1}$ NAA, i.e. 33 and 29.5 mg $g^{-1}$ FW, respectively (Table 4, Fig. 4).

Most plantlets were adapted, had normal growth, and 70% of them survived. The obtained results agree with the findings of Beura *et al*. (2006) on *D. sanderiana,* Debergh and Maene (1981) on *D. deremensi*s Engl, and Ying *et al*. (2008) on *D. cambodiana* Pierre ex Gagnep.

Junaid *et al.* (2013) reported the micropropagation of only one cultivar of *D. sanderiana.* However, in the present study, high plant regeneration was achieved and this is the first report on mass production of two cultivars of *D. sanderiana.* The proposed protocol can be used in commercial mass propagation of this plant.

## 4. Conclusions

The highest mean value for parameters such as rooting percentage, the number of roots, and root length were observed on media supplemented with 2 mg $l^{-1}$. Plantlets were subsequently planted in a mixture of perlite and vermiculite (1:1) under 95% relative humidity and were then trans-

Table 3 - Effects of different treatments on some characteristics of acclimatized 'Green' plantlets, 60 days after transfer

| BA (mg $l^{-1}$) | NAA (mg $l^{-1}$) | Mean length of shoots (cm) | Mean number of leaves | Mean stem diameter (mm) | Mean chlorophyll content (mg $g^{-1}$ FW) |
|---|---|---|---|---|---|
| 0 | 0.0 | 3.40 b [(z)] | 4.50 c | 2.70 c | 26.40 bc |
| | 0.25 | 3.60 b | 8.80 a | 2.72 c | 33.20 b |
| 1 | 0.50 | 3.40 b | 5.20 c | 2.77 c | 26.68 bc |
| | 0.25 | 4.00 b | 5.20 c | 3.85 b | 43.94 a |
| 2 | 0.50 | 5.80 a | 7.20 b | 5.17 a | 52.16 a |
| | 0.25 | 6.70 a | 5.00 c | 3.15 bc | 31.82 bc |
| 3 | 0.50 | 3.50 b | 4.50 c | 2.69 c | 26.76 bc |
| | 0.25 | 3.90 b | 4.40 c | 2.76 c | 26.58 bc |
| 4 | 0.50 | 3.80 b | 4.40 c | 3.02 bc | 22.42 c |
| | 0.25 | 3.80 b | 5.40 c | 2.79 c | 24.24 bc |
| 5 | 0.50 | 3.60 b | 4.40 c | 3.03 bc | 29.30 bc |

[(z)] In each column, means followed by the same letter(s) are not significantly different using LSD test at 5% level.

Fig. 3 - 'Green' plantlets transferred to the pots.

Table 4 - Effects of different treatments on some characteristics of acclimatized 'Variegated' plantlets, 60 days after transfer

| BA (mg $l^{-1}$) | NAA (mg $l^{-1}$) | Mean length of shoots (cm) | Mean number of leaves | Mean stem diameter (mm) | Mean chlorophyll content (mg $g^{-1}$ FW) |
|---|---|---|---|---|---|
| 0 | 0.0 | 3.20 a-d [z] | 3.35 b | 2.50 c | 25.53 abc |
| | 0.25 | 2.66 cd | 3.66 ab | 2.276 c | 28.56 a |
| 1 | 0.50 | 3.50 abc | 5.50 a | 2.106 c | 27.23 ab |
| | 0.25 | 2.33 d | 3.45 b | 2.32 c | 20.80 bc |
| 2 | 0.50 | 3.00 a-d | 3.30 b | 2.85 abc | 27.76 ab |
| | 0.25 | 3.83 a | 3.41 b | 2.67 bc | 33.00 a |
| 3 | 0.50 | 3.66 ab | 3.66 ab | 2.43 ab | 29.50 a |
| | 0.25 | 3.00 a-d | 3.40 b | 3.56 a | 26.43 abc |
| 4 | 0.50 | 3.16a-d | 3.33 b | 2.54 c | 27.96 ab |
| | 0.25 | 2.83 bcd | 3.33 b | 2.76 abc | 27.63 ab |
| 5 | 0.50 | 2.66 cd | 3.66 ab | 2.72 bc | 19.36 c |

[z] In each column, means followed by the same letter(s) are not significantly different using LSD test at 5% level.

Fig. 4 - Acclimated 'Variegated' plantlets.

ferred to greenhouse conditions. The proposed protocol can be used in commercial mass production of *D. sanderiana*.

## References


BADAWY E.M., HABIB A.M.A., BANA A.E., YOSRY G.M., 2005 - *Propagation of* Dracaena fragrans *plants by tissue culture technique*. - Arab J. Biotech., 8: 329-342.

BEURA S., SAMAL P., JAGADEV P.N., 2006 - *Preliminary studies of* in vitro *cloning of Dracaena (*Dracaena sanderiana*)*. - Acta Hort. Sin., 80: 123-153.

BHATTACHARJEE S.K., 2006 - *Advances in ornamental horticulture*. - Pointer Publishers, Jaipur, India, pp. 2065.

CHAND S., SINGH A.J., 2004 - In vitro *shoot regeneration from cotyledonary node explants of a multipurpose leguminous tree,* Pterocarpus marsupium r*oxb*. - In Vitro Cell. Dev. Biol. Plant, 40: 167-170.

CHILDERS C.C., RODRIGUES J.C.V., 2005 - *Potential pest mite species collected on ornamental plants from Central America at port of entry to the United States*. - Fla. Entomol, 88: 408-414.

DEBERGH P., 1975 - *Intensified vegetative multiplication of* Dracaena deremensis*.* - Acta Hort. Sin., 54: 83-92.

DEBERGH P., 1976 - *An* in vitro *technique for the vegetative multiplication of chimeral plants of* Dracaena *and Cordyline*. - Acta Horticulturae, 64: 17-19.

DEBERGH P., MAENE L., 1981 - *"A scheme for commercial propagation of ornamental plants by tissue culture"*. - Scientia Hort., 14: 335-345.

JUNAID A., ABDUL M., MAHESHWAR P.S., 2013 - In vitro *micropropagation of* Dracaena sanderiana *Sander ex Mast: An important indoor ornamental plant.* - Saud. J. Biol. Sci., 20: 63-68.

MIMAKI Y., KURODA M., IDE A., KAMEYAMA A., YOKOSUKA A., SASHIDA Y., 1999 - *Steroidal saponin from the aerial parts of* Dracaena draco *and their cytostatic activity on HL 60 cells.* - Phytochemistry, 50(5): 805-813.

MIMAKI Y., KURODA M., TAKAASHI Y., SASHIDA Y., 1998 - *Steroidal saponins from the stems of* Dracaena concinna. - Phytochemistry, 47(7): 1351-1356.

NORMAN D.J., YUEN J.M.F., 1998 - *A distinct pathotype of Ralstonia (Pseudomonas)* solanacearum *race 1, biovar 1 entering Florida in pothos (*Epipremnum aureum*) cuttings*. - Can. J. Plant Pathol., 20: 171-175.

PAEK K.Y., OH M.O., CHOI J.K., 1985 - *Mass propagation of Cordyline and Scindapsus* in vitro. - Korean J. Hortic. Sci., 26: 83-92.

PALM M.E., ROSSMAN A.Y., 2003 - *Invasion pathways of terrestrial plant-inhabiting fungi*, p. 31-43. - In: RUIZ G.M., and CARLTON J.T. (eds). *Invasive species: Vectors and management strategies.* - Island Press, Washington, DC.

PRADO J., CASANOVES F., HILDAGO E., BENJAMIN T., SADOF C., 2008 - *Effects of production practices on the abundance of quarantine pests in* Dracaena marginata *in Costa Rican production fields*. - J. Econ. Entomol., 101: 1779-1785.

THROPE T.A., 1990 - *The current status of plant tissue culture*, pp. 1-22. - In: BHOJWANI S.S. (ed.) *Plant tissue culture: Applications and limitation.* - Elsevier Sc Publishing Co., NY, USA, pp. 470.

VENKATARAMAIAH V., PRASAD S.V., RAJESWARA R.G., SWAMY P.M., 1980 - *Levels of phenolic acids in* Pterocarpus santalinus *L.* - Indian J. Exp. Biol.,18: 887-889.

VONK NOORDEGRAAF C., 1998 - *Trends and requirements in floriculture in Europe*. - Acta Horticulturae, 454: 39-48.

YING X., BAO-LING H., CHENG-QUN L., YIN-PING Z., CHUANMING Z., YING L., 2008 - *Rapid propagation of* Dracaena cambodiana in vitro. - J. Anhui Agric. Sci., 32: 112-120.

YOKODUK A., MIMAKI Y., SASHIDA Y., 2000 - *Steroidal saponins from* Dracaena surculosa. - J. Nat. Prod., 63: 1239-1243.

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# Dormancy removal in pistachio nut: Influences of Hydrogen Cyanamid (Dormex®) as compared to ordinary seed chemical pre-treatments

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*Key words*: dormancy, germination, gibberellin, $KNO_3$, hydrogen cyanamid, pistachio.

**Abstract: Seed germination is considered an important phenological stage of plant growth during which the viable, non-dormant embryos develop into seedlings under favorable conditions. Currently, various seed priming treatments may be used to enhance germination values (germination percentage, Lag phase, time to reach 50% germination) and breaking dormancy; gibberellins and $KNO_3$ are applied as common treatments in this regard. In the present study, efficacy of Hydrogen Cyanamid (HCN, Dormex®) on seed germination of two cultivated pistachio varieties (Abasali, Shahpasand) were studied and the results were compared to ordinary ingredients, namely gibberellic acid ($GA_3$) and $KNO_3$. The extraction of parameters was carried out using four-parameter Hill function. The results reveal that HCN may significantly improve critical parameters involved in pistachio seed germination. In Shahpasand variety, seeds treated with HCN germinated prior to other treatments (150.53 hours after treatment) and achieved 50% germination earlier than other treatments (199.00 hours only). According to the results, utilization of HCN in seed testing experiments may be encouraged.**

## 1. Introduction

In agriculture, seeds are considered as starting points of plant growth. Assuming seeds are non-dormant, germination is the key component of seedling emergence (Forcella *et al.,* 2000). Germination may be defined as the process associated with the initiation and completion of embryo emergence; it refers to the progress of a seed from imbibition through radicle emergence (Gniazdowska *et al.,* 2010). Germination parameters are useful for estimating the conversion of seeds to seedlings and, thus, the suitability of a seed lot for commercial seedling production. Germination parameters are also useful in determining the type of seed pre-treatment as well as nursery management practices needed to attain a high level of germination (Kolotelo *et al.,* 2001). Abscisic acid (ABA) induces dormancy during maturation, and gibberellins (GAs) play a key role in dormancy release and in the promotion of germination (Kaur *et al.,* 2006). In many cases, endogenous levels of these hormones (GA, ABA) are associated with the ability of the seeds to germinate (Seo *et al.,* 2006); seed dormancy must be broken to induce germination. In morpho-physiological dormancy, seeds must be exposed to cold, heat, gibberellic acid, or chemical materials for dormancy breaking (Hilton, 1984; Dehghanpour *et al*., 2011). The use of potassium nitrate has been an important seed treatment in seed-testing laboratories for many years without a good explanation for its action mechanism (Çetinbaş and Koyuncu, 2006). Hydrogen cyanamide (HCN Dormex®) has been identified as one of the most effective dormancy-breaking agents in many deciduous plant species (Siller-Cepeda *et al.,* 1992) and has been exploited mainly as an artificial rest-breaking agent to stimulate budbreak in mild winter climates (Ben Mohamed *et al.,* 2012). However, the emission of HCN has been detected during the pre-germination period of many seeds (Gniazdowska *et al,*. 2010).

Pistachio (*Pistacia vera* L.), a member of Anacardiaceae family (Khan *et al.,* 1999), is a dioecious species and for this reason it has acquired large genetic diversity (Behboodi, 2002). Seed dormancy, oily nature of cotyledons leading to early spoilage, risk of heterozygosis, and production of male/female plantlets are major problems associated with pistachio sexual propagation (Jangali Baygi, 2012). Therefore, under natural conditions, an erratic and irregular germination over a quite long period. On the other hand, Pistachio plants cannot be readily propagated from cuttings taken from mature trees and are currently propagated by grafting on seedling rootstocks (Behboodi, 2002). Further-

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more breeding programs and development of hybrid seedlings/superior rootstocks require a rapid and reliable method for seed germination (Ghazaeian *et al*., 2012). Hence, in the present study, effects of HCN on dormancy removal and seed germination of two pistachio varieties were studied and the results were compared to widespread chemical treatments such as $GA_3$ and $KNO_3$ as well as cold stratification.

## 2. Materials and Methods

The present study was undertaken in the physiology laboratory at the Horticulture Department, Gorgan University of Agricultural Sciences and Natural Resources, Golestan, Gorgan, Iran. Two Iranian cultivated pistachio varieties, ('Abbasali' and 'Shahpasand') were procured from a commercial orchard located in Damghan, Semnan Province.

Initially, the healthy and bold seeds were selected and the extracted kernels were treated for 24 h with different chemicals, *i.e.* $GA_3$ (50, 100 mg/l), $KNO_3$ (1.0, 1.5 %), and Dormex® (50, 100 mg/l), all procured from Sigma. Dormex is a mineral component with chemical formula $H_2CN_2$ (Siller-cepeda *et al.,* 1992). The chemical component utilized in the present study was aqueous Hydrogen Cyanamid ($H_2CN_2$ 187364 Sigma, F.W.= 42.04 g/mole). Initially the required amount of HCN was dissolved in a few drops of ethanol to prepare the stock solution from which the concentrations of 50-100 mg/l HCN were prepared with distilled water. The control seeds were also soaked in distilled water only and some seeds of the same lot were kept in a refrigerator (5°C) as cold stratification treatment. In order to prevent fungal contamination, the treated kernels were surface sterilized with sodium hypochlorite 10% and then rinsed at least three times with autoclaved water. These were then cultured in plastic containers filled with autoclaved sand (diameter 2 mm). The samples were observed daily for a period of 54 days and the number of germinated seeds was recorded. Radicle emergence (5 mm in length) was taken as germinated seed. The cumulative germination count of each pistachio variety seed lot was fitted to a four-parameter Hill function (4-PHF; Equation 1) already reported by El-Kassaby *et al.* (2008) as a mathematical representation and parameter extraction of seed germination:

$$(1) \qquad y = y_0 + \frac{ax^b}{c^b + x^b}$$

In the equation 1, y is considered as the percentage of cumulative germination at time *x*, $y_0$ is the intercept on the *y* axis (≤0), *a* is the asymptote, or maximum cumulative germination percentage, which is equivalent to germination capacity, *b* is a mathematical parameter controlling the shape and steepness of the germination curve and *c* is the "half-maximal activation level" measured in days and represents the time required for 50% of viable seeds to germinate ($G_{50\%}$). Following extraction of a, b, c and $Y_0$, from 4-PHF, the time at germination onset (lag) and the final germination percentage ($G_{max}$) were also computed using the following equations:

$$(2) \qquad lag = b\sqrt{\frac{-y_0\ c^b}{a + y_0}}$$

$$(3) \qquad G_{max} = y_0 + a$$

The entire research work was designed as a completely randomized design (CRD) with factorial arrangement including seven treatments and three replications. The data were analyzed using SAS software.

## 3. Results and Discussion

Pistachio plants cannot be readily propagated from cuttings procured from mature trees and are currently propagated through budding/grafting on seedling rootstocks. Though tissue culture techniques were proposed as new and advanced methods for multiplication of this species (Behboodi, 2002) seedling development followed by budding is still considered a superior method for pistachio orchard establishment. However, seed dormancy and erratic germination cause significant trouble for growers, nurserymen, and fruit breeders (Jangali Baygi, 2012). Dormancy release is accomplished by diverse mechanisms that include complex interactions with the environment mediated by plant hormones and other small molecules, and is supposed to select conditions for germination that are most favorable for plant survival (Finkelstein *et al*., 2008). Application of $GA_3$. Thiourea, and $KNO_3$ as common priming agents were formerly reported in dormancy removal of some nut species such as wild pistachio (Khan *et al*., 1999), walnut (Kaur *et al.,* 2006), and pecan (Ghazaeian *et al*., 2012). Hence, the present study was also undertaken on two cultivated pistachio varieties and the following results were found.

The results of analysis of variance of the effects of chemical treatments on dormancy removal and germination parameters of pistachio varieties are shown in Table 1. The data demonstrate a sharp and considerable effect of chemical priming on germination percentage, the time at germination onset (lag), and time taken to 50% germination in each seed lot. Furthermore, the results demonstrate the effectiveness of Dormex® to release pistachio seeds from dormancy.

The lag phase is an important parameter to determine quality of seed lots. In Abbasali variety the control seeds germinated 731.28 h after culture, while the chemical priming considerably reduced this period. For example, seeds treated with 1.5% $KNO_3$ germinated at 263.26 h only. As shown in figure 1, application of Dormex® at the rate of 100 mg/l had the similar effect on lag phase and the germination at onset was recorded at 385 h following treatment. In the case of Shahpasand variety, Dormex® was found to be the best treatment to remove seed dormancy and the lag period from 150.35 h in control seeds was reduced to 52.78 h only. This result indicates that the priming treatment with Dormex® may lead to precocious and early seed

Table 1 - The results of analysis of variance of the effects of chemical treatments on dormancy removal and germination parameters of two cultivated pistachio varieties

| Sorces of variations | d.f. | Mean square | | |
|---|---|---|---|---|
| | | $D_{50}$ | $G_{max}$ | lag |
| Variety | 1 | 2508961.5** | 1138.38** | 1328137.5** |
| Treatments | 6 | 66477.16** | 911.36** | 49107.3** |
| Variety x treatment | 6 | 33053.44* | 528.47** | 21142.09* |
| Error | 28 | 12569.26 | 87.34 | 17639.87 |
| cv | | 19.68 | 12.28 | 52.83 |

$G_{max=}$ germination percentage.
$D_{50}$= time to reach 50% germination.
Lag= time to germination onset.

germination. An application of calcium cyanamide was previously proposed to overcome a lack of winter chilling in many plants (Walton *et al.,* 1991). Ben-Mohamed *et al.*, (2012) investigated the effect of HCN on dormancy release and carbohydrate metabolism in the buds of grapevine.

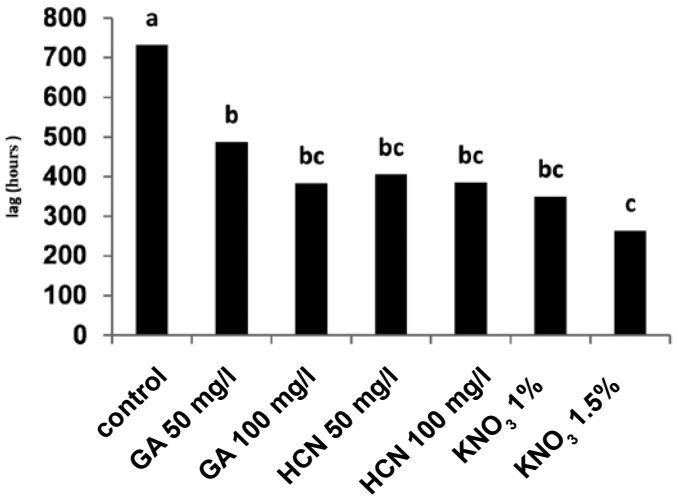


Fig. 1 - Effect of different priming agents on time to germination at onset (lag phase) of Abbasali pistachio variety.

Their results showed that application of HCN to dormant grape buds advanced budbreak to 10 days after treatment while the untreated buds opened within 14 days after treatment. Furthermore, HCN caused significant differences in the rate and percentage of budbreak (85% as compared to 42% budbreak in the controls). However, there are quite a few reports on the effect of this compound on seed dormancy. Gniazdowska *et al.* (2010) studied the role of HCN on breaking dormancy of apple embryos. Interestingly, they found that the deep dormancy of apple embryos may be removed by a quite short pre-treatment (3-6 h) of HCN. Furthermore, the development of abnormal seedlings was also lower than control, non treated seeds. Such effect of HCN in apple embryos is comparable with the response of Shahpasand pistachio variety in the present study (Fig. 2).

The time taken to reach 50% seed germination is also considered as a remarkable parameter in seed germination experiments. According to figures 3 and 4, application of chemical treatments significantly reduced this

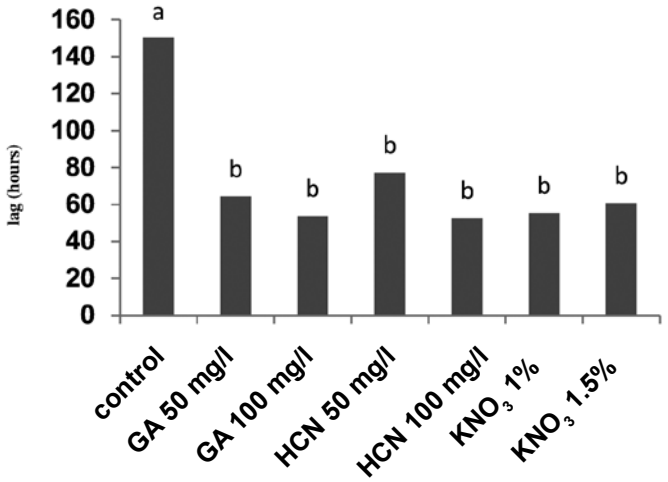


Fig. 2 - Effect of different priming agents on time to germination at onset (lag phase) of Shahpasand pistachio variety.

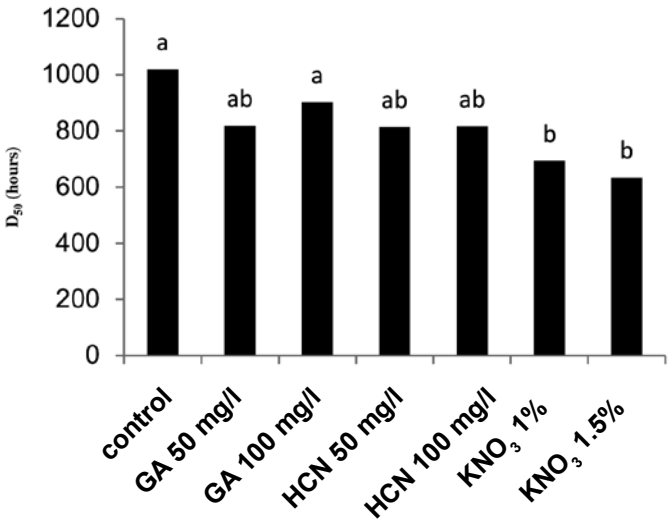


Fig. 3 - Effect of different priming agents on time to reach 50% germination ($G_{50\%}$) of Abbasali pistachio variety.

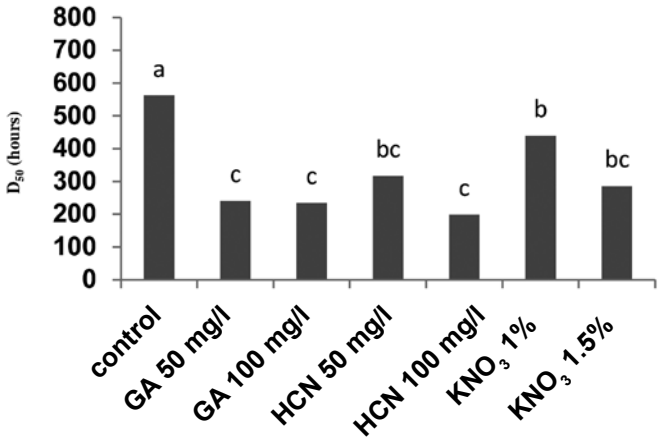


Fig. 4 - Effect of different priming agents on time to reach 50% germination ($G_{50\%}$) of Shapasand pistachio variety.

period. In Shahpasnd variety, the time taken to $G_{50\%}$ in control seeds was recorded as 562 h while in the case of priming treatments it was effectively reduced to 199 h (Dormex® 100 mg/l), 234 h ($GA_3$ 100 mg/l) and 286 h ($KNO_3$ 1.5%). In the case of Abbasali, the same trend was observed and $KNO_3$ 1.5% was found to be the most effective treatment.

In both varieties, cumulative germination in control seeds was low but it was enhanced following chemical priming (Fig. 5 and 6). This means that $G_{max}$ in 'Abbasali' increased from 46.23% in control seeds to 86.30% in seeds treated with $KNO_3$. The same variety had 81.91% of $G_{max}$ when treated with Dormex®. Application of $KNO_3$ in 'Shahpasand' seeds led to 99.25% germination. Hence, the results clearly reveal that Dormex® was effective in dormancy removal and germination of pistachio nuts and that in some cases it was comparable or even better than $GA_3$ and $KNO_3$ treatments.

The effectiveness of gibberellins in seed germination has been reported in numerous studies (Bewley and Black, 1994; Seo *et al.*, 2006). *In vitro* germination of walnut (*Juglans regia* L.) embryos was undertaken by Kaur *et al.* (2006) and they used components such as $GA_3$, Benzyl adenine, and kinetin to improve embryo growth and development. They reported $GA_3$ along with low temperature, as the best treatment for walnut *in vitro* embryo germination. In another experiment for dormancy breaking of tulip seeds, Rouhi *et al.* (2010) also applied $GA_3$ at rates from 250 to 500 mg/l. They observed an enhancement of seed germination as $GA_3$ levels were increased. In general, two main functions have been proposed for gibberellins during seed germination process. First, GA increases the growth potential of the embryo. Secondly, it is necessary to overcome the mechanical restraint conferred by the seed-covering layers, by weakening the tissues surrounding the radicle (Kucera *et al.*, 2005). Gibberellins stimulate germination by inducing hydrolytic enzymes that weaken the barrier tissues such as the endosperm or seed coat, inducing mobilization of seed storage reserves, and stimulating expansion of the embryo (Finkelstein *et al.,* 2008).

Potassium nitrate is considered to be one of the most effective and widely used components with respect to breaking seed dormancy (Çetinbaş and Koyuncu, 2006). As far as pistachio seed germination is concerned, Khan *et al.* (1999) applied $KNO_3$ at the rate of 1% to wild pistachio and they observed 26% seed germination as compared to control with only 2%. In an experiment carried out on *Prunus avium* L., the stratified seeds were treated with $GA_3$, $KNO_3$ and thiourea (Çetinbaş and Koyuncu, 2006). Treatments with 7,500 mg/l $KNO_3$ after 120 days of stratification were more effective, yielding 64.54% germination of seeds with coat. However, in case of seeds without coat (kernels), $GA_3$ was found to be more effective than other chemicals.

The role of HCN in dormancy removal may be attributed to its stimulatory effects on endogenous ethylene production. Ethylene increases respiration as well as starch degradation of seed reserves and the embryo then starts germination. A similar result was also reported for tobacco in which ethylene was involved in endosperm rupture and

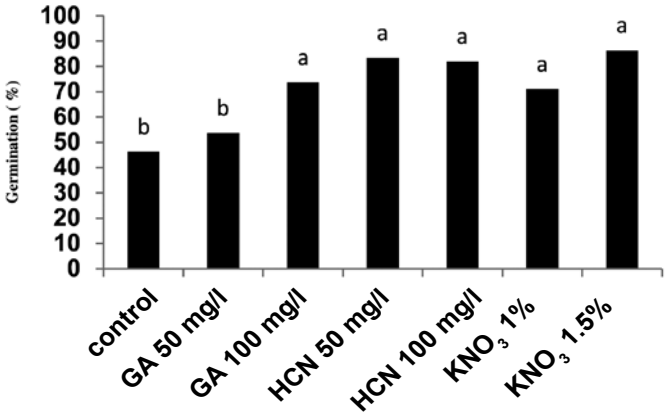


Fig. 5 - Effect of different priming agents on cumulative germination percentage ($G_{max}$) of Abbasali pistachio variety.

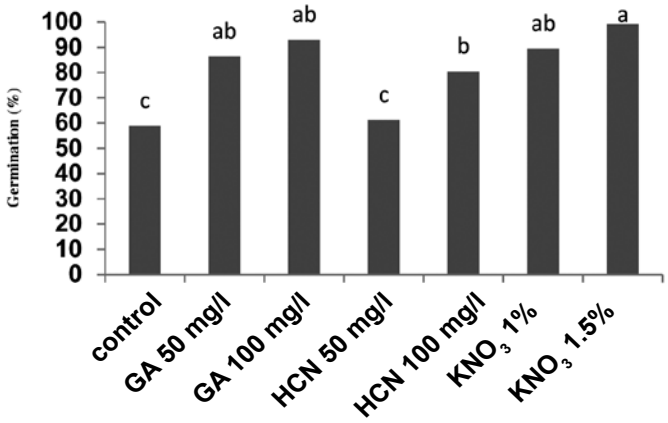


Fig. 6 - Effect of different priming agents on cumulative germination percentage ($G_{max}$) of Shahpasand pistachio variety.

high bGlu I expression during seed germination (Kucera *et al*., 2005). Ben Mohamed *et al*. (2012) also stated that starch concentration was high in dormant buds of grapevine and declined rapidly to reach its lowest level following HCN application. The same process may occur in treated pistachio seeds leading to 99.25% germination in Shahpasand variety (Fig. 6). Another reason for the role of HCN in improvement of seed germination can be the activation of the pentose-phosphate pathway which is thought to be necessary for the breaking of dormancy in buds and seeds (Walton *et al*., 1991). They demonstrated that application of HCN to kiwifruit dormant buds led to production of high levels of proline amino acid. They stated that elevated levels of proline in HCN-treated plants could be associated with a greater stimulation of the pentose-phosphate pathway, resulting in a greater percentage of budbreak and increased bud fruitfulness.

In conclusion, it can be stated that HCN is an effective compound for pistachio seed germination. Moreover, the role of ordinary chemical seed pre-treatments such as $GA_3$ and $KNO_3$ to improve pistachio seed germination was reconfirmed. Application of HCN also leads to precocious germination and early seedling development. This parameter may be useful for nurseries or fruit breeders as a useful measure to develop hybrid seedlings in a shorter time. Overall, although more experiments with other species should be carried out to confirm the results of the present research work, it can be said that utilization of HCN in seed testing experiments should be encouraged as an effective pre-treatment and dormancy breaking agent.

## References


BEHBOODI B. SH., 2002 - *Tissue culture results of all wild pistachio species and some cultivars in Iran*. - Acta Horticulturae, 591: 399-409.

BEN MOHAMED H., VADEL A.M., GEUNS J.M.C., KHEMIRA H., 2012 - *Carbohydrate changes during dormancy release in superior seedless grapevine cuttings following hydrogen cyanamide treatment.* - Scientia Horticulturae, 140: 19-25.

BEWLEY J.D., BLACK M., 1994 - *Seeds: Physiology of development and germination*. - Plenum, NY, USA, pp. 367.

ÇETINBAŞ M., KOYUNCU F., 2006 - *Improving germination of* Prunus avium *L. seeds by gibberellic acid, potassium nitrate and thiourea.* - HortScience., 33(3): 119-123.

DEHGHANPOUR FARADHAH H., TAVAKKOL AFSHARI R., SHARIFZADEH F., CHAVOSHINASAB S., 2011 - *Germination improvement and α-amylase and β-1,3-glucanase activity in dormant and non-dormant seeds of Oregano (*Origanum vulgare*).* - Australian Journal of Crop Science, 5(4): 421-427.

EL-KASSABY Y.A., MOSS I., KOLOTELO D., STOEHR M., 2008 - *Seed germination: Mathematical representation and parameters extraction*. - Forest Science, 54(2): 220-227.

FINKELSTEIN R., REEVES W., ARIIZUMI T., STEBER C., 2008 - *Molecular aspects of seed dormancy.* - Annu. Rev. Plant Biol., 59: 387-415.

FORCELLA F., BENECH ARNOLD R.L., SANCHEZ R., GHERSA C.M., 2000 - *Modeling seedling emergence.* - Field Crop Res., 67: 123-139.

GHAZAEIAN M., ZAMANI S., SAFARNEJAD A., KHERADMAND G., 2012 - *Effects of stratification treatments on germination of pecan seeds (*Carya illinoensis*).* - International Journal of AgriScience, 2(7): 588-591.

GNIAZDOWSKA A., KRASUSKA U., BOGATEK R., 2010 - *Dormancy removal in apple embryos by nitric oxide or cyanide involves modifications in ethylene biosynthetic pathway.* - Planta., 232: 1397-1407.

HILTON J.R., 1984 - *The influence of light and potassium nitrate on the dormancy and germination of* Avena fatua *L. (wild oat) seed and its ecological significance*. - New Phytol., 96: 31-34.

JANGALI BAYGI M., 2012 - *Analysis of seed germination using Hill function: A case study of Pistachio*. - MSc thesis, Horticulture Dep., Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Golestan, Iran.

KAUR R., SHARMA N., KUMAR K., SHARMA D.R., SHARMA S.D., 2006 - In vitro *germination of walnut (*Juglans regia *L.) embryos*. - Scientia Horticulturae, 109: 385-388.

KHAN J., RAUF M.A., HARUN-UR-RASHID A.Z., KHATTACK M.S., 1999 - *Different stratification techniques on seed germination of pistachio cv. Wild.* - Pakistan Journal of Biological Sciences, 2(4): 1412-1414.

KOLOTELO D., VAN STEENIS E., PETERSON M., BENNETT R., TROTTER D., DENNIS J., 2001 - *Seed handling guidebook*. - BC Ministry of Forests, Tree Improvement Branch, Victoria, BC, Canada, pp. 106.

KUCERA B., COHN M.A., LEUBNER-METZGER G., 2005 - *Plant hormone interactions during seed dormancy release and germination*. - Seed Science Research, 15: 281-307.

ROUHI H.R., SHAKARAMI K., TAVAKKOL AFSHARI R., 2010 - *Seed treatments to overcome dormancy of waterlily tulip (Tulipa kaufmanniana Regel.)*. - Australian Journal of Crop Science, 4(9): 718-721.

SEO M., HANADA A., KUWAHARA A., ENDO A., OKAMOTO M., YAMAUCHI Y., NORTH H., MARION-POLL A., SUN T., KOSHIBA T., KAMIYA Y., YAMAGUCHI S., NAMBARA A., 2006 - *Regulation of hormone metabolism in* Arabidopsis *seeds:phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism*. - The Plant Journal, 48: 354-366.

SILLER-CEPEDA J.H., FUCHIGAMI L.H., CHEN T.H.H., 1992 - *Hydrogen Cyanamide-induced Budbreak and Phytotoxicity in 'Redhaven' Peach Buds*. - Hortscience., 27(8): 874-876.

WALTON E.F., CLARK C.J., BOLDINGH A.L., 1991 - *Effect of Hydrogen Cyanamide on amino acid profiles in kiwifruit buds during budbreak*. - Plant Physiol., 97: 1256-1259.

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*Short note*

# Non-invasive acoustic sensing of tuberous roots of sweet potato (*Ipomoea batatas*) growing belowground

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*Key words*: sweet potatoes, undersoil detection, water-proof speaker.

**Abstract: The study applies acoustic means for the detection of belowground tuberous roots of sweet potato by developing a novel non-invasive sensing technology based on propagation of frequency-modulated sound through the soil and its detection with acoustic band-pass filtering devices. The presence of tuberous roots hidden in the bed of sandy soil was successfully detected with the proposed acoustic approach, which is still primitive. However, this investigation may induce further studies and developments for belowground sensing and imaging techniques as novel ecological research tools and to support the introduction of vegetables into so-called "plant factories".**

## 1. Introduction

Petty-Clark's law of economics (formally known as Petty's law) (Tsuchiya, 1993) suggests that the proportion of primary industries declines as the economy of a country develops, and in turn, those of secondary and tertiary industries must increase (Kawata, 2011). Today in Japan, agricultural, fishery, and forestry workers make up about 3.51% of the total working population in the nation. In the past six decades, the agricultural force in Japan has been markedly weakened and thinned, partly due to a nation-wide decline in birth rates and a decrease in new employment in the field (http://www.stat.go.jp/data/roudou/longtime/03roudou.htm).

These factors eventually caused highly advanced aging of the agricultural population in Japan. From a macroscopic point of view, many may expect that the decreasing trend in the agricultural working population may continue based on predictions by the Petty-Clark's law (Nakamura, 2009). In contrast, there would be microscopic growth in the working population in specific forms of agriculture. One of the authors of this paper (TK) is currently proposing two models in which agriculture can be converted to higher industries, i.e. as leisure and as a high-tech food production industry (Kawano, 2014).

In the last two decades, prototypic high-tech agricultural farms (so-called "plant factories") aimed at automating the production of fresh produce, chiefly vegetables, under precisely controlled environments have been developed (Ikeda, 2011; Nishida *et al*., 2012; Nishimura *et al*., 2012). Today, such commercially launched "plant factories" cover sprouts, leafy vegetables, and tomatoes (Morimoto, 2005; Shimizu *et al*., 2008). In order to expand the scope of "plant factories" and enable the automated cultivation of a wide range of agriculturally important plant species, the agricultural and horticultural processes which are a successive series of necessary maintenance steps must be translated or re-documented into automatable protocols based on the records of both plant physiological and mechanical data (Kawano *et al*., 2012).

Despite their agricultural importance, twining plants have not been included in the trends of "plant factory" development, possibly due to the belief that handling of growing vines must be achieved manually by experienced specialists (Kawano *et al*., 2012). *Ipomoea* cultivars are examples of such twining crops, whose aerial parts are herbaceous perennial vines and bear alternate heart-shaped green leaves and sympetalous flowers (Sher *et al*., 2001). In addition, root vegetables or crops bearing root products belowground have not been produced in "plant factories" yet, despite the fact that the growth of roots are central

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to the physiology and ecology of higher terrestrial plants including forest trees and agricultural crops.

In soil- or sand-based conventional agricultural fields, a variety of root vegetables are produced. Such vegetables having highly developed roots include beet (*Beta vulgaris* ssp. *vulgaris*), turnip known as *Kabu* in Japan (*Brassica rapa* L.), carrot (*Daucus carota* L.), radish and *Daikon*-Japanese white radish (*Raphanus sativus* L.), Chinese yam known as *Nagaimo* in Japan (*Dioscorea oppositifolia* L.), and burdock known as *Gobō* in Japan (*Arctium lappa* L.). Among the root vegetables, sweet potato (*Ipomoea batatas* L.), cassava (*Manihot esculenta* Crantz), and yacón or so-called Andes potato (*Smallanthus sonchifolius*) are typical examples of vegetables bearing starchy tuberous roots. In addition to the above true root vegetables, there are groups of undersoil vegetables that develop root-like organs as storage of starch, sugars, and nutrition. Economically important crops with undersoil organs such as corms of taro (*Colocasia esculenta*), tubers of potato (*Solanum tuberosum*), and rhizomes of lotus (*Nelumbo nucifera*), ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), and ginseng (*Panax ginseng*), are examples of vegetables with specialized stems. Furthermore, vegetables producing bulbs can be included in the undersoil vegetables, mostly belonging to *Allium* such as garlic (*Allium sativum* L.), onion, and shallot (*Allium cepa* L.).

Today, soils or sands are hardly manipulated in sensor-equipped "plant factories" while hydroponic cultures are mostly favored. In order to introduce such root vegetables in "plant factories", it is necessary to monitor the belowground properties and processes where plant roots take place, mainly spatial occupation by the growing root system and changes in water, salts, and other elements that can influence productivity and functioning of the soil ecosystem. However, dynamic growth and development of a root system are poorly understood since roots growing belowground are invisible to conventional optical means.

Before facing the challenges to growing under-soil vegetables in the controlled environment inside "plant factories", appropriate non-invasive sensing technologies allowing real-time monitoring of belowground plant growth must be developed and applied. Due to the light absorbing nature of the soils, optical measurements are difficult to apply. Three-dimensional computed tomography with X-ray scanning technology might be a candidate technology if plants are grown in laboratory-scaled small pots (We have developed non-invasive scanning protocols for garlic bulbs, data not shown). However, X-ray scanning cannot be employed on-site in plant producing facilities.

In this study, we attempted to detect the presence of tuberous roots which were hidden in the sandy bed. For this purpose, a novel instrumental set-up was designed for modulation and demodulation of sound signals passed through soil or sand with and without growing plant tissues.

Sweet potato, known as Satsuma-imo in Japanese, is a dicotyledonous crop that belongs to the family of *Convolvulaceae*. Due to its starchy and sweet-tasting tuberous roots, this plant is often considered both a starch producing crop and a fiber-rich root vegetable (Purseglove, 1968; Woolfe, 1992).

## 2. Materials and Methods

*Acoustic sensing system*

Frequency-modulated sound signals propagated through sandy soil containing plant tissue samples or not were monitored using a pair of acoustic probes, a flat sound radiator, and sensing microphones (Fig. 1A, B). In order to eliminate the background sound noise, a signal from the reference (ground) microphone was used. To generate the modulated sound signal at the frequency of interest, a function generator (DFG-6020 20MHz DDS, EZ Digital Co. Ltd., Gwangju, Korea) was used (Fig. 1C). Signals received by the sensing microphone were amplified by a AT-MA2 microphone amplifier (Audio-Technica, Tokyo, Japan) and band-filtered to demodulate using an acoustic *band-pass filter* (Multi-function filter 3611 NF Corporation, Yokohama, Japan), and the amplitude of the sound at specific frequency captured through the system was analyzed using an oscilloscope (TBS 1064, Tektronix Inc., OR, USA).

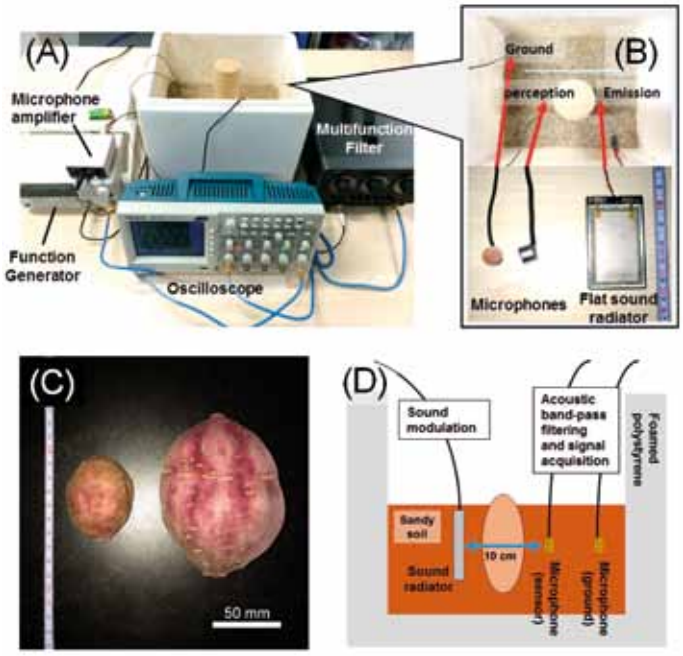


Fig. 1 - Equipment for the acoustic sensing of belowground plant tissues. (A) Experimental configuration. Modulation and demodulation of acoustic signal propagated in a sandy soil bed were preliminarily tuned with and without wooden stick. (B) A pair of acoustic probes designed for emitting and receiving the modulated and band-pass filtered sound signals in the soil or sand. Left, sensing and reference microphones. Right, flat-shaped sound radiator with directional sound propagation. (C) Sweet potato tuberous roots used for demonstration. (D) Experimental design.

*Experimental procedure*

An experimental set-up (Fig. 1) inside the sand-filled container of polystylene foam with or without model tissues embeded in the sand, was used for acoustic measurements. Band-modulated sound signals were emitted and detected at a constant distance of 100 mm, by a pair of acoustic probes, namely, a directional sound radiator and receivers (microphones), both positioned in the bed of dry (*ca*. 1% of water content) or wet (*ca*. 10% of water content) sands.

The given sound frequency was in the range between 100 and 20,000 Hz. Changes in the height of voltage signal between the peaks and valleys of the recorded sound waves were recorded (Fig. 2A) and expressed as the extent of signal intensity, reflecting the perception of band-pass filtered sound after propagation of band-modulated sound. Mean values of the height of voltage signal obtained at each frequency (n=5) were used to obtain a series of regression lines with the least-square method. Data were recorded in the absence (blank control) and presence of tubers.

*Plant materials*

Since *Ipomoea* plants naturally grow in sandy fields, and sand-culture is often employed for cultivation of sweet potatoes (Leonard *et al*., 1948; Spence and Humphries, 1971), commercially available river-sand mixture was chosen as tuber root supporting medium. Assuming there is the necessity to monitor tuberous roots inside a plant growth container, a planter made of foamed polystyrene filled with dry or wet river-sand mixture was employed as a model platform for acoustic sensing demonstrations.

Inside the container, two types of sandy soil (volume: 4L; area: 220 mm x 262 mm, depth: 100 mm) with known water content (dry at *ca*. 1 or wet at *ca*. 10% w/w) were tested. Portions or intact sweet potato tuberous roots of different sizes were detected and compared with the absence of any vegetable tissue (blank control). In particular, two intact tuberous roots with a central swelled portion (45 and 82 mm in diameter) (Fig. 1C) were obtained from a local market and used as model vegetable tissues. As a model for sampling tissues with smaller mass, a cut plug (cylindrical section of 20 mm in diameter) of tuberous root was prepared and used for acoustic measurements.

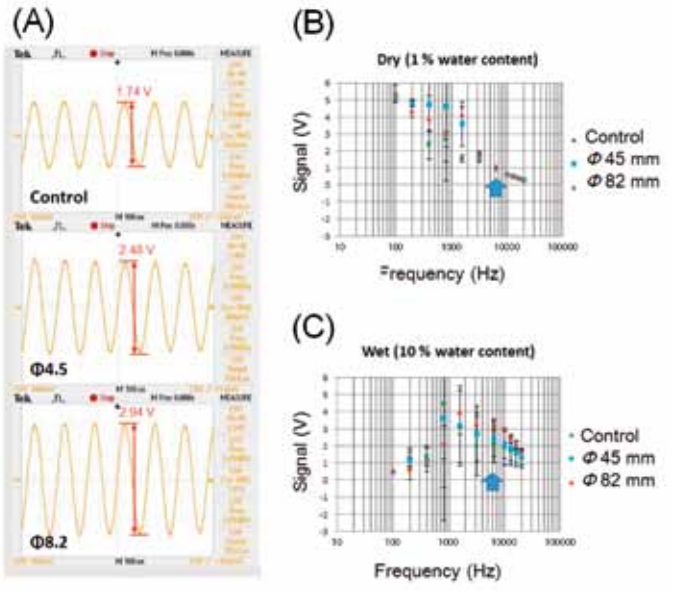


Fig. 2 - Detection of sound signals propagated in the sand layer with and without sweet potato tuberous toots. (A) Typical raw records of signal modulated at *ca*. 6.4 kHz (6.386-6.413 kHz). Changes in signal intensity (voltage changes between the peaks and valleys of the waves) were monitored. Relationship between the frequency and yield of sound signal in the beds of dry sand (B; water content, *ca*. 1%) and wet sand (C; water content, *ca*. 10%) were compared. Arrows in (B) and (C) indicate signal yields at 6.4 kHz. Error bars, S.D. (n= 5).

## 3. Results and Discussion

*Sound signal detection*

The intensity of sound signals propagated through sand differed noticeably depending on the sound frequency (Fig. 2). Fractuations of signal intensities were shown to be higher in the relatively lower range of frequency examined (100-1,600 Hz) in both sand samples. Compared to the dry sand, the wet sand likely enhanced the yield of sound signals in the presence of sweet potato tissues, especially at the higher range of frequency examined (3,200-20,000 Hz). Figure 2A reports typical raw records of sound signals showing a size-dependent enhancement at 6.4 kHz in the wet condition. Thus, data from the wet sand bed was used for further analysis to elucidate the effect of tissue size on signal acquisition.

*Sound signal relationships*

Relationships between sound signal frequency and yield in the beds of dry and wet sands were compared by obtaining a series of regression lines with the least-square method (Fig. 3A). Correlation coefficients ($r^2$) for four regressions were between 0.8825 and 0.9991. To evaluate tissue size-dependent changes in the yield of sound signals in the wet sand bed, two indices (i.e. index of subtraction and index of ratio) were proposed and the processed data were used to obtain the linear regressions and were compared with other linear regressions obtained for raw signal data (Fig. 3B, C). With the index of subtraction (sample-blank) (Fig. 3B), correlation coefficients ($r^2$) for three regressions were between 0.7148 and 0.9623. With the index of ratio (sample / blank) (Fig. 3C), correlation coefficients ($r^2$) for three regressions were between 0.7829 and 0.8885. The regressions obtained for the index of subtraction highlighted the relationship between the signal yield and tissue size increases since the coefficients for $x$ in the linear regressions increased with size: $2{\cdot}10^5\ x$ for the ø20 mm sample, $3{\cdot}10^5\ x$ for the ø45 mm sample, and $5{\cdot}10^5\ x$ for the ø82 mm sample (Fig. 3B).

*Further studies*

During frequency-modulated sensing of plant tissues, the impact of acoustic signals on plant growth must be

minimized, as recently demonstrated by Gagliano *et al*. (2012 a, b) who suggested that the growing tips of plant roots could be attracted by acoustic signals with specific spectra raging between 200 and 400 Hz, if plant roots were continuously exposed to the sound stimulus. One possible way to avoid such influence during acoustic sensing is to use signals in a frequency range higher or lower than the plant activating range of sound (<200 Hz, >400 Hz), or to use sound only for short periods when required. Since the range of frequency recommended for monitoring belowground plant tissues was shown to be between 3,200 and 20,000 Hz (Fig. 3), the impact of applied sound on plant growth could be avoided.

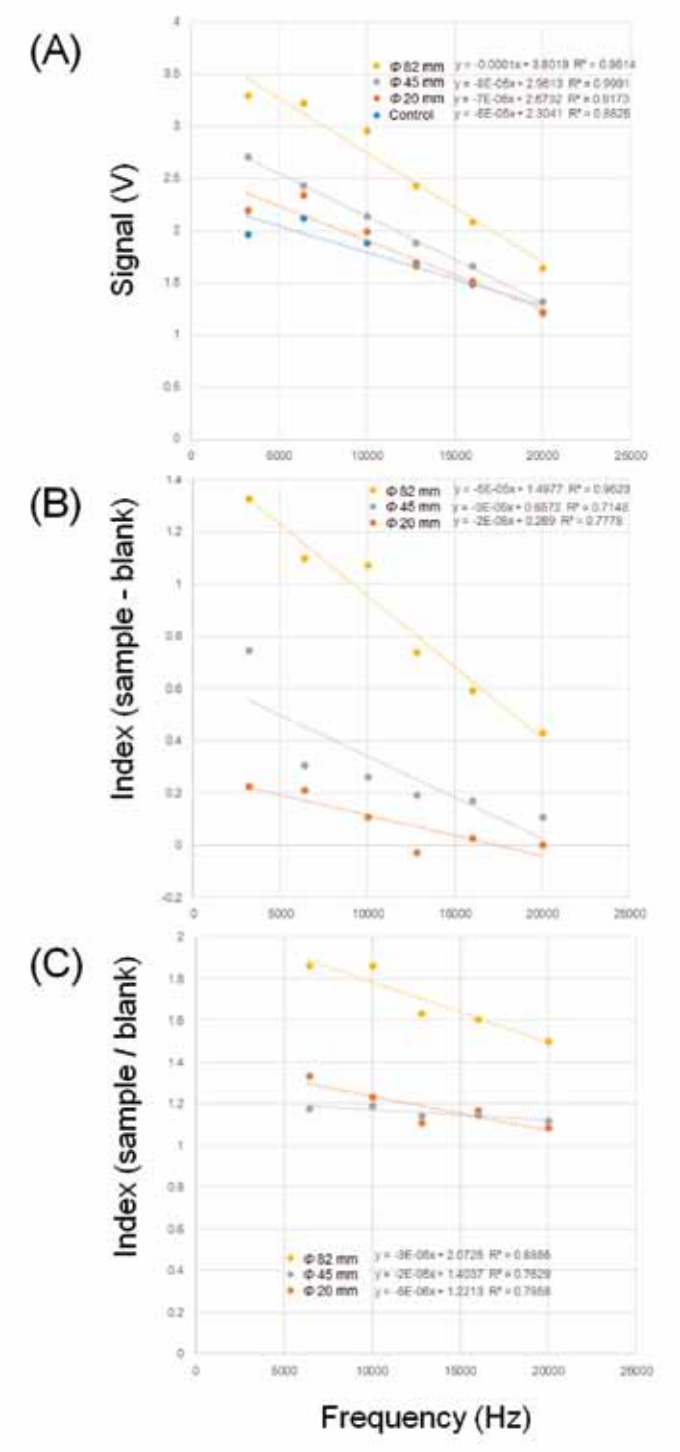


Fig. 3 - Signals for belowground tuberous roots differed in size based on the acoustic recording at the higher range of frequency (between 3,200 and 20,000 Hz). Data recorded in the absence (blank control) and presence of tuberous roots of sweet potato (20, 45 and 85 mm in diameter) were plotted. Mean values obtained at each frequency were used (n = 5). (A) Plots and linear regression of raw signal. (B) Evaluation of the signal with the subtraction index (sample-blank). (C) Evaluation of the signal with the ratio index (sample/blank).

In the present work we have developed a novel, non-invasive sensing technology for the detection of belowground plant tissues based on sound propagation in the soil. We employed tuberous root tissue of sweet potato as model material to test the instrumental set-up which was specifically designed for modulation and demodulation of sound signals through soil or sand with and without plant tissues.

It is well known that growth of tuberous roots of sweet potato is highly sensitive to changes in temperature (Spence and Humphries, 1971), moisture (Spence and Humphries, 1971; Eguchi *et al*., 2012), nutrient (Leonard *et al*., 1948), phytohormone levels (Spence and Humphries, 1971), and oxygen supplies (Eguchi and Yoshida, 2011) in the supporting sand. However, to date, underground growth of sweet potatoes' tuberous root was rarely studied with non-invasive real-time monitoring approaches. Further studies are still needed, although there are several technical problems to be overcome, chiefly, sensitivity and resolution of the sensing units.

Ecologists, ecohydrologists, and biogeochemists need detailed insights into plant properties and processes where plant roots reside, including changes in water, salts, and other elements that can influence ecosystem productivity and functioning (Jayawickreme *et al*., 2014). However, limitations in data and some confusion over terminology, together with a strong dependence on a small set of conceptual frameworks, have limited the exploration of root function in terrestrial models (Smithwick *et al*., 2014). Relying on traditional mechanical sampling and observation techniques can be costly, time consuming, and poorly feasible, especially if the spatial scales involved are large (Jayawickreme *et al*., 2014). Therefore, non-invasive real-time monitoring may largely benefit both the ecological research community and the field of "plant factory" research and development.

Although the present study succeeded in only the primary test case, we hope it will stimulate further studies and development by scientists and engineers.

## References

EGUCHI T., ITO Y., YOSHIDA S., 2012 - *Periodical wetting increases α-tocopherol content in the tuberous roots of sweetpotato (*Ipomoea batatas *(L.) Lam.)*. - Environ. Control Biol., 50: 297-303.

EGUCHI T., YOSHIDA S., 2011 - *Tuberous root thickening: a rapid response to hypoxia in sweetpotato (*Ipomoea batatas (L.) Lam.). - Environ. Control Biol., 49: 47-50.

GAGLIANO M., MANCUSO S., ROBERT D., 2012 a - *Towards understanding plant bioacoustics*. - Trends Plant Sci., 17: 323-325.

GAGLIANO M., RENTON M., DUVDEVANI N., TIMMINS M., MANCUSO S., 2012 b - *Acoustic and magnetic communication in plants. Is it possible?* - Plant Signal. Behav., 7: 1346-1348.

IKEDA H., 2011 - *Plant factory*. - IEEJ Trans. Sens. Micromachin., 131: 200-205.

JAYAWICKREME D.H., JOBBÁGY E.G., JACKSON R.B., 2014 - *Geophysical surface imaging for ecological applications*. - New Phytol., 201: 1170-1175.

KAWANO T., 2014 - *Plant pigmentation control under LEDs: Economic views and technological achievements behind the emergence of plant factories*. - Proceeding of Plant Factory Conference, A-12.

KAWANO T., KAWANO A., KAWANO M., 2012 - *Simplified exercise for mechanical repositioning of growing runners of forcing-cultured cucumber plants against the supporting tendril tensile strength*. - Environ. Control Biol., 50: 415-423.

KAWATA Y., 2011 - *Economic growth and trend changes in wildlife hunting*. - Acta Agriculturae Slovenica, 97: 115-123.

LEONARD O.A., ANDERSON W.S., GIEGER M., 1948 - *Effect of nutrient level on the growth and chemical composition of sweet potatoes in sand cultures*. - Plant Physiol., 23: 223-237.

MORIMOTO T., 2005 - *Fundamental research for intelligent control in cultivating and storage systems*. - Environ. Control Biol., 43: 299-303.

NAKAMURA Y., 2009 - *"Agriculture as an occupational choice" has yet to come*. - Chuokoron, 11: 42-60. In Japanese.

NISHIDA H., ARIMA S., HATOU K., TAKAYAMA K., 2012 - *Research centers for intelligent plant production systems in Japan (1) Ehime University*. - J. Sci. High Technol Agric. 24: 5-9. Japanese with abstract in English.

NISHIMURA Y., FUKUDA K., WADA T., 2012 - *Research centers for intelligent plant production systems in Japan (2) Osaka Prefecture University*. - J. Sci. High Technol Agric., 24: 10-15. Japanese with abstract in English.

PURSEGLOVE J.W., 1968 - *Tropical crops: Dicotyledons*. - Longman Scientific and Technical, NY, USA, pp. 332.

SHER J.L., HOLBROOK N.M., SILK W.K., 2001 - *Temporal and spatial patterns of twining force and lignification in stems of* Ipomoea purpurea. - Planta, 213: 192-198.

SHIMIZU H., KUSHIDA M., FUJINUMA W., 2008 - *A growth model for leaf lettuce under greenhouse environments*. - Environ. Control Biol., 46: 211-219.

SMITHWICK E.A.H., LUCASH M.S., McCORMACK M.L., SIVANDRAN G., 2014 - *Improving the representation of roots in terrestrial models*. - Ecol. Model., 291: 193-204.

SPENCE J.A., HUMPHRIES E.C., 1971 - *Effect of moisture supply, root temperature, and growth regulators on photosynthesis of isolated rooted leaves of sweet potato* (Ipomoea batatas). - Ann. Bot., 36: 115-121.

TSUCHIYA K., 1993 - *Agricultural economy (4th ed.)*. - Toyo Keizai Shinpo Sha, Tokyo, Japan. In Japanese.

WOOLFE J.A., 1992 - *Sweet potato: An untapped food resource*. - Cambridge University Press and the International Potato Center (CIP), Cambridge, UK.

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# Lipase production by *Fusarium culmorum* in solid state fermentation

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*Key words: Fusarium culmorum*, lipase activity, solid-state fermentation.

**Abstract: *Fusarium* is a large genus of filamentous fungi which cause some of the most important diseases in agricultural and horticultural crops. This fungus is considered to be a useful producer of enzymes from an industrial point of view. In the present study, lipase production by *Fusarium culmorum* SY6 was investigated under solid-state fermentation (SSF). Among the several agronomic wastes, corn cob hulls and tomato pulp supported the highest yield of lipase (170 and 165 U/g of dry substrate, respectively) after five days of incubation. It was determined that pH 9 and 60°C gave optimum enzyme activity. The *F. culmorum* SY6 strain grown in SSF in a simple medium proved to be a promising microorganism for lipase production.**

## 1. Introduction

Lipase (triacylglycerol acylhidrolases, EC 3.1.1.3) is an extra cellular enzyme which catalyses the hydrolysis of triglycerides to free fatty acids and glycerol (Singh and Mukhopadhyay, 2012). According to its various industrial applications, biotechnological uses for lipases are steadily increasing (Stamatis *et al.,* 1999). Although lipases can be obtained from bacteria and yeasts (Jaeger *et al.,* 2000; Kulkarni *et al.,* 2002), the enzymes from fungi generally meet industrial demand since they are usually excreted extracellularly, facilitating extraction from fermentation media (Hiol *et al.,* 2000; Abbas *et al.,* 2002).

*Fusarium* is a large genus of filamentous fungi, and most of *Fusarium* species are harmless saprobes and relatively abundant members of the soil microbial community (Summerell *et al.,* 2001). This ecological habitat of the fungus implies that *Fusarium* would be a useful resource of extracellular enzymes. Several different enzymatic activities were investigated in isolates of *Fusarium* species, including lipase (Burkert *et al.,* 2004; Bakri *et al.,* 2013, 2014).

Solid-state fermentation (SSF) technique involves the growth and metabolism of microorganisms on moist solids without any free flowing water. SSF has many advantages over submerged fermentation, including an economical use of space that is required for fermentation, simplification of the fermentation media, superior yields, and no requirement for complex machinery (Pandey, 1994). However, enzyme production is related to the type and concentrations of nutrients and growth conditions (Prazeres *et al.,* 2006).

To reach commercial feasibility, enzyme production must be increased by introducing more potent strains, and by optimising culture conditions (Singh and Mukhopadhyay, 2012). Since the effect of carbon sources on lipase production by the fungus *F. culmorum* has not been investigated so far, a study toward this aim was conducted on the new *F. culmorum* strain SY6 cultured under solid state fermentation (SSF).

## 2. Materials and Methods

*Microorganism*

The organism used was *F. culmorum* SY6, isolated in our laboratory and having the ability to produce lipase enzyme (Bakri *et al*., 2014). The strain was grown on Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) with 13 mg/l kanamycin sulphate added after autoclaving and incubated at 23°C for 10 days in the dark to allow mycelial growth and sporulation. The cultures were maintained on silica gel at 4°C until needed.

*Cultural conditions*

The strain *F. culmorum* SY6 was grown in 250-ml Erlenmeyer flasks containing (g/l): $Na_2HPO_4 \cdot 2H_2O$ 10; KCl 0.5; $MgSO_4 \cdot 7H_2O$ 0.15 and yeast extract 5, as nitrogen source. The mineral salt was added in such a way that the final substrate-to-moisture ratio was 1:5. The pH was adjusted to 6.5 before sterilization. The influences of different carbon sources (wheat bran, corn cobs hulles, beet pulp, tomato pulp, soya cake, cotton seed cake and wheat

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straw) on lipase production were tested (Fig. 1). The contents were sterilized by autoclaving at 121°C for 15 min. After cooling, the sterilized medium was inoculated with spores ($10^6$/ml) from a seven-day-old culture. The flasks were incubated at 30°C for five days.

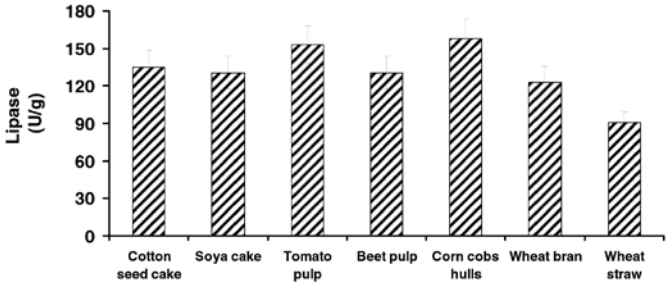


Fig. 1 - Effect of some agro-industrial wastes as carbon source on lipase production by *F. culmorum* SY6.

*Enzyme assay*

Liplolytic activity was determined titirmetrically on the basis of olive oil hydrolysis (Macedo *et al*., 1997) with some modifications. Olive oil emulsion substrate was prepared by mixing 10 mL olive oil and 90 ml from 5% Arabic gum solution. The reaction mixture contained 5 mL olive oil emulsion substrate and 1 mL of crude enzyme. The enzyme substrate mixture was incubated on an orbital shaker with a shaking speed of 150 rpm at 50°C for 20 min. To stop the reaction, 10 ml ethanol acetone mixture (1:1) was added to the reaction mixture. Liberated fatty acids were titrated with 0.05 mol/L NaOH. One lipase unit (U) was defined as the amount of the enzyme that released one µmol fatty acid per min.

*Effect of pH*

The effect of pH on lipase activity was measured at different pH values ranging from 4 to 10. The pH of the reaction mixture was varied using various buffers (citrate buffer, phosphate buffer, and borate buffer).

*Effect of temperature*

Temperature effect on lipase activity was determined at different temperatures in the range 40-80°C. Crude enzyme and substrate were tested by pre-incubating at various reaction temperatures to determine the optimal incubation temperature.

*Statistical analysis*

All the experiments were repeated twice, and the means were analyzed statistically with the analysis of variance using the STAT-ITCF program; LSD= least significant differences at $P<0.05$.

## 3. Results and Discussion

*Effect of carbon sources and lipase production*

The results showed that significant differences ($P<0.05$) in the mean lipase yield values existed among carbon sources, with values being consistently higher on corn cob hulls and tomato pulp (170 and 165 U/g, respectively) after five days of incubation, whereas, wheat straw exhibited lowest activity. These results might be attributed to the fact that the presence of more available carbon increases both mycelium growth and its activity. Moreover, corn cob granulate shows excellent adsorption characteristics (Damaso *et al.,* 2008). However, findings these agree with those of Abbas *et al*. (2002), but not with those of Burkert *et al*. (2004). In addition, our results are in agreement with those of Gombert *et al.* (1999) and Falcony et *al.* (2006) in SSF, who reported that lipase enzyme produced by fungi, could be enhanced under SSF.

Although quantitative comparison of lipase activities reported in literature is not always possible because no standard enzyme substrate has been adopted yet, the lipase productivity from *F. culmorum* SY6 observed in this work was higher than optimum productivities reported in the literature for some microorganisms grown in SSF (Table 1).

*Effect of PH*

Generally, enzymes are sensitive to the concentration of hydrogen ions present in the reaction mixture; therefore, pH is considered an important factor that determines the enzyme activity. The pH- relative activity of *F. culmorum* SY6 lipase was determined in the range 4.0-10.0 pH (Fig. 2). The optimum pH was found to be 9. Most microbial lipases have their optimum activity at a pH range of 7.0-9.0 (Zhang *et al*., 2005; Ulker *et al*., 2011). An optimum pH of 7.0 for *Rhizopus oryzae* lipase (Hiol *et al*., 2000), pH of

Table 1 - Optimum lipase activities produced by filamentous fungi grown in SS

| Microorganism | Substrate | Lipase (U/g) | Reference |
|---|---|---|---|
| *Aspergillus niger* J-1 | wheat bran | 9.14 | Falcony *et al.,* 2006 |
| *Penicillium restrictum* | babassu oil cake | 30.3 | Gombert *et al.,* 1999 |
| Fusarium culmorum | cobs hulls | 170 | This work |
| *Rhizopus oligosporous* | almond meal | 48 | Ul-Ha *et al.,* 2002 |
| *Rhizomucor pusillus* | olive oil cake and sugar cane | 79.6 | Cordova, 1998 |

8.5 for *Trichoderma harziamum* (Ulker *et al*., 2011), and pH 9.0 for *Penicillium caseicolum* lipase (Saxena *et al*., 2003) has been reported.

*Effect of temperature*

Optimization of temperature is vital for enzyme activity. In our work, the activity of F. culmorum SY6 lipase was investigated at different temperatures and the results obtained are shown in figure 3. The data reveal that the optimum temperature for lipase activity was at 60°C, followed by 70 and 80°C However, when the temperature increased or decreased from 60°C, the activity of lipase gradually reduced. Prazeres et al. (2006) and (Ulker et al., 2011) reported that Fusarium oxysporum and Trichoderma harzianum presented lipase with maximum activity at 55 and 40°C respectively.

## 4. Conclusions

The present study reveals that *F. culmorum* SY6 strain is a potential and promising microorganism as it produced a high level of lipase under solid state fermentation. Adding corn cob hulls and tomato pulp significantly increased the enzyme production (170 and 165 U/g, respectively) after five days of incubation compared to 117 U/g reported by Bakri *et al.* (2014) in which this compound was not used. Moreover, the basic parameters such as pH and temperature were found to exert a marked influence on the activity of lipase.

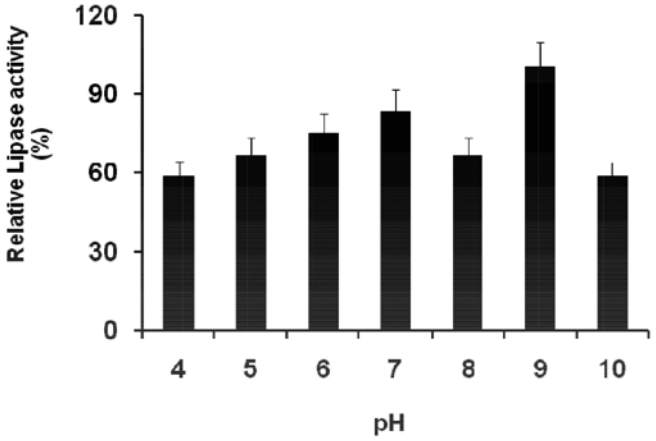


Fig. 2 - Effect of different pH values on the activity of *F. culmorum* SY6 lipase.

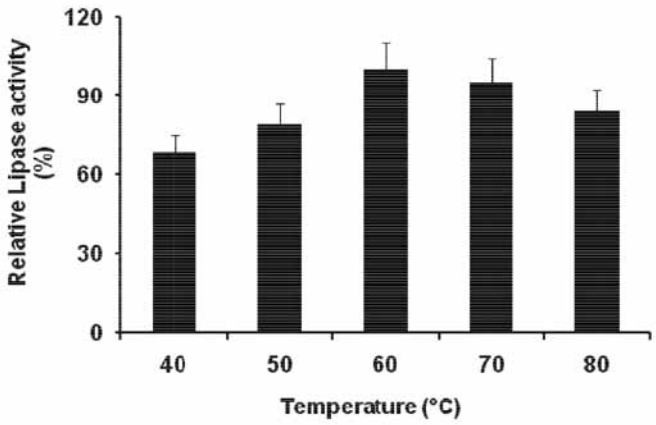


Fig. 3 - Effect of temperature on the activity of *F. culmorum* SY6 lipase.

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## References

ABBAS H., HIOL A., DEYRIS V., COMEAU L., 2002 - *Isolation and characterization of an extracellular lipase from* Mucor *spp. strain isolated from palm fruit*. - Enzyme Micob. Technol., 31: 968-975.

BAKRI Y., JAWHAR M., ARABI M.I.E., 2013 - *Xylanase production by* Fusarium solani *in solid state fermentation.* - Res. Biotech., 4: 31-37.

BAKRI Y., JAWHAR M., ARABI M.I.E., 2014 - *Enzymatic activity of the endophytic* Fusarium *species strains isolated from wheat.* - Adv. Hort. Sci., 28: 129-132.

BURKERT T.F., MAUGERI F., RODRIGUES M.I., 2004 - *Optimization of extracellular lipase production by* Geotrichum *sp. using factorial design.* - Biore. Technol., 910: 77-84.

CORDOVA J., 1998 - *Isolement identification et physiologie des champignons thermophiles en vue de la production de lipases par fermentation en milieu solide Montpellier.* - Université de Montpellier II, France.

DAMASO M.C.T., PASSIANOTO M.A., FREITAS S.C., FREIRE D.M.G., LAGO R.C.A., COURI S., 2008 - *Utilization of agroindustrial residues for lipase production by solid-state fermentation.* - Brazillian J. Microbiol., 39: 676-681.

FALCONY G., ARMAS J.C., JULIO C., MENDOZA D., JOSE L., HERMANDEZ M., 2006 - *Production of extracellular lipase from* Aspergillus niger *by solid-state fermentation.* - Food Technol. Biotechnol., 44: 235-240.

JAEGER K.E., DIJKSTRA B.W., REETZ M.T., 2000 - *Bacterial Biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications.* - Annu. Rev. Microbiol., 53: 315-351.

HIOL A., JONZO M.D., DRUET D., COMEAU L.C., 2000 - *Purification and characterization of an extracellular lipase from a thermophilic* Rhizopus oryzae *strain isolated from palm fruit.* - Enzyme and Microbial Technol., 26: 421-430.

GOMBERT A.K., PINTO A.L., CASTILHO L.R., FREIRE D.M.G., 1999 - *Lipase production by* Penicillium restrictum *in solid-state fermentation using babassu oil cake as substrate.* - Process Biochem., 35: 85-90.

KULKARNI N., GADRE R.V., 2002 - *Production and properties of alkaline, thermophilic lipase from* Pseudomonas fluorescens *NS2W.* - J. Ind. Microbiol. Biotechnol., 28: 344-348.

MACEDO G.A., PARK Y.K., PASTORE G.M., 1997 - *Partial purification and characterization of an extracellular lipase from a newly isolated strain of* Geotrichum *sp.* - Rev. Microbiol., 28: 90-95.

PANDEY A., 1994 - *Solid state fermentation: an overview, In: Solid state fermentation.* - Ashok Pandey, Wiley Eastern, New Deli, India.

PRAZERES J.N.D., CRUZ J.A.B., PASTORE G.M., 2006 - *Characterization of alkaline lipase from* Fusarium oxysporum *and the effect of different surfactants and detergents on the enzyme activity.* - Braz. J. Microb., 37: 505-509.

SAXENA R.K, DAVIDSON W.S., SHEORAN A., GIRI, B., 2003 - *Purification and characterization of an alkaline thermostable lipase from* Aspergillus carneus. - Process Biochem., 39: 239-247.

SINGH A.K., MUKHOPADHYAY M., 2012 - *Overview of fungal lipase: a review.* - Appl. Biochem. Biotechnol., 166: 486-520.

STAMATIS H., XENAKIS A., KOLISIS F., ENANTIOMERIC N., 1999 - *Selectivity of a lipase from* Penicillium simplicissimum *in the esterification of menthol in microemulsions.* - Biotechnol. Lett., 15: 471-476.

SUMMERELL B.A., LESLIE J.F., BACKOUSE D., BRYDEN W.L., BURGESS L.W., 2001 - *Fusarium*: Paul E. Nelson Memorial Symposium. 1st Ed. - American Phytopathological Society Press, St. Paul Minnesota, USA.

UL-HA I., IDREES S., RAJOKA M.I., 2002 - *Production of lipases by* Rhizopus oligosporous *by solid-state fermentation.* - Process Biochem., 37: 637-641.

ULKER S., OZEL A., COLAK A., KARAOGLU S.A., 2011 - *Isolation, production, and characterization of an extracellular lipase from* Trichoderma harzianum *isolated from soil.* - Turkish J. Biol., 35: 543-550.

ZHANG H.Y., WANG X., CHING C.B., WU G.C., 2005 - *Experimental optimization of enzymic kinetic resolution of* racemic flurbiprofen. - Biotechnol. Appl. Biochem., 42: 67-71.

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# Influence of seed provenance on the propagation of *Picea abies* (L.) Karst

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*Key words*: germination, growth, *Picea abies*, somatic embryogenesis, substrate.

**Abstract: *Picea abies* (L.) Karst. is the most common species cultivated in Italy for sale as Christmas trees. The aim of this study was to identify the best seed provenance to improve plant production. Three Italian provenances - Gran Bosco di Salbertrand, Pezzel e Fochino, and Val di Fiemme - were examined. A part of seeds were sown on benches in a greenhouse after cold stratification, and another part was used for induction of somatic embryogenesis. In the first experiment, two different amounts of organic matter (manure), as components of growing media, were evaluated, while in the second one different levels and combinations of growth regulators in the medium were tested. The seed provenance and composition of the growing medium influenced seed germination and seedling growth. The best performance on both growing media was achieved by the Val di Fiemme provenance, and in general seedling emergence and growth were the most favourable in the growing medium with a lower organic matter amount. Also, the *in vitro* cultures evidenced differences in the efficiency of somatic embryogenesis among the provenances.**

## 1. Introduction

Norway spruce [*Picea abies* (L.) Karst.] is an economically important tree species that plays a key role in natural ecosystems in the boreal zone from Norway to Siberia, and is one of the most valuable European forest tree species. The natural distribution of *P. abies* in Italy spans the entire Alpine range, where it ascends to an altitude of 2,000 m, and it is also found in part of the northern Apennines. A large portion of the spruce forests are used commercially and managed with varying levels of intensity. In Italy, this species is also the most cultivated for the production of Christmas trees for its conical shape, compact, intense green foliage, and rapid growth. The Christmas trees present on the Italian market are certified because they derived from 90% cultivations in forest nurseries and the trees are usually obtained by seed. Seeds are collected from selected stands, and the obtained seedlings are cultivated in a nursery for almost four years: two years as "seedlings" in seedbeds, and two to three years as "transplantations" in fields depending on the desired size. Christmas tree cultivation is concentrated mainly in Veneto and Tuscany (Arezzo and Pistoia districts) where about 800 hectares are devoted to this crop. Nevertheless, a considerable amount of the seedlings are imported from Belgium, Denmark, Holland, and Germany for the establishment of Christmas tree plantations. Recently, increased importation from Hungary, Romania and Moldavia has evidenced problems related to the genetic control of propagation material. Thus, to avoid genetic mixing between autochthonous species and those coming from abroad, the imported seedlings carry a special label which certifies their origin from specialized cultivations, nationality, and indication as not intended for reforestation.

A sustainable Christmas tree market should aim to increase the production of plants from autochthonous species characterized by high levels of germinability and growth, and to produce seedlings of adequate size and quality in a short time for transplantation.

The commercial importance of genetic characteristics and origin of the propagation material for plantation quality are well documented. In particular, rates of early growth and morphological and physiological characteristics are among the features that differ between populations. Consequently, the provenance of seeds in relation to the choice of planting site is extremely important. Furthermore, identification of both the best seed provenance and environmental conditions for germination and growth of seedlings could be the first objective to improve nursery production. The influence of provenance has not been widely reported on seed germination of *Picea*, while it is well documented for seedling growth. At provenance level, strong relationships are gener-

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ally observed between traits that characterise the duration of the growth period and the degree of lignification. Variation in bud flushing and initiation of shoot growth among plants derived from different Norway spruce seed provenances is assumed to be regulated both by differential responses to the accumulated temperature sum in spring and by conditions during acclimation in the preceding year (Schmidt-Vogt, 1976; Dormling, 1982). Accordingly, trees from with northern latitude or high altitude provenances, which are adapted to a short growing season, have inferior growth potential compared with those adapted to a longer growing season. Variation between plants from different provenances has been demonstrated for a number of other traits, such as nutrient demands, respiratory activity, shade tolerance, and differences in crown shape, and resistance to snow and ice damage (Schmidt-Vogt, 1976).

On the other hand, seedling morphological characteristics before planting were found to highly affect seedling growth during the first years after transplantation (Tsakaldimi, 2006). Furthermore, fertilizer application during two years improved survival and increased annual height of *Betula pubescens* and *Larix sibirica* (O´Skarsson *et al*., 2006). Many studies have reported the effect of manure on increase diameter and height growth of some species of *Pinus* (Nourshad and Ghorani, 1990), and biomass (root and shoot dry weight) of potted and bare rooted *P. taeda* seedlings (Kiani *et al*., 2005). Moreover, combinations of types of soil in different ratios of nutrients have also influenced seed germination of important forest species (Selivanovskaya and Latypova, 2006). Germination, survival, growth, and biomass of *Cupressus arizonica* and *Cupressus sempervirens* are enhanced using organic matter and in particular, manure has increased the maximum quality index (Dickson *et al*., 1960; Ahmadloo *et al*., 2012).

Therefore, the aim of this study was to assess the influence of seed provenance on emergence and seedling growth of *Picea* and, as an ancillary purpose, to evaluate if an increase of the amount of organic matter (dust manure) in the growing media compared to that commonly used can improve or affect the emergence and seedling growth of *Picea* seeds of different provenance. Further, considering that somatic embryogenesis is potentially the most promising method for vegetative propagation of coniferous genera (Dunstan *et al*., 1995), the influence of seed provenance has also been evaluated on somatic embryo germination starting from embryogenic tissues.

## 2. Materials and Methods

*Seed lots*

The seeds were provided by the National Centre for the Study and Maintenance of Forest Biodiversity (CNBF) of Peri (Verona, Italy). CNBF is primarily involved in the production, selection, and conservation of forest seeds (of trees and shrubs). Specifically, the forest seeds produced by the Centre come from forests enrolled in the "Libro Nazionale Boschi da Seme" (L.N.B.S.), and traditional and "special" harvesting methods are used in order to preserve the genetic diversity of the species. All the material is certified and traceable through Global Positioning System (GPS) technology (http://www3.corpoforestale.it). In this study, mature seeds of *Picea abies* (L.) Karst. harvested in October 2011 from selected populations of the following three Italian provenances were evaluated: Gran Bosco di Salbertrand - GS (L.N.B.S n. 088, CSR n. 034-Pie; 45°N; altitude 1030-1900 m); Pezzel e Fochino - PF (L.N.B.S n. 137 SO 037; 46°N; 1350-1540 m); and Val di Fiemme - VF (L.N.B.S n. 023 Val di Fiemme TN; 46°N; 1600-2000 m).

Certification of seed quality (purity, weight of 1000 seeds, germinability, cut test, viability, value crop) in accordance with the ISTA (International Seed Testing Association) was carried out by CNBF and is reported in Table 1.

*In vivo* experiment

The seeds from each provenance were stored separately in plastic bags at 5°C. Later, two sowing beds (each 1.60 m × 5.0 m) were prepared in a greenhouse. A common nursery soil, composed of a mixture of 75% sand, 10% perlite, and 15% organic material (mixture of peat, manure) for a total organic C content of 6.26%, was utilized for the first bed (Substrate 1). The same substrate with a rate higher than one-and-a-half of organic material (total organic C 9.82% ) was used for the second bed (Substrate 2) (Table 2). Chemical parameters of growing media were determined according to the SISS methods (SISS, 1985), whereas the ones of organic carbon (OC), nitrogen (N), and inorganic carbon (IC) by using NA 1500 CHNS Analyzer, Carlo Erba (Milan, Italy) coupled with the procedure reported by Santi *et al*. (2006). In March 2012, an average of 20 and 15 g seeds, respectively, for GS, PF, and VF provenances were sown on each sowing bed. Before the trials, the seeds were soaked in water for 12 h. The seeds were sown in seven rows, and a thin layer

Table 1 - Certification of seed quality

| Seed provenance | Purity (%) | Weight 1000 seeds | Germinability (%) | Cut Test (%) | No. viable seeds/g | Value crop (%) |
|---|---|---|---|---|---|---|
| Gran Bosco Salbertrand (LNBS n.088 (SR n.054- Pie.) | 94.5 | 7 | 71 | 91 | 96 | 67.1 |
| Pezzel e Fochino (LNBS n.137 SO037) | 92 | 6.4 | 74 | 88 | 113 | 68.1 |
| Val di Fiemme (LNBS n.023 Val di Fiemme) | 97.2 | 8.8 | 92 | 97 | 119 | 89.4 |

Table 2 - Chemical characteristics of growing media

| Growing media | Substrate 1 | Substrate 2 |
|---|---|---|
| C[(z)] (%) | 6.26 | 9.82 |
| Organic matter %) | 10.91 | 17.25 |
| N [(y)] (%) | 0.2 | 0.39 |
| C/N | 31.3 | 25.17 |
| IC [(x)] (%) | 2.83 | 1.47 |
| CEC [(w)] ( $mol_c kg^{-1}$ ) | 14.4±2.7 | 32.0±3.9 |
| pH | 7.3±0.2 | 7.1±0.2 |

[(z)] C= Organic carbon;
[(y)] N= Nitrogen;
[(x)] IC= Inorganic carbon;
[(w)] CEC= Cationic exchange capacity.

of sand was applied to cover the seeds. The sowing beds were watered as necessary. After emergence, the seedlings were fertilised twice at intervals of 15 days with 50 ppm Flory 4 (Agrochimica). Emergence rates were recorded in March, April, and May. The final stem height (cm) was determined for all seedlings at 90 days, the time of their transfer to pots. In June 2012 the seedlings were transferred to pots (volume 80 ml) filled with the same growing media. Two hundred seedlings per provenance were distributed in five multipot PVC pots of 40 places each. The seedlings were transferred to a shaded (50% light reduction) location outdoors and cultivated until the end of July 2013. Growth was evaluated after 4, 12 and 52 weeks. On the final date, 15 seedlings per provenance were randomly selected for root and shoot biomass measurements. The fresh weight (FW) and dry weight (DW), obtained by drying in an oven at 70°C for 48 h, were determined. The root-to-shoot DW ratio was also calculated for each seedling.

*In vitro* experiment

For induction of embryogenic tissues, the seeds were sterilized in 2% (v/v) sodium hypochlorite solution for 20 min and rinsed three times with sterile distilled water. The zygotic embryo was dissected from each seed and placed on solid BM1 medium (Gupta *et al.*, 1987) supplemented with 1% sucrose, hydrolysed casein (500 mg/l), L-glutamine (450 mg/l), and myo-inositol (1g/l), and solidified with 0.3% Phytogel. Five or 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 2.5 or 5 µM 6-benzyladenine (BA) were included in the medium. The trial was carried out in Petri dishes (100 mm × 15 mm) with 40 embryos per provenance, divided into five replicates of eight embryos each. The embryos were incubated in the dark at 20°C. The cultures were assessed after incubation for four weeks.

For culture proliferation, the embryogenic tissues were subcultured three times on fresh medium and kept in the dark for development of early-stage somatic embryos. The fresh weight of embryogenic tissues was measured at the end of each subculture. For somatic embryo maturation, embryogenic tissues from material derived from each provenance were transferred to medium containing abscisic acid (ABA) to allow maturation of the somatic embryos. The maturation medium was BM1 medium supplemented with 20 µM ABA, 2% sucrose, and 7.5% polyethylene glycol (PEG-4000). Embryogenic tissue clumps growing vigorously and of about the same size (about 1 $cm^3$ in volume) were selected to monitor the embryo maturation process. The cultures were maintained in the dark at 20°C for about five weeks until the somatic embryos reached cotyledonary stage. Thirty randomly selected mature somatic embryos per provenance were transferred to germination BM1 basal medium supplemented with 0.2% activated carbon (AC), 2% sucrose and 0.4% Phytogel. The embryos were cultured under photoperiod (16h/8h, light/dark) for eight weeks, after which time the plantlets were transferred to *ex vitro* conditions. They were placed in multipot PVC pots filled with a mix of pumice, peat, and organic fertilizer, and they were transferred to greenhouse conditions.

*Collection data and statistical analysis*

With regard to seedling emergence in the greenhouse, the number of seeds that were sown on each seed bed for each provenance was calculated using the proportion:

Weight in grams of 1000 seeds:1000=Weight in grams of seeds sown on each bed:x

For each provenance, seedling emergences were normalized with respect to the number of seeds put to germinate on each bed. Therefore, the data on the emergence of seedlings from different provenances were normalized to 100 according to this proportion:

Seedling emergence:Total seeds put to germinate x row of seed bed=x:100

Seedling data (emergence and height) were arranged in a completely randomized design, and the statistical significance of all differences in emergence and height of the seedlings was tested using factorial analysis of variance (ANOVA), with the substrate and provenance as the main factors. Differences among provenances were evaluated using Tukey's Multiple-Range Tests (Snedecor and Cochran, 1980) at the 5% level of significance. For seedling growth parameters, means and standard errors were calculated for material derived from each provenance. All statistical analyses were performed with STATISTIX software version 8 (Analytical Software, Tallahassee, FL, USA).

## 3. Results

In vivo *experiment*

The three provenances significantly ($p<0.05$) varied in emergence and seedling height (cm) in the two different

substrates used in the seed beds (Table 3). Seed provenance significantly affected all these processes. In addition, the substrate effect was also significant except at 15 days after sowing, and the substrate x provenance interaction effect was also significant. More precisely, seedling emergence in the greenhouse occurred 15 to 30 days after sowing, and thereafter it was very low or non-existent (data not shown). With regard to substrate 1, which contained a lower organic matter content, seedling emergence differed significantly among the three provenances at both 15 and 30 days after sowing. At 15 days, VF showed 58% seedling emergence, whereas GS and PF showed 38 and 20% seedling emergence, respectively. After 30 days, the percentage of seedling emergence for PF increased to 25%, whereas GS and VF (Fig. 1A) showed the highest seedling emergences of 62 and 91%, respectively. As for substrate 2, which contained the highest organic matter content, the emergence percentage ranged from 34% for GS to 36 and 38% for VF and PF, respectively, after 15 days. Subsequently, a large percentage of seedlings for GS and PF appeared flaccid and twisted as if suffering from wilt. These seedlings continued to decline, assumed a dark colour and greasy appearance, and then quickly disintegrated to leave areas of bare soil in the seed bed (Fig. 1B). After 30 days, seedling emergence was reduced to 20%, whereas that for VF increased to 47%.

Seeds from the VF provenance generated the most seedlings on both substrates; seeds from PF displayed the worst performance on the same substrates at the end of culture in the greenhouse (Table 3). In contrast, GS provenance produced the most seedlings only on substrate 1. The final stem height was determined for all seedlings at 90 days from sowing. The stem height of seedlings ranged from 2.4 to 4.3 cm on substrate 1, and from 2.1 to 3.2 cm on substrate 2. VF seedlings showed the greatest stem height on substrate 1; PF seedlings exhibited the lowest

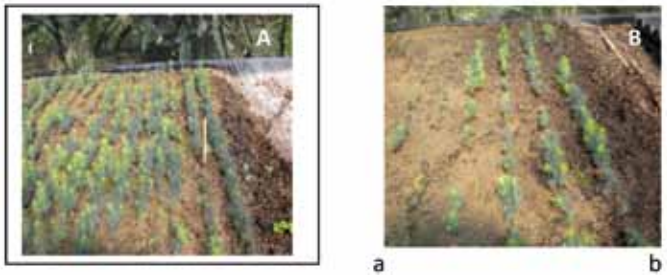


Fig. 1 - Emergence and growth of seedlings on different seed beds at 30 days after sowing: (A) seedlings from the provenance Val di Fiemme on substrate 1; (B) at left, seedlings from the provenance Pezzel and Fochino, showing areas of bare soil, and at right, seedlings from the provenance Val di Fiemme on substrate 2.

on both substrates. The stem height of GS seedlings was highest on substrate 1.

The growth trends were not different for seedlings of the provenances measured after 4, 12 and 52 weeks cultivation in pots (Fig. 2). At the time of initial measurement (4 weeks), PF (7.9 cm) and GS (7.4 cm) seedlings showed superior stem growth compared to VF seedlings (6.4 cm). At the end of the cultivation period (52 weeks), GS and PF seedlings had mean stem heights of 14.7 and 13.8 cm, respectively, whereas VF seedlings attained a mean height of 10 cm (Fig. 2). The minimum standard defined by the European Economic Community for growth of three-year-old seedlings is 14 cm in height (E.E. C. Minimum Standards) (Magini, 1977). Therefore, the recorded growth in seedlings of 15 months old from GS and PF provenance exceeded the minimum standard. Among the provenances, seedlings grown in pots exhibited changes in early growth, and fresh and dry matter allocation (Table 4). GS seedlings showed significantly higher values in fresh shoot biomass. On the other hand, VF provenance, compared with GS,

Table 3 - Seedling emergence and early growth of three provenances of *P. abies* in two different substrates

| Growing medium | Provenance | Seedling emergence (%) | | Seedling height [(Z)] (cm) |
|---|---|---|---|---|
| | | 15 days | 30 days | |
| Substrate 1 | Gran Bosco di Salbertrand | 38 b | 62 b | 4.3 a |
| | Pezzel e Fochino | 20 c | 25 cd | 2.4 d |
| | Val di Fiemme | 58 a | 91 a | 3.7 b |
| Substrate 2 | Gran Bosco di Salbertrand | 34 bc | 20 d | 2.6 d |
| | Pezzel e Fochino | 38 b | 20 d | 2.1 d |
| | Val di Fiemme | 36 bc | 47 bc | 3.2 c |
| Two-way ANOVA (P values) | | | | |
| Substrate (S) | | 0.4152 | 0.0000 | 0.0000 |
| Provenance (P) | | 0.0005 | 0.0000 | 0.0000 |
| S x P | | 0.0001 | 0.0013 | 0.0000 |

Values for each parameter, column, and factor followed by different letters are significantly different (Tukey's multiple range test, $p < 0.05$).
[(z)] The final stem height was determined for all seedlings at 90 days from sowing: the average for Gran Bosco di Salbertrand, Pezzel e Fochino and Val di Fiemme was calculated on 200, 123 and 331 seedlings for the substrate 1 and on 867, 257 and 801 seedlings for the substrate 2, respectively.

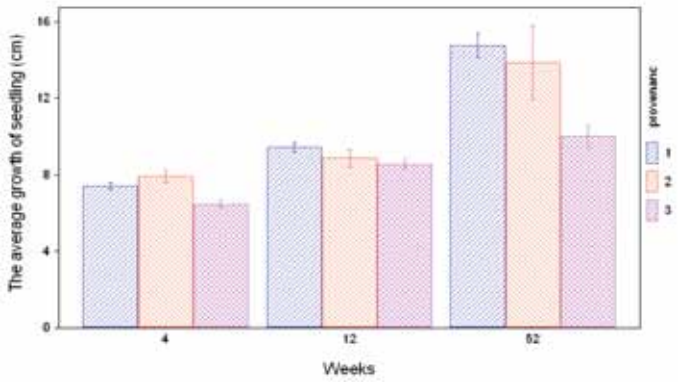


Fig. 2 - Stem heights of *Picea abies* seedlings grown in pots outdoors measured after 4, 12, and 52 weeks of cultivation in pots. Data represent the means (±SE) for seedlings from the Gran Bosco di Salbertrand (1), Pezzel e Fochino (2), and Val di Fiemme (3) provenances. A minimum of 200 seedlings were measured for each provenance.

showed similar values in dry shoot biomass, root biomass, and root/shoot ratio, but lower values were always found in PF provenance.

In vitro *experiment*

Explants from all examined provenances give rise to embryogenic tissues (Table 5). The mature zygotic embryos from GS seeds yielded a higher frequency (13.5%) of embryogenic tissue induction, compared with those for PF (8.6%) and VF (5.5%). A higher frequency (13.4%) of embryogenic tissue induction, independent of provenance, was achieved on medium supplemented with low concentrations of growth regulators (5 µM 2,4-D and 2.5 µM BA) than on medium with higher concentrations of growth regulators (10 µM 2,4-D and 5µ M BA). With regard to the proliferation of embryogenic tissues, only the provenance GS showed the highest increase in fresh weight of embryogenic tissue and the capacity for embryo maturation in the presence of low concentrations of growth regulators. In contrast, a greater increase in embryogenic tissue fresh weight was achieved for all provenances on medium supplemented with the higher concentrations of 2,4-D and BA. These cultures were whitish to translucent, and were characterised by vigorous growth (Fig. 3 A, B); maturation of embryogenic tissues for each provenance is reported in Table 5. Specifically, GS and PF embryogenic tissues showed the highest maturation efficiency (mean of 52 and 73 somatic embryos per clump, respectively), whereas the VF embryogenic tissues produced an average of only 24 somatic embryos per clump. The maturation process up to the cotyledonary stage required about 5 weeks of culture on the maturation medium (Fig. 3 C-E). Over 74% of the somatic embryos germinated rapidly after transfer to the germination medium. The development of plantlets with true needles was observed within 1 to 2 weeks (Fig. 3F).

Table 4 - Morphological characteristics of *Picea abies* seedlings grown in pots outdoors

| Provenance | Mean number | | Shoot biomass (g) | | Root biomass (g) | | Root to shoot ratio |
|---|---|---|---|---|---|---|---|
| | Branche | Roots | Fresh | Dry | Fresh | Dry | |
| G S | 3.0±0.46 a | 9.7±1.34 a | 5.15±0.09 a | 2.71±0.08 a | 1.23±0.12 a | 1.06±0.07 a | 0.395 a |
| P F | 1.3±0.44 a | 7.0±1.29 a | 2.15±0.18 c | 1.88±0.16 b | 0.52±0.12 b | 0.44±0.07 b | 0.257 a |
| V F | 1.8±0.63 a | 7.0±1.29 a | 4.45±0.10 b | 2.66±0.09 a | 1.16±0.14 a | 1.05±0.09 a | 0.390 a |

All parameters of growth were evaluated after 52 weeks of growth in pots. The data were subjected to analysis of variance. Values are the mean of a minimum of 15 seedlings for each provenance. Means within a column followed by a different letter are significantly different (Tukey's multiple range test, P< 0.05).
GS= Gran Bosco di Salbertrand;
PF= Pezzel e Fochino;
VF= Val di Fiemme.

Table 5 - Induction of embryogenic cultures, proliferation, and somatic embryo maturation

| Provenance | Embryogenic cultures (%) | Increase of mass embryogenic (g) | | Embryo maturation | |
|---|---|---|---|---|---|
| | | 5µM 2.4D+ 2.5µM BA | 10µM 2.4D+ 5µM BA | No. somatic embryos/clump | Total no. embryos/clump |
| Gran Bosco Salbertrand | 13.5±2.35 a | 1.09±0.60 a [z] | 1.51±0.26 a | 52.0±15.0 b | 619 |
| Pezzel e Fochino | 8.6±2.72 a | 0.22±0.15 a | 1.66±0.39 a | 73.0±11.5 a | 881 |
| Val di Fiemme | 5.5±2.11 a | 0.30±0.10 a | 1.13±0.48 a | 24.0±9.0 c | 288 |
| Growth regulators | | | | | |
| 5µM 2.4D+ 2.5µM BA | 13.4±1.57 a | | | | |
| 10µM 2.4D+ 5µM BA | 5.0±2.72 b | | | | |

[z] Data represent increase of fresh weight over time. The data were subjected to analysis of variance. Values within a column followed by a different letter are significantly different (Tukey's multiple range test, P < 0.05).

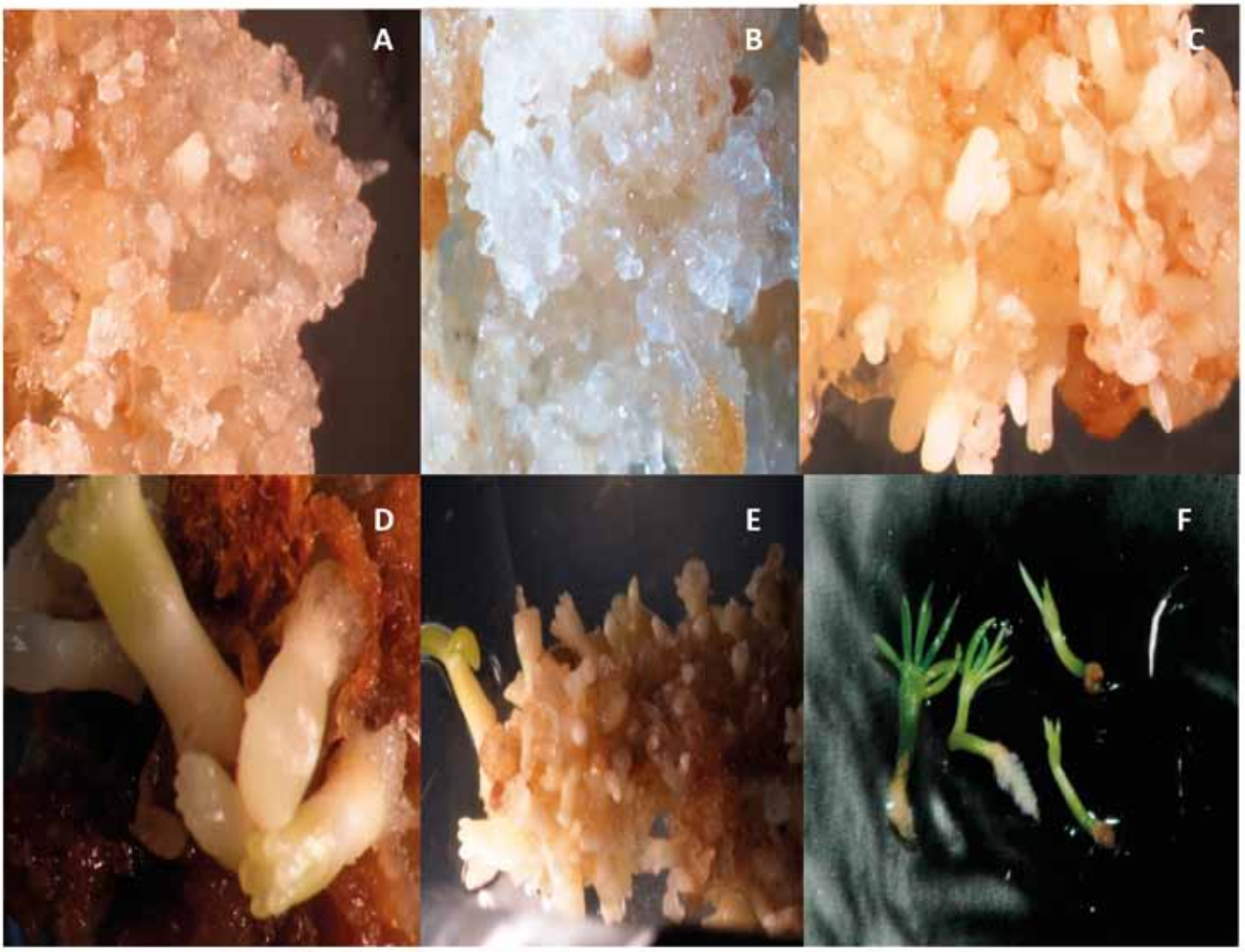


Fig. 3 - Somatic embryogenesis from mature zygotic embryos of *Picea abies*; proliferation of embryogenic tissues (A and B); maturation (C-E) and germination (F) of somatic embryos.

## 4. Discussion and Conclusions

The efficiency of *P. abies* propagation from seeds and by somatic embryogenesis was closely related to the provenance of the plant material. Furthermore, the emergence and growth of seedlings propagated in seed beds, and the capacity for somatic embryogenesis were influenced by the composition of the growing medium. With regard to seed propagation, the best performance in terms of production and seedling growth was achieved by the Val di Fiemme provenance on both growing media, whereas the Pezzel e Fochino provenance showed the worst performance. Moreover, the positive results of VF provenance on emergence and growth of seedlings in seed beds are also confirmed by the best values regarding seed quality of this provenance. In addition, the organic matter added to growth medium and its content should be taken into account. Malakouti and Homaei (2004) reported that organic matter improved seed germination and seedling growth, providing suitable conditions for seedling production. On the contrary, this study shows that the highest content of organic matter seems to induce a detrimental effect on growth and seedling survival. Probably high amounts of organic matter in the nursery environment promotes favourable conditions for the development of pathogens living in the growing medium. Therefore, the mortality of Gran Bosco di Salbertrand and Pezzel e Fochino seedlings could be due to a disease, likely damping off, as evidenced from areas of bare soil in the seed beds. In contrast, the VF seedlings showed superior growth and survival and seem to be more resistant to pathogens. Consequently, control of organic-matter content, humidity level in seed beds, and reduction in the duration of cultivation under greenhouse conditions are important factors to achieve a high percentage of seedling emergence and growth. At the end of the cultivation period in pots, the fifteen-month seedlings of GS provenance showed a height growth in excess of the minimum standard for three-year-old seedlings. However, the VF and GS provenances showed similar values in dry matter,

while lower values were always found in PF. On basis of these results, the production of Christmas trees could be increased by the use of Val di Fiemme seeds, which showed higher germinability and growth of seedlings and by shortening the time for transplanting.

To the authors' knowledge, this is the first report of the application of somatic embryogenesis technology to *P. abies* seeds from different Italian provenances. Our results demonstrate a low production of embryogenic tissues, and among the provenances a different efficiency was evident with regard to the proliferation and maturation of somatic embryos. Only the GS provenance was able to develop somatic embryos capable of germination on both proliferation media; the other provenances gave rise only to somatic embryos on medium with a high concentration of growth regulators. Specifically, GS and PF provenances showed the highest maturation efficiency in terms of the number of embryos per embryogenic clump. These results obtained *in vitro* are in line with previous studies conducted on conifers. Chen *et al*. (2010) reported low frequency of embryo initiation and genetic specificity of explants as serious problems associated with embryogenesis induction. Moreover, the induction of somatic embryogenesis varies greatly among different genotypes under identical culture protocols and proliferation and maturation levels have been identified as major constraints to somatic embryogenesis (Tang *et al*., 2001).

In order to improve the efficiency of propagation from seeds and by somatic embryogenesis, further studies will be finalized to manipulate the composition of the growing medium to improve the emergence and growth of seedlings and frequency of embryogenic tissues.

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## References

AHMADLOO F., TABARI M., HAMED Y., KOOCH Y., AHMAD R., 2012 - *Effects of soil nutrient on seedling performance of arizona cypress and medite cypress*. - Annals of Biological Research, 3(3): 1369-1380.

CHEN S., CHEN S., CHEN F., WU T., WANG Y., SHANJUN Y., 2010 - *Somatic embryogenesis in mature zygotic embryos of* Picea likiangensis*.* - Biologia, 65(5): 853-858.

DICKSON A., LEAF A.L., HOSNER J.F., 1960 - Q*uality appraisal of white spruce and white pine seedling stock in nurseries.* - The Forestry Chronicle, 36(1): 10-13.

DORMLING I., 1982 - *Frost resistance during bud flushing and shoot elongation in* Picea abies. - Silva Fennica, 16: 167-177.

DUNSTAN D.I., TAUTORUS T.E., THORPE T.A., 1995 - *Somatic embryogenesis in woody plants,* pp. 471-538. - In: THORPE T.A. (ed.) In vitro *embryogenesis in plants*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 566.

GUPTA P.K., DURZAN D.J., FINKLE B.J., 1987 - *Somatic polyembryogenesis in embryogenic cell masses of* Picea abies *(Norway spruce ) and* Pinus taeda *(loblolly pine) after thawing from liquid nitrogen.* - Canadian Journal of Forest Research, 17: 1130-1134.

ITALIAN SOCIETY FOR SOIL SCIENCE, 1985 - *Metodi Normalizzati di Analisi del Suolo*. - Edagricole, Bologna, Italy.

KIANI B., SHARAJI ROSTAMII T., TAHERI F., 2005 - *Studying ability rhizogenesis* Pinus taeda *seedlings in bare root and potting condition.* - Iranian Journal Natural Resources, 58(2): 333-338.

MAGINI E., 1977 - *Appunti di vivaistica forestale*. - Clusf Cooperativa Editrice Universitaria.

MALAKOUTI M.J., HOMAEI M., 2004 - *Soil fertility of arid and semi-arid regions (Difficulties and Solutions).* - Tarbiat Modares University Press, Tehran, Iran, pp. 482.

NOURSHAD M., GHORANI M., 1990 - *Research projects selection the best soil mixture in order to container seedlings production.* - Afforestation and Park Bureau Press, Mazandaran, pp. 57.

O´SKARSSON H., SIGURGEIRSSON A., RAULUND-RASMUSSEN K., 2006 - *Survival, growth, and nutrition of tree seedlings fertilized at planting on Andisol soils in Iceland: Six-year results*. - Forest Ecology and Management, 229(1-3): 88-97.

SANTI C.A., CERTINI G., D'ACQUI L.P., 2006 - *Direct determination of organic carbon by dry combustion in soils with carbonates.* - Communications in Soil Science and Plant Analysis, 37: 155-162.

SCHMIDT-VOGT H., 1976 - *Die Fichte. Band I. Taxomomie, Verbreitung, Morphologie, Okologie, Waldgesellschaften.* - Verlag Paul Parey, Hamburg and Berlin.

SELIVANOVSKAYA S.Y.U., LATYPOVA V.Z., 2006 - *Effects of composted sewage sludge on microbial biomass, activity and pine seedlings in nursery forest.* - Waste Management, 26(11): 1253-1258.

SNEDECOR G.W., COCHRAN W.G., 1980 - *Statistical methods*. - Iowa University Press, Ames, Iowa, USA.

TANG W., WHETTEN R., SEDEROFF R., 2001 - *Genotypic control of high-frequency adventitious shoot regeneration via somatic organogenesis in loblolly pine.* - Plant Science, 161: 267-272.

TSAKALDIMI M., 2006 - *Kenaf (*Hibiscus canna*binus L.) core and rice hulls as components of container media for growing (*P. halepensis *M.) seedlings*. - Bioresource Technology, 97(14): 1631-1639.

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# Effects of 1-MCP and ethylene on antioxidant enzyme activity and postharvest physio-biochemical characteristics of cut carnation flower cv. Fortune

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*Key word*: Ethylene, peroxidase enzyme, postharvest longevity, senescence, superoxide dismutase.

**Abstract: Carnation (*Dianthus caryophyllus* L.) flowers are one of the most important cut flowers in the world. The majority of carnation cultivars are sensitive to ethylene which affects the physiological and biochemical postharvest characteristics of these flowers. Applying inhibitors of biosynthesis and action of ethylene is important factor to protect the display quality and extend postharvest life. In order to evaluate the effects of 1-methylcyclopropene (1-MCP) and ethylene on the antioxidant enzyme activity of cut carnation cv. Fortune and subsequently on the extension of vase-life, this experiment was designed as a Completely Randomized Design (CRD) with three replications. Carnation cut flowers were treated with 1-MCP at concentrations of 0, 0.5, 1 and 1.5 µl/l for 24 h and subsequently exposed to ethylene (1 µl/l) for 16 h. Data were analyzed using MSTAT-C statistical software and means were compared based on Least Significant Differences (LSD) test ($p< 0.01$). Our results showed that 1-MCP treatment had significant effects on vase life and biochemical characteristics like contents of leaf chlorophyll, petal anthocyanin, petal cell membrane stability and antioxidant enzymes activity such as catalase, peroxidase, and superoxide dismutase. The highest vase life and cell membrane stability were appeared in samples treated with 1.5 µl/l 1-MCP which was significantly higher than 0 and 0.5 treatments, although there was no significant difference with 1 µl treatment. The highest chlorophyll and anthocyanin contents were also measured under 1.5 µl/l 1-MCP which was significantly higher than other treatments. The highest and lowest catalase and peroxidase activity were related to 1 and 0 µl/l 1-MCP, respectively. The highest and lowest superoxide dismutase activity was observed in 1.5 and 0 µl/l 1-MCP. In conclusion, application of 1-MCP improved and delayed the onset of senescence symptoms resulted in extending the vase life of cut carnation cv. Fortune.**

## 1. Introduction

Carnation (*Dianthus caryophyllus* L.) flowers are one of the most important cut flowers cultivated commercially in the world and they are also used as ornamental plants for decorative purposes in orchards and landscapes (Singh *et al*., 2005). Vase life of cut flowers is an important characteristics in crop quality evaluation and it depends on genetic and environmental factors (Seglie *et al*., 2011). Ethylene, as a gaseous plant hormone, plays a prominent role in accelerating senescence phenomena of most plant organs such as fruits, flowers, and floral buds (Yang and Hoffman, 1984). When plants produce ethylene, or are exposed to an external source of ethylene, receptors perceive its presence and the signal is transferred by downstream active genes. Ethylene, by inducing expression of senescence-related genes, affects the physiological characteristics vegetative and reproductive organs resulting in organ abscission and yellowing (Ahmadi *et al*., 2008). It has been found that exogenous ethylene in some plants like miniature roses, geranium, and begonia has undesirable effects on flower quality and results in accelerating senescence and decreasing flower life (Ahmadi *et al*., 2009; Seglie *et al*., 2011). Application of external ethylene in rose petals causes a considerable increase in ethylene production and activity of ACC-synthase and ACC-oxidase genes (Ma *et al*., 2006; Ahmadi *et al*., 2009), although ethylene receptor genes are not affected by external ethylene (Ahmadi *et al*., 2009). Senescence is an oxidative process in which reactive oxygen species and antioxidants play an important role. In general, reactive oxygen species accelerate flower senescence by increasing cell membrane permeability due to decreasing proteins and nucleic acids resulting from different protease and nuclease enzyme activities (Barth *et al*., 2006). Decreasing antioxidant enzyme activity and increasing peroxidation of cell membrane lipids have been indicated as possible reasons of senescence in different plant species (Buchanan-Wollaston, 1997). In addition, cell death is accelerated in this phase because of a boost in ethylene production during the senescence period (Ebeles *et al*., 1992). Plants gain from antioxidant mechanisms to

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alleviate the effects of free radicals. These mechanisms include changes in content of defense-related enzymes such as peroxidase, catalase, polyphenol oxidase and other compounds like phenols (Staskawicz *et al*., 1995). Since postharvest senescence is an important restricting factor in crop presentation and marketability of many cut-flowers, using high confidence methods to delay crop senescence is of great importance. 1-MCP as an anti-ethylene compound has been proved to be effective in inhibiting ethylene response by competing with ethylene for placing on the site of ethylene receptors (Seglie *et al*., 2011; Daneshi Nergi and Ahmadi, 2014). Studies have shown that 1-MCP has inhibited the phenomena of petal fall in geranium, considering that its effectiveness depends on transport conditions, storage temperature, and application times (Cameron and Reid, 2001). Studies on cut carnation showed that all concentrations of 1-MCP decreased ethylene production and chlorophyll destruction was delayed in comparison with control plants (Asil *et al*., 2013). Black tulip flowers treated with 1-MCP for 8 h showed maximum anthocyanin till the twelfth day of vase life (Chutichudet *et al*., 2010). Application of 1-MCP on soya plants decreased hydrogen peroxide in comparison with untreated plants as well as production of ethylene and free radicals but increased the activity of antioxidant enzymes (Djanaguiraman *et al*., 2011).

Considering the role of 1-MCP as an ethylene inhibitor, the purpose of the current study was to evaluate 1-MCP efficacy in extending display-quality 'Fortune' carnation flowers. To gain a deep understanding of biochemical characteristics of cut-flowers, enzyme assays were evaluated in this experiment.

## 2. Materials and Methods

Carnation cut-flowers of 'Fortune' cultivar were harvested from commercial greenhouses in Pakdasht (Iran) according to standard indexes. Flowers were immediately transferred to the laboratory of post harvest physiology of the Horticulture Department, Faculty of Agriculture, Tarbiat Modares University. Healthy and uniform flowers were selected for the considered treatments. Cut-flowers were placed in the vase solution and treated with 1-MCP (0, 0.5, 1 and 1.5 µl/l) for 24 h in 200 L glass aquarium chambers. After 1 h of ventilation, the aquarium lids were re-sealed and ethylene was injected inside each chamber using a Hamilton syringe to expose cut flowers to 1 µl/l ethylene for 16 h (Daneshi Nergi and Ahmadi, 2014). After termination of the ethylene treatment, the lids of the glass chambers were removed and the vases were placed on the lab bench. Experiments were run under the following conditions: temperature 20±2°C, relative humidity of 60-65%, light intensity of 15 µmol/$m^2s^{-1}$ with 12 h light and 12 h darkness (Daneshi Nergi and Ahmadi, 2014). Experimentation was conducted in a completely randomized design with three replications and four treatments. Sampling was carried out to evaluate physiological and biochemical characteristics at desired times and data were analysed using MSTAT-C statistical software; means were compared according to the Least Significant Differences (LSD) test ($P<0.01$) and graphs were designed using Excel software.

*Vase life*

In this study, vase life was considered the time during which cut-flowers can keep their market quality and before senescence symptoms, including bending of petal margins and wilting, appear (Singh, 1994). Cut-flower durability was based on day distance after the end of cut-flower treatment till their ornamental value disappeared.

*Leaf chlorophyll analysis*

To measure chlorophyll, leaf samples of 0.5 g were ground using a mortar and pestle with liquid nitrogen, dipped in 15 ml 80% acetone in test tubes and centrifuged at 6000 rpm and 4°C. The absorbance of the solutions was read against blank (solvent) at 663 and 646 nm using a spectrophotometer (BIO-RAD) (Richardson *et al*., 2002). Chlorophyll content was calculated as follows:

Chlorophyll a (µg.ml) = $12/5A_{663}$ -$2/79A_{646}$

Chlorophyll b (µg.ml) = $21/51A_{646}$ -$5/1A_{663}$

Total Chlorophyll (µg.ml) = Chlorophyll a + Chlorophyll b

*Petal anthocyanin*

Petal samples of 200 mg were pulverized in 3 ml 99:1 (v/v) methanol and hydrochloric acid and the obtained extracts were centrifuged at 12000 rpm for 20 min at 4°C. Supernatants were kept at 4°C under darkness for 24 h. Light absorption was then estimated by spectrophotometer at 550 nm wavelength and using silence coefficient (=33000 $mol^2$ $cm^{-1}$) (Krizek *et al*., 1993).

*Petal membrane stability index*

To determine petal membrane stability, two petal samples, each including 200 mg of each replication, were weighted and dipped in 10 ml double distilled water. One sample was placed in 40°C Benmary for 30 min and the other 100°C Benmary for 15 min. After cooling the sample of 40°C Benmary and reaching room temperature, electrical conductivity of the solutions was measured with a conductivity meter and the stability percent of the membrane was determined according Ezhilmathi *et al*. (2007) as follows:

$$\text{Membrane stability index (percent)} = [1-(C1/C2)] \times 100$$

*Enzyme assays*

*Peroxidase (POD) enzyme.* Peroxidase (POD) was extracted from 200 mg homogenized samples in 25 mM Na-phosphate buffer (pH 6.8) followed by centrifugation at 12000 rpm for 30 min at 4°C. For assay, a mixture consisting of 25 mM Na-phosphate buffer (pH 6.1), 28 mM Guaiacol, 5 mM hydrogen peroxide and crude extract was prepared and its absorbance at 470 nm was detected for 1 min, using spectrophotometer (BIO-RAD). Enzyme activ-

ity was expressed as absorption delta of 470 nm per mg protein (Chance and Maehly, 1955).

*Catalase (CAT) enzyme*. Catalase (CAT) was extracted from 200 mg samples homogenized in 25 mM Na-phosphate buffer (pH 6.8) followed by centrifugation at 12000 rpm for 30 min at 4°C. The supernatant was transferred to 15 ml tubes and referred to enzyme extract. For assay, a mixture consisting of 25 mM Na-phosphate buffer (pH 6.1), 10 mM hydrogen peroxide and crude extract was prepared and its absorbance at 240 nm was detected using a spectrophotometer (BIO-RAD). Enzyme activity was described by measuring the conversion rate of hydrogen peroxide to water and oxygen molecules, as the decrease of absorbance per time per mg of protein (Cakmak and Horst, 1991). Enzyme activity was expressed as absorption delta of 240 nm per mg protein. All steps of enzyme extraction were performed on ice.

*Superoxide dismutase (SOD) enzyme*. 200 mg of plant tissues were extracted in 50 mM HEPES-KOH buffer (pH 7.8) containing 0.1 mM EDTA. The homogenate was transferred to centrifuge tubes and was centrifuged at 12000 rpm for 30 min at 4°C. The supernatant was transferred to 15 ml tubes and referred to enzyme extract. For assay, a mixture consisting of 50 mM HEPES-KOH (PH 7.8) containing 0.1 mM Na-EDTA, 50 mM $Na_2CO_3$ (PH 10.2), 12 mM L-methionine, 75 µM Nitro Blue Tetrazolium (NBT), 1µM Riboflavin and crude extract was prepared and enzymatic extract as a unit of SOD activity was considered as enzymatic amount which resulted in 50% inhibition of NBT in 560 nm (Chance and Maehly, 1955). Reaction mix absorption was measured by spectrophotometer.

Total soluble protein was measured using the Bradford (1976) method. Absorption of 1 ml Bradford reagent along with 100 µl enzymatic extract were mixed completely and registered in 595 nm. Protein content was estimated using calibration curve of cow albumin serum (BSA) (Bradford, 1976).

## 3. Results and Discussion

*Vase life*

Vase life is one of the important post harvest characteristics of ornamental plants especially for cut carnation, which is highly affected by ethylene. Reduction of unfavorable effects of ethylene is an appropriate method for enhancing postharvest durability of plants or plant organs and compounds like 1-MCP are used extensively in order to alleviate the undesired effects of ethylene in horticultural crops. Results showed that the effect of 1-MCP on characteristics of cut carnation cv. Fortune was significant (P<0.01). Increasing 1-MCP concentration enhanced vase life of cut flowers so that the longest vase life (11.8 days) was related to 1.5 µl/l 1-MCP which was significantly (P<0.01) higher than control and 0.5 µl/l 1-MCP, while there was no significant (P<0.01) difference with 1 µl/l 1-MCP. By preventing external ethylene action, treatment with 1-MCP increased vase life (Fig. 1). In accordance with our results, Yamane *et al*. (2004) showed that 1-MCP increased vase life of cattleya flowers. It seems that 1-MCP prevents ACC-oxidase and ACC synthase expression, subsequently decreasing ethylene production in primary days of treatment with 1-MCP, resulting in increased vase life (Yamane *et al*., 2004; In *et al.,* 2013; Yang *et al*., 2013). This is also in agreement with the results of Chutichudet *et al*. (2010) who reported that 1-MCP protects tulip cut-flower quality by preventing ethylene production (Chutichudet *et al*., 2010). Hence, increasing vase life of cut-flowers treated with 1-MCP is related to inhibiting ethylene action and ethylene biosynthesis (Serek *et al*., 1994; Serek and Sisler, 2001).

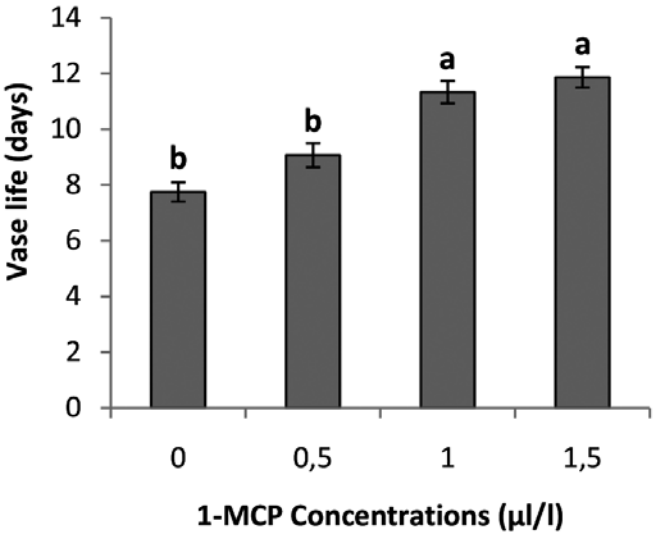


Fig. 1 - Effect of 1-MCP on vase life of carnation cv. Fortune. Data are means with standard errors (n=3).

*Membrane stability index*

1-MCP treatment preserved membrane stability in carnation cut flowers. The greatest membrane stability on the seventh day after treatment was related to 1.5 µl/l 1-MCP with no significant (P<0.01) difference toward 1 µl/l, but it was significantly (P<0.01) higher than control and 0.5 µl/l 1-MCP. 1-MCP can prevent membrane degradation by decreasing the lipid peroxidation which is regulated by ethylene (Yuan *et al*., 2010) (Fig. 2). Since ethylene is the main factor for increasing respiration rate in climacteric crops and causes an acceleration of physical characteristic changes and cell membrane phospholipids degradation, it seems that 1-MCP treatment protects membrane stability by preventing ethylene action.

*Chlorophyll content*

Based on the results, the highest chlorophyll content on the seventh day after treatment measured in 1.5 µl/l 1-MCP showed a significant (P<0.01) difference with the other treatments. The effect of 1-MCP on protecting chlorophyll content is a result of ethylene action and consequently inhibition of ethylene biosynthesis, which is considered the

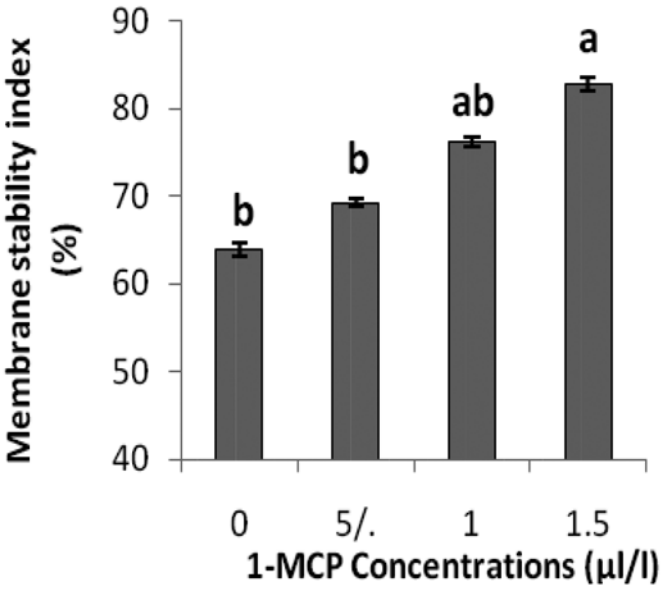


Fig. 2 - Effect of 1-MCP on petal membrane stability of carnation cv. Fortune. Data are means with standard errors (n=3).

most important factor of leaf chlorosis in ornamental plants. In accordance with the present results, 1-MCP treatment at all concentrations decreased ethylene biosynthesis which was followed by reduction of chlorophyll destruction compared to control plants (Asil *et al*., 2013) (Fig. 3). According to Serek *et al*. (1998) 1-MCP inhibited leaf chlorosis in chrysanthemum and geranium cv. Isable (Serek *et al*., 1998). In this case, the effect of 1-MCP was attributed to binding ethylene receptors. A recent study showed that 1- MCP can keep carbon assimilation in a condition of good efficiency and prevent tricarboxylic acid cycle. Under these conditions, by provoking the biosynthesis of gibberellins, the postharvest senescence process is retarded which appeared as preserving the postharvest quality (Wang *et al*., 2014).

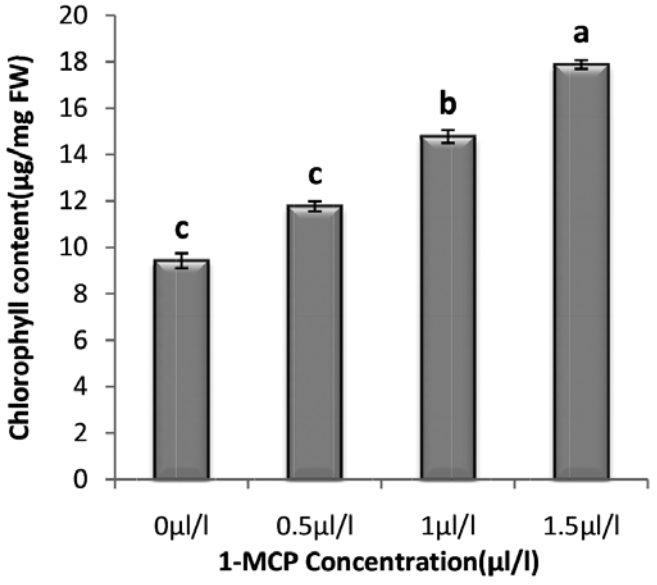


Fig. 3 - Effect of 1-MCP on petal chlorophyll content of carnation cv. Fortune. Data are means with standard errors (n=3).

*Anthocyanin content*

Our results showed that the highest anthocyanin content on the seventh day after treatment was related to 1.5 µl/l 1-MCP with significant (P<0.01) differences with the other treatments (Fig. 4). Positive effects of 1-MCP on inhibition of external ethylene action, delaying senescence and accordingly protecting suitable cell pH were the factors of anthocyanin photosynthesis pigments. Chutichudet *et al*. (2010) found that Black tulip cut flowers treated with 300 ppb 1-MCP for 8 h had the highest anthocyanin content till the twelfth day of vase life (Chutichudet *et al*., 2010). Usually, postharvest destruction of anthocyanin pigments is as a result of bracteoles' membrane function destruction (Jiang and Chen 1995; Jiang *et al*., 2004). The stability of anthocyanin may be due to the role of 1-MCP, which can decrease membrane destruction of fresh crops (Hershkovitz *et al*., 2005). Vacuole pH is enhanced during senescence and anthocyanin gets free of color before destruction (Zhang *et al*., 2001). Furthermore, anthocyanin destruction occurs as a result of polyphenol oxidase activity (Francis, 1989). The preventing from anthocyanins accumulation in tissues could be ascribed to ethylene action, based on research on *Arabidopsis thaliana* showed negative self-regulation in relationship of ethylene, carbohydrates and anthocyanins. Stress induced by ethylene resulted in reduced absorption of carbohydrates and consequently decreased accumulation of pigments (Das *et al*., 2011).

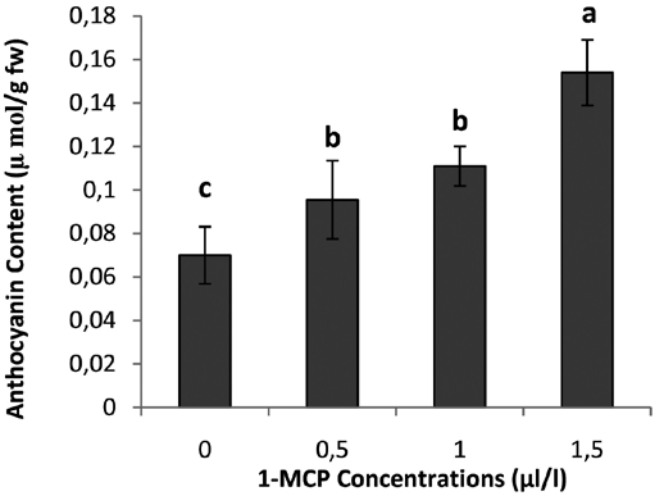


Fig. 4 - Effect of 1-MCP on petal anthocyanin content of carnation cv. Fortune. Data are means with standard errors (n=3).

*Antioxidant enzymes*

The highest activity of catalase was found in flowers treated with 1 µl/l 1-MCP, however it was not significantly (P<0.01) higher than 1.5 µl/l but was significantly (P< 0.01) higher than control and 0.5 µl/l 1-MCP treatments (Fig. 5). The highest activity of peroxidase was detected in 1 µl/l 1-MCP with significant (P<0.01) differences toward the other treatments (Fig. 6). 1-MCP protected antioxidant enzyme activity by inhibiting ethylene action and biosynthe-

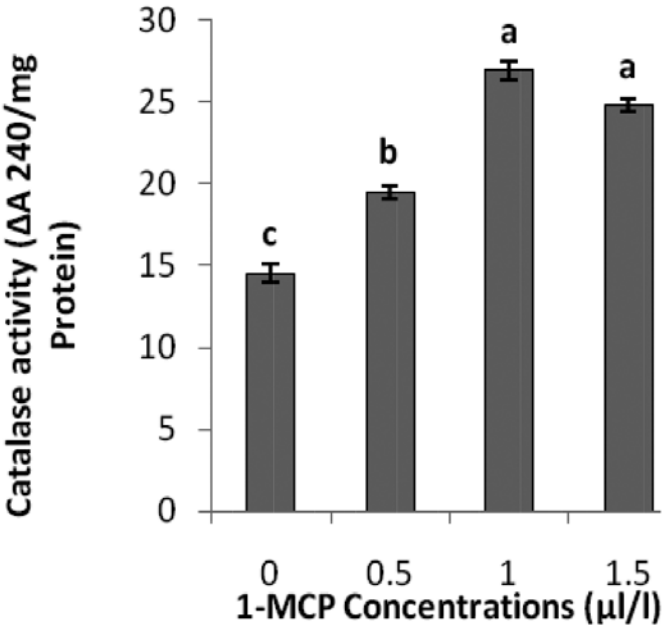


Fig. 5 - Effect of 1-MCP on petal catalase enzyme activity of carnation cv. Fortune.

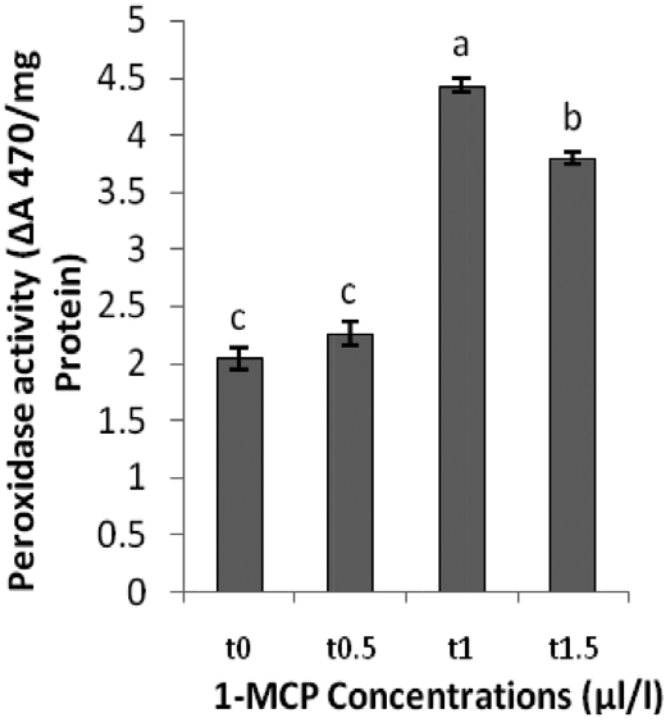


Fig. 6 - Effect of 1-MCP on petal peroxidase enzyme of carnation cv. Fortune.

sis, and subsequently decreased respiration rate. The petal senescence process causes metabolic and physiological changes, which result in the petal death. Senescence begins with the expression of a set of genes related to senescence and it emerges at metabolic level as oxidative processes and often the catabolic processes in senescence increase are irreversible (Buchanan-Wollaston, 1997). In microsomal membranes of carnation, many superoxides are generated during senescence (Mayak *et al*., 1983). Here also, increasing free radicals from ethylene stress in plants untreated with (0 µl/1) 1-MCP damaged to immunity system of antioxidant and reduction of immunity enzymes activity. When treated with different concentrations of 1-MCP, there was more antioxidant enzyme activity because of decreasing effects of external ethylene. Peroxidase has different biological functions, such as detoxification of hydrogen peroxide, lignin biosynthesis, hormonal signaling and response to stress (Gao *et al*., 2010). Catalase is considered an important biological factor and its major function is in the process of superoxide metabolism by playing role in releasing oxygen and hydrogen peroxide free radicals and preventing creation of hydroxyl radicals (Spanou *et al*., 2012). Superoxide dismutase like Cu-Zn superoxide dismutase, Mn superoxide dismutase and outside cell superoxide dismutase play a critical role in inhibition of superoxide (Miao and St Clair, 2009). In fact, peroxidase, catalase and superoxide dismutase play roles in protecting the metabolism balance of oxygen in plant tissues (Xie *et al*., 2003). Superoxide causes lipid peroxidation, cell membrane damage and finally senescence; 1-MCP can affect enzyme activities, which remove superoxide (Li *et al*., 2007). In accordance with our obtained results, increasing antioxidant enzyme (catalase, superoxide dismutase and peroxidase) activity of gladiola florets treated with 1-MCP has been reported (Fig. 7). It seems that this treatment decreases oxidative stresses in cut-flowers (Hassan and Ali, 2014). In other words, activity of these enzymes is a factor for the protection of cells against oxidative stresses (Zhou *et al*., 2014). It should be mentioned that even if ethylene decreases in response to 1-MCP, the activity of antioxidant enzymes will increase. In addition, a considerable increase in peroxidase, catalase and superoxide dismutase activity was observed in petals of carnation flower cv. Lilacon purple treated with 0.5 µl/l 1-MCP. The 1-MCP treatment

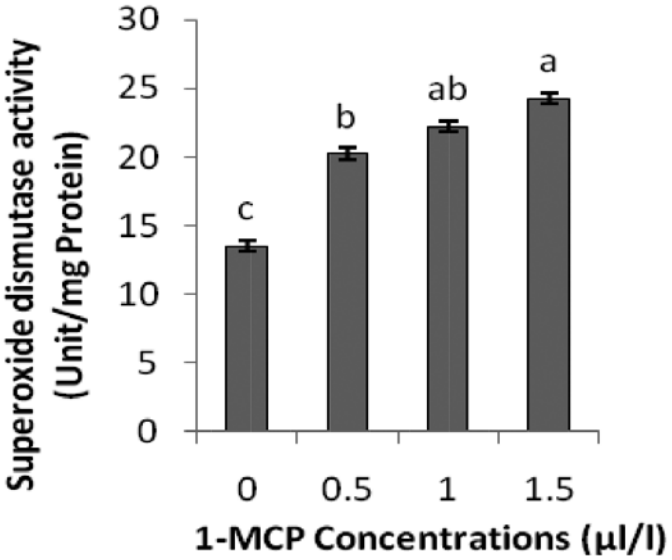


Fig. 7 - Effect of 1-MCP on petal superoxide dismutase enzyme of carnation cv. Fortune.

decreased hydrogen peroxide and superoxide (peroxide anion) compared to control plants (Karimi, 2014). This decreasing may be a result of low ethylene biosynthesis and inhibition of hydrogen peroxide and peroxide anion by peroxide and superoxide dismutase enzymes (Larrigaudiere *et al*., 2004). In asparagus, 1-MCP hindered the ethylene signal transduction and resulted in a delay by affecting ethylene biosynthesis, and enhancing superoxide dismutase activity (Zhang *et al*., 2012). In brief, our study has shown that 1-MCP treatment can delay senescence and increase flower vase life by protecting photosynthesis pigments and increasing antioxidant enzyme activity.

In conclusion, 1-MC prevented the effects of exogenous ethylene and alleviated the stress conditions induced by ethylene in cut carnation flowers. It seems that 1-MC treatment increased the resistance capacity of tissue/organs by boosting the activity of antioxidant enzymes resulting in favorable physiological and biochemical organ activities.

## References


AHMADI N., MIBUS H., SEREK M., 2008 - *Isolation of an ethylene induced putative nucleotide laccase in miniature roses (*Rosa hybrida *L.).* - Plant Growth Regulation, 27: 320-330.

AHMADI N., MIBUS H., SEREK M., 2009 - *Characterization of ethylene-induced organ abscission in F1 breeding lines of miniature roses (*Rosa hybrida *L.).* - Postharvest Biology and Technology, 52: 260-266.

ASIL M., KARIMI M., ZAKIZADEH H., 2013 - *1-MCP improves the postharvest quality of cut spray carnation (*Dianthus *caryophyllus L.) 'Optima'flowers*. - Horticulture, Environment and Biotechnology, 54(1): 58-62.

BARTH C., DE TULLIO M., CONKLIN P.L., 2006 - *The role of ascorbic acid in the control of flowering time and the onset of senescence*. - Journal of Experimental botany, 57(8): 1657-1665.

BRADFORD M.M., 1976 - *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. - Analytical biochemistry, 72: 248-254.

BUCHANAN-WOLLASTON V., 1997 - *The molecular biology of leaf senescence*. Journal of Experimental Botany, 48(2):181-199.

CAKMAK I., HORST W.J., 1991 - *Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (Glycine max).* - Physiologia Plantarum, 83: 463-468.

CAMERON A.C., REID M.S., 2001 - *1-MCP blocks ethylene-induced petal abscission of Pelargonium peltatum but the effect is transient*. - Postharvest Biology and Technology, 22: 169-177.

CHANCE B., MAEHLY A.C., 1955 - *Assay of catalase and peroxidase*. - Methods Enzymol., 2: 764-775.

CHUTICHUDET P., CHUTICHUDET B., BOONTIANG K., 2010 - *Effect of 1-MCP fumigation on vase life and other postharvest qualities of siam tulip (*Curcuma aeruqinosa *Roxb.) cv. laddawan.* - International Journal of Agricultural Research, 5(1): 1-10.

DANESHI NERGI M.A., AHMADI N., 2014 - *Effects of 1-MCP and ethylene on postharvest quality and expression of senescence-associated genes in cut rose cv. Sparkle.* - Scientia Horticulturae, 166: 78-83.

DAS P.K., GEUL B., CHOI S.B., YOO S.D., PARK Y.I., 2011 - *Photosynthesis-dependent anthocyanin pigmentation in arabidopsis*. - Plant Signal., 61: 23.

DJANAGUIRAMAN M., PRASAD P., AL-KHATIB K., 2011 - *Ethylene perception inhibitor 1-MCP decreases oxidative damage of leaves through enhanced antioxidant defense mechanisms in soybean plants grown under high temperature stress*. - Environmental and experimental botany, 71(2): 215-223.

EBELES F.B., MORGAN P.W., SAHVEIT M.E., 1992 - *Ethylen in plant biology.* 2nd ed. - Academic Press, New York, USA.

EZHILMATHI K., SINGH V., ARORA A., SAIRAM R., 2007 - *Effect of 5-sulfosalicylic acid on antioxidant activity in relation to vase life of Gladiolus cut flowers*. - Plant Growth Regulation, 51: 99-108.

FRANCIS F.J., 1989 - *Food colorant. Anthocyanins. Critical Reviews*. - Food Science and Nutrition, 28: 273-314.

GAO C., WANG Y., LIU G., WANG C., JIANG J., YANG C., 2010 - *Cloning of ten peroxidase (POD) genes from* Tamarix hispida *and characterization of their responses to abiotic stress*. - Plant molecular biology reporter, 28(1): 77-89.

HASSAN F., ALI E., 2014 - *Protective effects of 1-methylcyclopropene and salicylic acid on senescence regulation of gladiolus cut spikes.* - Scientia horticulturae, 179: 146-152.

HERSHKOVITZ V., SAGUY S.I., PESIS E., 2005 - *Postharvest application of 1-MCP to improve the quality of various avocado cultivars.* - Postharvest biology and technology, 37(3): 252-264.

IN B.C., STRABLE J., BINDER B.M., FALBEL T.G., PATTERSON S.E., 2013 - *Morphological and molecular characterization of ethylene binding inhibition in carnations*. - Postharvest Biology and Technology, 86: 272-279.

JIANG Y., DUAN X., JOYCE D., ZHANG Z., LI J., 2004 - *Advance in understanding of enzymatic browning in harvested litchi fruit.* - Food Chemistry, 88(3): 443-446.

JIANG Y.M., CHEN F., 1995 - *A study on polyamine change and browning of fruit during cold storage of litchi (*Litchi chinensis *Sonn.).* - Postharvest biology and technology, 5(3): 245-250.

KARIMI M., 2014 - *Change in ethylene production and ACC content of potted carnation in response to anti-ethylene treatments.* - International Journal of Biosciences, 4(8): 116-123.

KRIZEK D.T., KRAMER G.F., UPADHYAYA A., MIRECKI R.M., 1993 - *UV-B response of cucumber seedlings grown under metal halide and high pressure sodium/deluxe lamps.* - Physiologia Plantarum, 88: 350-358.

LARRIGAUDIERE C., VILAPLANA R., SORIA Y., RECASENS I., 2004 - *Oxidative behaviour of Blanquilla pears treated with 1-methylcyclopropene during cold storage*. - Journal of the Science of Food and Agriculture, 84(14): 1871-1877.

LI Z., WANG L., WANG W., ZHU Y., 2007 - *Physiological effect and application of 1-MCP on delaying fruit senescence*. - Plant Physiology Journal, 43: 201-206.

MA N., TAN H., LIU X., XUE J., LI Y., GAO J., 2006 - *Tran-*

*scriptional regulation of ethylene receptor and CTR genes involved in ethylene-induced flower opening in cut rose (Rosa hybrida) cv. Samantha.* - Journal of Experimental Botany, 57(11): 2763-2773.

MAYAK S., LEGGE R.L., THOMPSON J.E., 1983 - *Superoxide radical production by microsomal membranes from senescing carnation flowers: an effect on membrane fluidity*. - Phytochemistry, 22(6): 1375-1380.

MIAO L., ST CLAIR D.K., 2009 - *Regulation of superoxide dismutase genes: implications in disease*. - Free Radical Biology and Medicine, 47(4): 344-356.

RICHARDSON A.D., DUIGAN S.P., BERLYN G.P., 2002 - *An evaluation of noninvasive methods to estimate foliar chlorophyll content*. - New Phytologist, 153: 185-194.

SEGLIE L., MARTINA K., DEVECCHI D., ROGGERO C., TROTTA F., SCARIOT V., 2011 - *The effects of 1-MCP in cyclodextrin-based nanosponges to improve the vase life of* Dianthus caryophyllus *cut flowers.* - Postharvest Biology and Technology, 59: 200-205.

SEREK M., JONES R.B., REID M.S., 1994 - *Role of ethylene in opening and senescence of Gladiolus sp. Flowers*. - American Society for Horticultural Science, 119: 1014-1019.

SEREK M., PRADUCKI A., SISLER E.C., 1998 - *Inhibitors of ethylene action affect final quality and rooting of cuttings before and after storage*. - Hortscience, 33: 153-155.

SEREK M., SISLER E.C., 2001 - *Efficacy of inhibitors of ethylene binding in improvement of the postharvest characteristics of potted flowering plants*. - Postharvest Biology and Technology, 23: 161-166.

SINGH H., HALLAN V., RAIKHY G., KULSHRESTHA S., SHARMA M., RAM R., GARG I., ZAIDI A., 2005 - *Characterization of an Indian isolate of carnation mottle virus infecting carnations*. - Current Science. 88: 594-601.

SINGH K., 1994 - *Effects of spermidine, IAA, ACC and ethylene on petal longevity in carnation (*Dianthus caryophyllus *L.)*. - Phyton, 34: 309-313.

SPANOU C.I., VESKOUKIS A.S., STAGOS D., LIADAKI K., ALIGIANNIS N., ANGELIS A., SKALTSOUNIS A.L., ANASTASIADI M., HAROUTOUNIAN S.A., KOURETAS D., 2012 - *Effects of Greek legume plant of extracts on xanthine oxidase, catalase and superoxide dismutase activities.* - Journal Physiology and Biochemistry, 68(1): 37-45.

STASKAWICZ B.J., AUSUBEL F.M., BAKER B.J., ELLIS J.G., JONES J.D., 1995 - *Molecular genetics of plant disease resistance*. - Science, 268(5211): 661-667.

WANG Y., ZHANG L., ZHU S., 2014 - *1-Methylcyclopropene (1-MCP)-induced protein expression associated with changes in Tsai Tai (Brassica chinensis) leaves during low temperature storage*. - Postharvest Biology and Technology, 87: 120-125.

XIE M., ZHANG J., XIE J., 2003 - *Relationships between some physio-biochemical changes and senescence during storage in bitter gourd*. - Acta Botanica Boreali-Occidentalia Sinica, 24(4): 716-719.

YAMANE K., YAMAKI Y., FUJISHIGE N., 2004 - *Effects of exogenous ethylene and 1-MCP on ACC oxidase activity, ethylene production and vase life in* Cattleya alliances. - Journal of the Japanese Society for Horticultural Science, 73: 128-133.

YANG S.F., HOFFMAN N.E., 1984 - *Ethylene biosynthesis and its regulation in higher plants*. - Annual Review of Plant Physiology, 35: 155-189.

YANG X., SONG J., CAMPBELL-PALMER L., FILLMORE S., ZHANG Z., 2013 - *Effect of ethylene and 1-MCP on expression of genes involved in ethylene biosynthesis and perception during ripening of apple fruit*. - Postharvest Biology and Technology, 78: 55-66.

YUAN G., SUN B., YUAN J., WANG Q., 2010 - *Effect of 1-methylcyclopropene on shelf life, visual quality, antioxidant enzymes and health-promoting compounds in broccoli florets*. - Food Chemistry, 118(3): 774-781.

ZHANG P., ZHANG M., WANG S., WU Z., 2012 - *Effect of 1-methylcyclopropene treatment on green asparagus quality during cold storage*. - International Agrophysics, 26(4): 407-411.

ZHANG Z., PANG X., JI Z., JIANG Y., 2001 - *Role of anthocyanin degradation in litchi pericarp browning*. - Food Chemistry, 75(2): 217-221.

ZHOU Q., MA C., CHENG S., WEI B., LIU X., JI S., 2014 - *Changes in antioxidative metabolism accompanying pitting development in stored blueberry fruit*. - Postharvest biology and technology, 88: 88-95.

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# Seed oil content, fatty acids composition and antioxidant properties as affected by genotype in *Allium cepa* L. and perennial onion species

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*Key words*: oil fatty acids composition, onion seed oil, proteins, selenium, total phenolics.

**Abstract: The antioxidant content in plant seeds is deemed to affect seed oil protection against auto-oxidation to a large extent, whereas the relationship between a strong antioxidant element such as selenium (Se) and either seed oil accumulation or fatty acids composition has not been investigated so far. The aim of the present work was to assess Se concentrations in seeds and their relationships with oil content and fatty acids composition in: a) ten *Allium cepa* cultivars, i. e. eight Russian and two Italian; and b) six perennial onion species (*A. schoenoprasum*, *A. obliquum*, *A. altaicum*, *A. fistulosum*, *A. nutans*, *A. ramnósum*). Fatty acids composition of *Allium* seed oil was determined by gas chromatography method, whereas total and water soluble Se concentration was assessed by microfluorimetric method. The oil content of *Allium cepa* seeds was 1.7 fold higher (10.7-16.5%) than that recorded in perennial onions (4.0-10.8%) and it was positively correlated with the total Se concentration. Within *A. cepa*, the seeds of the two Italian cultivars Ramata di Montoro and Rossa di Tropea were characterized by the highest oil content (16.5-16.6%) and oleic acid (25-27%). Linoleic (C18:2) acid was the main fatty acid, followed by oleic (C18:1) and palmitic acids (C16:0) in all cultivars. Among the perennial onion seeds, the highest oil percentage was detected in *A. schoenoprasum* (10.8%) and the lowest in *A. ramnósum* (4.0%). Compared to *A. cepa* cultivars, the perennial onion species showed a similar oil fatty acid composition, with the main acids being C18:2, C18:1 and C16:0 in decreasing order, a lower level of C16:0, and enhanced levels of minor SFA, such as C20:0, C22:0 and C24:0. Further differences also included decreased levels of C16:1, 11-trance C18:1 and a higher concentration of C22:1. The concentration of water soluble forms of Se in seeds was positively correlated with linoleic acid and with total phenolics. Conversely, oleic acid was negatively correlated with water soluble forms of Se.**

## 1. Introduction

*Alliaceae* plants belong to a secondary selenium-accumulator group. They are stable at high concentrations of this element and are able to accumulate up to 1 mg of Se per g of dry weight during selenium uptake without causing growth inhibition (Terry *et al*., 2000). Although Se is not regarded as an essential trace element for most of agricultural crops, including onion, a moderate selenium concentration is reportedly a powerful natural antioxidant, capable of protecting plants against different types of stress, such as salinity, drought, UV-radiation, heavy metals, and attack by herbivores (Germ *et al*., 2007). Notably, the antioxidant role of Se in polyunsaturated fatty acid (PUFA) stability of seed oil seems to be of special interest.

Oils are a vital component of the human diet and are readily available in some vegetables species. Their accumulation in plant seeds, as well as their fatty acids composition, is affected by genetic, environmental, and farming factors (Sidlauskas and Bernotas, 2003). In addition, their antioxidant action is of great importance due to PUFA disposition against oxidation. An investigation of the germination of *Capsicum annuum* seeds demonstrated that fatty acids content and composition may play a major role on germination and seedling vigour at different temperatures (Kaymak, 2014). A significant decrease of seed germination during storage is reportedly connected with lipid auto-oxidation leading to PUFA degradation and an increase in cell membrane permeability (Harmann and Mattick, 1976). In this respect, the inhibitory effect of

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selenium on lipid peroxidation was described for ryegrass (Hartikainen *et al*., 2000) and germination was enhanced in lettuce seeds stored long-term as a result of Se application (Xue *et al*., 2001). Moreover, a direct correlation was found between the content of water soluble Se forms and germination rate of *Apiaceae* seeds (Dobrutskaya *et al*., 2010). Unlike the reports mentioned above, soybean biofortification with selenium did not affect the stability of seed oil PUFA against oxidation (Fallen *et al*., 2011).

A high oil content is typical in *Allium* species, with *Allium cepa* seeds containing about 26%, with the predominance of highly labile linoleic acid (Dini *et al*., 2008; Yalcin and Kavuncuoglu, 2014); a gradual germination decrease over time is however frequent in *Allium* species.

Actual perspectives of *Allium* seed oil utilization in the human diet as well as in phytotherapy of cardiovascular and dermatological diseases (Nakamura *et al.*, 2008) also require investigations into the relationship between selenium and either oil content or composition in *Allium* species, also connected to the genotype. Therefore, due to the lack of information reported in literature on these topics, research was carried out in order to assess selenium concentration, oil content and fatty acids composition, total phenolics in the seeds, as well as the significant correlations between these four parameters, in: a) ten *Allium cepa* L. cultivars, native of northern Europe (eight Russian cultivars) and the Mediterranean area (two Italian cultivars); and b) six perennial *Allium* species.

## 2. Materials and Methods

Research was carried out to compare the seeds obtained from northern Europe and Mediterranean onion cultivars with regard to selenium concentration, oil content and fatty acids composition, proteins, and total phenolics. In this context, seeds from eight Russian *Allium cepa* cultivars (Ledocol, Cherny prince, Alba, Sigma, Myachkovsky, Zolotnichock, Zolotie cupola, Globus) and six perennial onion species (*A. schoenoprasum*, cv. Medonos; *A. obliquum*, Novichock; *A. altaicum*, Alves; *A. fistulosum*, Troitsa; *A. nutans*, Lider; *A. ramnósum*, Aprior) were produced in the experimental fields of "All-Russian Institute of vegetable breeding and seeds production" in the Moscow region in 2014. In the same year, seeds were obtained from two Italian *Allium cepa* cultivars (Ramata di Montoro and Rossa di Tropea) grown in an experimental field arranged by the Department of Agricultural Sciences in a private farm sited in Pontecagnano (Salerno, Italy). In both locations, five onion bulbs per square meter of 46-70 mm caliber were planted in March and the infructescences containing physiologically ripe seeds were harvested in July-August. The farming practices before planting were: ploughing at 40 cm depth and hoeing at 15 cm; fertilization with 70 kg $ha^{-1}$ of N as ammonium sulphate, 80 kg $ha^{-1}$ of $P_2O_5$ as superphosphate and 130 kg $ha^{-1}$ of $K_2O$ as potassium sulphate. During the crop, the following practices were performed: hand weeding; fertilization with 130 kg $ha^{-1}$ of N and 140 kg $ha^{-1}$ of $K_2O$ as calcium nitrate and potassium nitrate; drip irrigation, activated when the soil available water capacity decreased to 70%; copper oxychloride application against rust.

The seed oil content was gravimetrically assessed after oil extraction with n-hexane (60°C) for 6 h in a Soxhlet extractor, according to AOAC (1990), and hexane removal from the oil by rotary evaporation under reduced pressure (20 mbar at 30°C). The fatty acids composition of *Allium* seed oil was detected by Gas Chromatography, according to AOCS (1999), via appropriate methyl ethers chromatography on capillary glass column, using an Agilent 6890 Gas Cromatograph (Agilent Technologies, Santa Clara, California, US), equipped with a Flame Ionization Detector. The carrier gas was helium and the total gas flow rate was 20 $mL \cdot min^{-1}$. The oven temperature was initially held at 80°C for 10 min, then sequentially increased to 175°C for 15 min, to 200°C for 5 min, and to 225°C for 70 min. The injector and detector temperatures were 260 and 240°C respectively. The peaks of fatty acids methyl ethers were identified by comparison to the retention times of reference standards (AOCS, 1999).

The microfluorimetric method was used to determine total and water soluble selenium content in seeds (Alfthan, 1984).

Total phenolics in water extracts were determined using Folin-Ciocalteu colorimetric method (Sagdic *et al*., 2011). Protein content was estimated by Kjeldahl method.

A randomized complete block design was used for the distribution of the treatments in the fields, with three replicates, and the plot size was 18 $m^2$ (4.0 x 4.0 m). Data were statistically processed by analysis of variance and mean separations were performed through the Duncan multiple range test, with reference to 0.05 probability level, using SPSS software version 17. Data expressed as percentage were subjected to angular transformation before processing. Standard deviation (SD) was assessed for the three replicate data per experimental treatment and coefficient of variation (CV) for the data set linked to each variable.

## 3. Results

As reported in Tables 1 and 2, the oil content recorded in *Allium cepa* seeds fell in the 10.7-16.6% range and was significantly higher than that detected in perennial onion species (4.0-10.8%).

Among *Allium cepa* cultivars, the two Italian ones (Ramata di Montoro and Rossa di Tropea) showed higher content of seed oil (16.5-16.6%) compared to the Russian cultivars (10.7-13.5%). *A. ramnosum* seeds were characterized by a 1.8-2.7 fold lower oil content than the other perennial onion seeds. Among the perennial onion species tested, the highest oil concentration was measured in *A. schoenoprasum* seeds (10.8%).

Selenium concentration in onion seeds varied greatly (108-476 and 112-261 μg·kg$^{-1}$ in perennial onions and *Allium cepa* respectively), giving the highest coefficient of variation (25% in *A. cepa* and 37% in perennial onions); the lowest CV was recorded for the oil content (9.2 and 11.2% in *A. cepa* and in perennial species seeds respectively). Moreover, only *Allium cepa* seeds showed a direct correlation between total Se concentration and oil percentage (r= 0.82; P < 0.05) (Fig. 1).

As reported in the Tables 3, 4, 5, the predominant fatty acids of seed oil in both *Allium cepa* and perennial onion species are the following in decreasing order: linoleic C18:2 (61.5-73.7%), oleic C18:1 (19.0-27.2%) and palmitic C16:0 (5.1-8.6%). Moreover, among the *Allium cepa* cultivars considered, the highest content of oil saturated fatty acids (SFA) was found in Ledokol seeds (11.3%), whereas the seed oil of *Allium cepa* Italian cultivars Ramata di Montoro and Rossa di Tropea showed the highest content of monounsaturated acids (MUFA). Among perennial onion species, the highest SFA value was detected in *A. ramnosum* (12.7%), whereas the highest content of oleic acid C18:1 was detected in *A. nutans* seed oil. Moreover, PUFA represented as much as 74% of

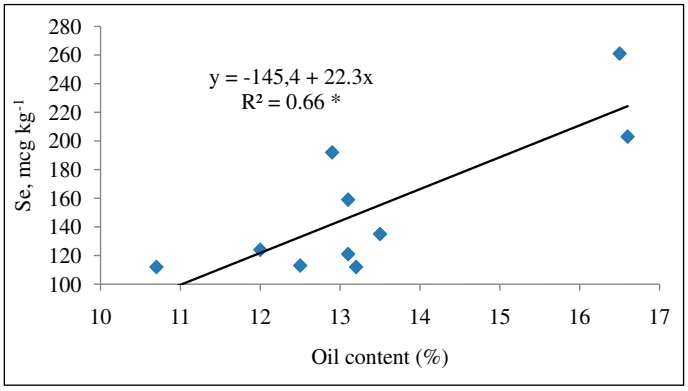


Fig. 1 - Relationship between selenium concentration and oil content in *Allium cepa* seeds (r = 0.82; P<0.05).

Table 1 - Chemical composition of *Allium cepa* seeds

| Cultivar | Oil content (%) | Proteins (%) | Total phenolics (mg g$^{-1}$ F.W.) | Selenium (μg kg$^{-1}$ F.W.) |
|---|---|---|---|---|
| Sigma | 10.7 ± 0.9 d | 22.4 ± 2.1 ce | 3.0 ± 0.2 bc | 112 ± 9 e |
| Globus | 12.0 ± 0.9 c | 22.1 ± 2.0 de | 2.8 ± 0.2 c | 124 ± 7 de |
| Alba | 12.5 ± 1.0 bc | 24.1 ± 2.2 bd | 1.9 ± 0.1 f | 113 ± 7 e |
| Ledocol | 12.9 ± 1.0 bc | 26.2 ± 2.2 b | 2.7 ± 0.2 cd | 192 ± 10 bc |
| Myachkovsky | 13.1 ± 1.1 bc | 19.1 ± 1.7 f | 3.8 ± 0.3 a | 121 ± 9 de |
| Zolotie cupola | 13.1 ± 1.0 bc | 34.7 ± 3.1 a | 3.2 ± 0.3 b | 159 ± 8 cd |
| Cherny prince | 13.2 ± 1.0 bc | 25.2 ± 2.0 bc | 2.1 ± 0.1 ef | 112 ± 9 e |
| Zolotnichock | 13.5 ± 1.1 b | 20.5 ± 1.8 e | 2.7 ± 0.2 cd | 135 ± 39 de |
| Ramata di Montoro | 16.5 ± 1.4 a | 22.0 ± 1.9 df | 2.4 ± 0.2 de | 261 ± 17 a |
| Rossa di Tropea | 16.6 ± 1.4 a | 22.3 ± 1.9 cd | 3.0 ± 0.3 bc | 203 ± 12 b |
| Mean ± SD | 13.5 ± 1.3 | 23.9 ± 3.0 | 2.8 ± 0.4 | 153 ± 40 |
| CV (%) | 9.2 | 12.4 | 14.6 | 25 |

Within each column: the data are reported as mean ± standard deviation; CV = coefficient of variation; means followed by different letters are significantly different according to the Duncan test at p≤0.05 (n=3).

Table 2 - Chemical composition of perennial onion species seeds

| Cultivar | Oil content (%) | Proteins (%) | Total phenolics (mg g$^{-1}$ F.W.) | Selenium (μg kg$^{-1}$ F.W.) |
|---|---|---|---|---|
| *A. ramnósum* (cultivar Aprior) | 4.0±0.3 d | 24.6±2.1 bc | 1.7±0.1 e | 223±25 b |
| *A. nutans* (Lider) | 7.0±0.6 c | 35.2±3.1 a | 3.0±0.3 bc | 108±12 c |
| *A. fistulosum* (Troitsa) | 7.1±0.6 c | 23.8±2.0 c | 3.8±0.3 a | 476±12 a |
| *A. altaicum* (Alves) | 8.6±0.7 bc | 26.4±2.2 bc | 3.3±0.3 ab | 182±7 bc |
| *A. obliquum* (Novichock) | 9.1±0.7 b | 19.9±1.7 d | 2.4±0.2 cd | 211±11 b |
| *A. schoenoprasum* (Medonos) | 10.8±0.9 a | 27.2±2.5 b | 2.1±0.2 de | 146±8 bc |
| Mean ± SD | 7.8±1.7 bc | 26.2±3.4 | 2.7±0.7 | 224±84 |
| CV (%) | 11.2 | 13.0 | 24.3 | 37 |

Within each column: the data are reported as mean ± standard deviation; CV = coefficient of variation; means followed by different letters are significantly different according to the Duncan test at p≤0.05 (n=3).

fatty acids in *A. altaicum* seed oil (Table 4), while 70.3% was the top value of these compounds in *Allium cepa* cultivars, i.e. in Myachkovsky and Zolotie cupola (Tables 3 and 4).

SFA content in seed oil of perennial onions decreased according to the following sequence: *A. ramnosum* > *A. nutans* > *A. fistulosum* = *A. obliquum* > *A. schoenoprasum* > *A. altaicum.* As regards MUFA, the ranking sequence was: *A. nutans* > *A. schoenoprasum* > *A. ramnosum* > *A. obliquum* > *A. fistulosum* > *A. altaicum.*

Compared to *Allium cepa*, the seed oil of perennial species was characterized by a decreased content in C16:0, 11-trans C18:1 and C16:1 fatty acids and high concentrations of C20:0, C22:0, C24:0 and C22:1, C 20:2 acids (Table 5).

Negative correlation was found between water soluble Se concentration and oleic acid (r= -0.64, P<0.05) and positive correlation between water soluble Se and linoleic acid (r= 0.63; P<0.05) (Fig. 2 and 3).

The highest content of total phenolics (3.8 mg $g^{-1}$) was detected in 'Myachrovsky' seeds among *Allium cepa* cultivars and in *A. fistulosum* among perennial onion species. Moreover, total phenolics were positively correlated with Se water soluble forms (r= 0.92; P<0.01), but they showed

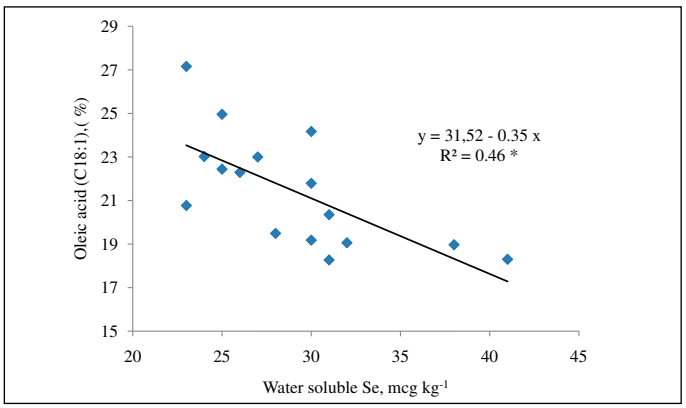


Fig. 2 - Correlation between water soluble selenium concentration and oleic acid concentration in *Allium* seeds (r = -0.64, P<0.05).

Table 3 - Fatty acids composition (%) of seed oil in *Allium cepa* cultivars

| Fatty acid | Onion cultivar | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Alba | Cherny prince | Sigma | Globus | Zolotnichock | Myachkovsky | Zolotie kupola | Ramata di Montoro | Rossa di Tropea | Ledokol |
| 14:0 | 0.14 b | 0.12 c | 0.11 c | 0.15 b | 0.12 c | 0.11 c | 0.12 c | 0.12 c | 0.12 c | 0.19 a |
| 16:0 | 8.62 a | 8.44 ab | 7.61 bc | 7.59 bc | 7.58 bc | 7.82 ac | 5.13d | 7.82 ac | 7.23 c | 8.53 a |
| 17:0 | 0.04 a | 0.01 c | 0.03 b | 0.04 a | 0.03 b | 0.03 b | 0.03 b | 0.04 a | 0.04 a | 0.04 a |
| 18:0 | 1.30 cd | 1.47 bc | 1.39 cd | 1.73 a | 1.50 b | 1.26 d | 1.43 bc | 1.69 a | 1.36 cd | 1.58 ab |
| 20:0 | 0.15e | 0.16 de | 0.14e | 0.22 c | 0.27 b | 0.17 de | 0.17 de | 0.22 c | 0.19 cd | 0.81 a |
| 22:0 | 0.10 ef | 0.10 ef | 0.14 c | 0.17 b | 0.19 a | 0.11 e | 0.13 d | 0.14 c | 0.14 c | 0.09 f |
| 24:0 | 0.00 d | 0.00 d | 0.00 d | 0.00 d | 0.00 d | 0.04 b | 0.05 a | 0.04 b | 0.05 a | 0.02 c |
| SFA | 10.35 ab | 10.30 ab | 9.42 b | 9.90 b | 9.69 b | 9.54 b | 7.06 c | 10.08 ab | 9.23 b | 11.26 a |
| 16:1 | 0.10 a | 0.06 cd | 0.05 d | 0.07 bc | 0.07 bc | 0.05 d | 0.08 b | 0.06 cd | 0.07 bc | 0.07 bc |
| 16:1 9 cis | 0.22 ab | 0.20 bd | 0.21 ac | 0.23 a | 0.19 ce | 0.17 e | 0.11 f | 0.18 de | 0.19 ce | 0.19 ce |
| 18:1 9 cis | 20.77 ce | 22.44 cd | 20.35 de | 21.79 cd | 19.18 e | 18.97 e | 19.06 e | 24.96 ab | 27.16 a | 23.00 bc |
| 18:1 11 trans | 1.00 cd | 1.05 bc | 1.06 bc | 1.17 ab | 1.11 bc | 0.91 de | 0.83 e | 1.25 a | 1.30 a | 1.09 bc |
| 20:1 | 0.20 a | 0.20 a | 0.20 a | 0.00 b | 0.00 b | 0.00 b | 0.00 b | 0.00 b | 0.00 b | 0.00 b |
| 22:1 | 0.02 d | 0.02 d | 0.02 d | 0.11 a | 0.05 c | 0.06 bc | 0.07 b | 0.03 d | 0.02 d | 0.05 c |
| MUFA | 22.31ce | 23.97 bc | 21.89 ce | 23.37 cd | 20.60 de | 20.16 e | 20.15 e | 26.48 ab | 28.74 a | 24.40 bc |
| 18::2i | 0.03 de | 0.06 ab | 0.06 ab | 0.07 a | 0.04 cd | 0.02 e | 0.05 bc | 0.07 a | 0.07 a | 0.07 a |
| 18:2 | 66.90 ab | 65.24 ab | 66.98 ab | 66.52 ab | 69.43 a | 69.91 a | 69.81 a | 62.99 b | 61.50 b | 63.77 ab |
| 20:2 | 0.21 cd | 0.21 cd | 0.23 bc | 0.19 d | 0.29 a | 0.19 d | 0.25 b | 0.25 b | 0.31 a | 0.25 b |
| 18:3 ω3 | 0.12 de | 0.12 de | 1.34 a | 0.19 b | 0.14 cd | 0.14 cd | 0.16 bd | 0.09 e | 0.10 | 0.17 bc |
| 20:3 | 0.05 a | 0.04 ab | 0.03 b | 0.04 ab | 0.04 ab | 0.03 b | 0.03 b | 0.05 a | 0.03 b | 0.05 a |
| PUFA | 67.26 ac | 65.67 ac | 68.64 ac | 67.01 ac | 69.94 ab | 70.29 a | 70.30 a | 63.45 c | 62.01 c | 64.31 bc |

Along each row, means followed by different letters are significantly different according to the Duncan test at p≤0.05 (n=3). SFA= saturated fatty acids; MUFA = mono unsaturated fatty acids; PUFA = poly unsaturated fatty acids.

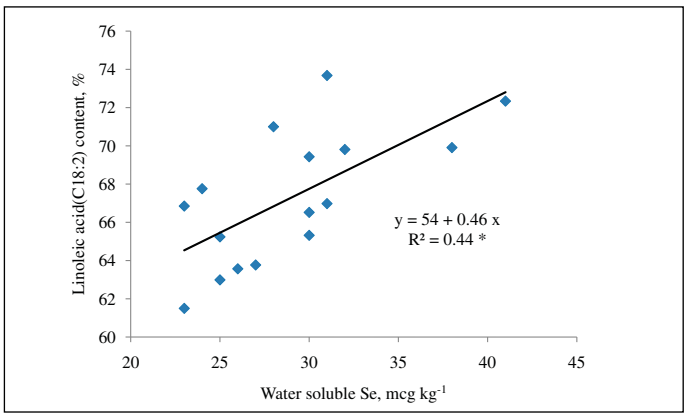


Fig. 3 - Correlation between selenium water soluble forms and linoleic content in *Allium* seeds (r = 0.66, P<0.05).

no correlation with total Se concentration (Fig. 4).

Finally, no significant correlation was recorded between proteins and Se, the latter considered either as total concentration or water soluble forms, in onion seeds.

## 4. Discussion and Conclusions

Seed aging and viability are affected by a number of factors, both during seed production and storage. The seed aging process depends on a seed's ability to resist degenerative changes as well as on its protection mechanisms, which are species-specific. Notably, seeds rich in lipids have limited longevity due to their specific chemical composition and, in fact, during seed storage of oily species, a declining trend of both total oil content and seed germination can be observed. However, fatty acids composition is

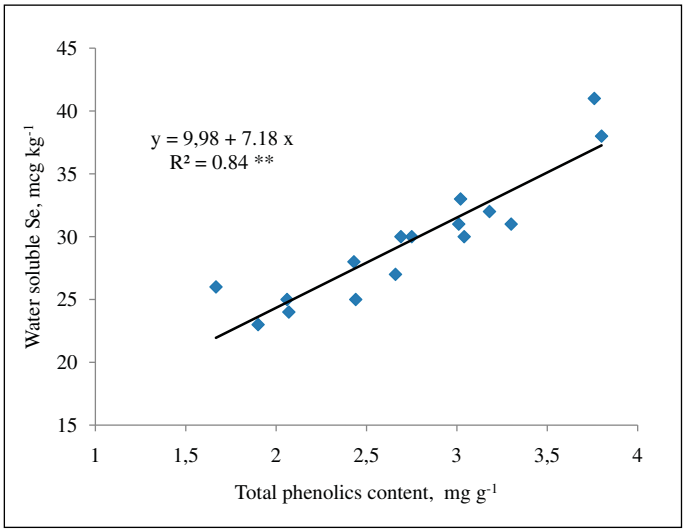


Fig. 4 - Correlation between water soluble selenium concentration and total phenolics in *Allium* seeds (r = 0.92; P< 0.01).

Table 4 - Fatty acids composition (%) of seed oil in perennial onion species

| Fatty acid | *A.schoenoprasum* Medonos | *A.altaicum* Alves | *A.obliquum* Novichock | *A.ramnosum* Aprior | *A.fistulosum* Troitsa | *A.nutans* Lider |
|---|---|---|---|---|---|---|
| 14:0 | 0.12 b | 0.08 d | 0.10 c | 0.17 a | 0.18 a | 0.09 cd |
| 16:0 | 5.13 c | 4.35 d | 5.89 b | 7.83 a | 5.25 bc | 5.62 bc |
| 17:0 | 0.02 d | 0.04 bc | 0.04 bc | 0.07 a | 0.03 cd | 0.05 b |
| 18:0 | 1.43 bc | 1.56 b | 1.32 c | 3.11 a | 1.47 bc | 1.61 b |
| 20:0 | 0.56 c | 0.71 b | 0.34 d | 0.96 a | 0.59 c | 0.94 a |
| 22:0 | 0.21 c | 0.30 b | 0.21 c | 0.38 a | 0.33 b | 0.38 a |
| 24:0 | 0.07 de | 0.09 c | 0.06 e | 0.16 a | 0.08 cd | 0.11 b |
| SFA | 7.54 c | 7.13 c | 7.96 bc | 12.68 a | 7.93 bc | 8.80 b |
| 16:1 | 0.08 b | 0.05 c | 0.10 a | 0.10 a | 0.07 b | 0.08 b |
| 16:1 9-cis | 0.11 a | 0.05 c | 0.05 c | 0.09 b | 0.06 c | 0.10 ab |
| 18:1 9-cis | 23.02 a | 18.27 b | 19.49 b | 22.29 a | 18.30 b | 24.17 a |
| 18:1 11-trance | 0.62 a | 0.40 b | 0.67 a | 0.44 b | 0.47 b | 0.61 a |
| 22:1 | 0.09 c | 0.10 bc | 0.07 d | 0.18 a | 0.11 b | 0.07 d |
| MUFA | 23.92 a | 18.87 b | 20.38 b | 23.10 a | 19.01 b | 25.03 a |
| 18::2i | 0.09 a | 0.05 c | 0.07 b | 0.05 c | 0.06 bc | 0.05 c |
| 18:2 | 67.76 ac | 73.68 a | 71.00 ab | 63.57 c | 72.34 a | 65.32 bc |
| 20:2 | 0.38 bc | 0.04 e | 0.32 d | 0.44 a | 0.34 cd | 0.41 ab |
| 18:3 ω-3 | 0.26 a | 0.20 bc | 0.21 bc | 0.19 c | 0.22 b | 0.27 a |
| 20:3 | 0.04 c | 0.02 d | 0.06 b | 0.02 d | 0.09 a | 0.10 a |
| PUFA | 68.53 ac | 73.99 a | 71.66 ab | 64.27 c | 73.05 a | 66.15 bc |

Along each row, means followed by different letters are significantly different according to the Duncan test at p≤0.05 (n=3). SFA= saturated fatty acids; MUFA = mono unsaturated fatty acids; PUFA = poly unsaturated fatty acids.

the main factor responsible for oil susceptibility to oxidation (Morello *et al*., 2004).

The results obtained in our research indicate that the oil contained in *Allium cepa* and perennial onion species seeds are similar in fatty acids composition and may be related to a group of natural oils, such as grape, poppy, sunflower and safflower oil, with a high content of linoleic acid (60-80%) (Fig. 5). Moreover, the high PUFA/SFA ratio detected in perennial onion seed oil is favourable for the treatment of atherosclerosis and cardiovascular diseases (Dini *et al*., 2008; Yalcin and Kavuncuoglu, 2014) and, in this respect, *A. altaicum* is a very interesting species with the highest PUFA/SFA ratio (10.4) in our trial. In addition, statistically significant differences in the content of oil fatty acids in seeds, both among *Allium cepa* cultivars and perennial onion species, have only been recorded for minor SFA and MUFA, such as C20:2, C22:1, C18:1 11-trans and C16:1 9 cis (Table 5). Notably, fatty acid profiles of these two groups of *Allium* species indicate deeper SFA synthesis in perennial onion seed oil, containing higher concentrations of C20:0, C22:0, C24:0 and a lower level of C18:0.

Other remarkable differences have been found in oil content, as *Allium cepa* seeds showed a 1.7 fold higher

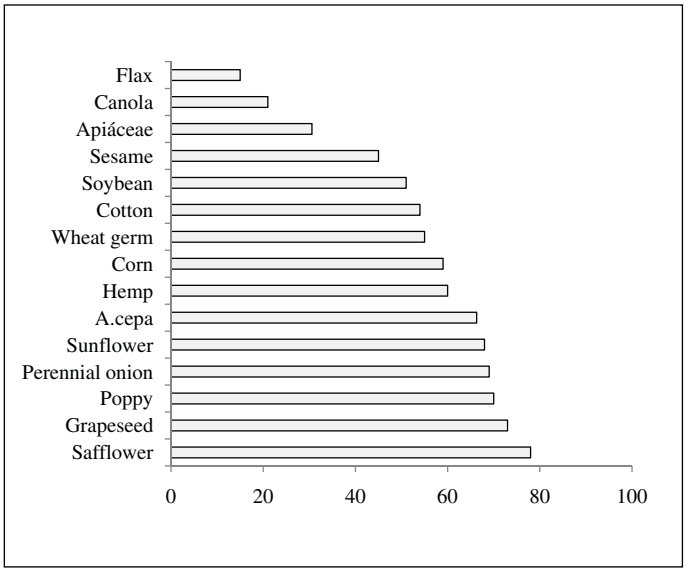


Fig. 5 - Content (%) of oleic acid in several natural oils.

Table 5 - Mean values of fatty acids composition of oil in *Allium cepa* and in perennial onion species seeds

| Fatty acid | Perennial onions (%) | *Allium cepa* (%) | Difference significance probability (P) |
|---|---|---|---|
| Myristinic 14:0 | 0.12±0.03 | 0.13±0.02 | > 0.5 |
| Palmitic 16:0 | 5.68±0.79 | 7.64±0.70 | < 0.001 |
| 17:0 | 0.04±0.01 | 0.03±0.01 | < 0.1 |
| Stearic 18:0 | 1.75±0.45 | 1.47±0.12 | < 0.1 |
| Eicosanoic 20:0 | 0.68±0.19 | 0.25±0.12 | < 0.001 |
| Behenic (docosanoic) 22:0 | 0.30±0.06 | 0.13±0.03 | < 0.001 |
| Lignoceric 24:0 | 0.10±0.03 | 0.02±0.02 | < 0.001 |
| SFA | 8.68±1.37 | 9.68±0.63 | < 0.1 |
| 16:1 | 0.08±0.01 | 0.07±0.01 | < 0.5 |
| 16:1 9-cis | 0.08±0.02 | 0.19±0.02 | < 0.01 |
| Palmitoleic ∑16:1 | 0.16±0.03 | 0.26±0.02 | < 0.002 |
| 18:1 9-cis | 20.90±2.24 | 21.77±2.10 | < 0.5 |
| 18:1 11-trans | 0.54±0.10 | 1.08±011 | < 0.001 |
| Oleic ∑ 18:1 | 21.46±2.26 | 22.95±2.10 | < 0.2 |
| Eicosaenic 20:1 | 0.00 | 0.06±0.08 | < 0.05 |
| Erucic 22:1 | 0.10±0.03 | 0.05±0.02 | < 0.001 |
| MUFA | 21.72±2.30 | 23.21±2.19 | < 0.2 |
| 18::2i | 0.06±0.01 | 0.05±0.02 | < 0.2 |
| 18:2 | 68.95±3.40 | 66.30±2.34 | < 0.1 |
| Linoleic ∑ 18:2 | 69.01±3.39 | 66.31±2.33 | < 0.1 |
| Eicosadienic 20:2 | 0.32±0.09 | 0.24±0.03 | < 0.05 |
| γ-linolenic 18:3 ω-3 | 0.23±0.03 | 0.30±0.26 | < 0.5 |
| Eicosatetraenic 20:3 | 0.06±0.03 | 0.04±0.01 | > 0.5 |
| PUFA | 69.61±3.29 | 66.89±2.43 | < 0.1 |
| UFA (MUFA+PUFA) | 91.33±1.37 | 91.20±1.70 | > 0.5 |

SFA= saturated fatty acids; MUFA = mono unsaturated fatty acids; PUFA = poly unsaturated fatty acids; UFA = unsaturated fatty acids.

level than perennial onions. Notably, the higher oil concentration detected in the two Italian cultivar seeds may be explained by both genotype attitude and the warmer climate in southern Italy.

*Allium* seeds showed a significant amount of Se (Tables 1 and 2), which suggests a possible active participation of this natural antioxidant in PUFA protection against peroxidation. A direct correlation between total Se concentration and oil content was found only in *Allium cepa*, which contained a higher seed oil percentage compared to perennial onion seeds, indicating a functional role of this element. A similar correlation was reported in previous research on flax seeds (Golubkina *et al*., 2012) where a stable protein-oil complex was identified. In this respect, it may be supposed that linseed proteins contain Se-aminoacids, such as selenomethionine or/and selenocystein due to the ability of Se to substitute sulphur in organic compounds (Pilon-Smith, 2015). The existence of a protein-oil complex in *Allium* seeds is unknown and this topic calls for special investigation, as no correlation has been demonstrated between Se and proteins nor between proteins and oil content, both in *Allium cepa* and in perennial onion seeds. Interesting focus has also arisen from Se and PUFA positive correlations. Oleic acid (C18:2), which predominates in *Allium* seed oil, is known to be 17 times more sensitive to oxidation than linoleic acid (C18:1) and, accordingly, antioxidant protection of their degradation has great importance. This is also true for the Italian cultivar seeds, grown in the Mediterranean area where high temperatures during vegetation stimulate the synthesis of oleic acid (C18:1) and inhibit linoleic acid biosynthesis (C18:2) (Bellaloui *et al*., 2013). Such protection may be achieved by Se via protein-oil interaction (Golubkina *et al*., 2012) or via activation of appropriate enzymes. Indeed, it is known that Se demonstrates antioxidant properties in plants by increasing the activity of glutathione peroxidase (Hartikainen *et al*., 2000) or of superoxide dismutase (Xue *et al*., 2001), thus leading to protection of tocopherol reduction; the activation of these enzymes suggests the involvement of Se water soluble forms.

Previous reports indicated that Se water soluble forms show the highest biological activity in the environment (Golubkina *et al.*, 2010). Moreover, it is known that the concentration of water soluble forms of the element increase significantly during seed germination due to auxine activation of hydrolytic enzymes (Golubkina and Papazyan, 2006). Indeed, the content of water soluble Se derivatives is shown to be directly proportional to germination value of *Apiáceae* family seeds (Golubkina *et al*., 2010). These data are in accordance with our research results, where a correlation with PUFA content is only concerned with water soluble forms of this element.

Theoretically, antioxidant defense against lipid peroxidation should be enhanced by Se in *Allium* species because of the relatively low content of oleic acid (C18:1) which shows a stabilizing effect on lipid peroxidation (North *et al*., 1994). In any case, it seems obvious that Se in *Allium* seeds does not independently act, but displays strict interaction with other antioxidants, especially with phenolics. In fact, antioxidants are considered to form a cooperative network, using a series of different redox reactions and this is confirmed by the significant positive correlation between Se water soluble forms and total phenolics content in *Allium* seeds recorded in our research (Fig. 4). Although the direct relationship between Se and total phenolics has already been noted in various agricultural crops enriched with the element (Du *et al*., 2009; Põldma *et al*., 2013; Bystrická *et al*., 2015), such relationship in untreated plant seeds has been demonstrated for the first time in our research. Notably, the highest phenolics content in *Allium* seeds was recorded in *A. fistulosum*, which also gave the highest total Se content (Table 1). Phenolics concentrations detected in *Allium* seeds in our research are close to the values previously reported for amaranth (Vollmannova *et al*., 2013) and soybean (Malencic *et al*., 2012) and underline the high nutritional value of *Allium* seeds. Phenolic antioxidant properties reportedly improve both sunflower seed oil stability during storage (Žilić *et al*., 2010) and, following exogenous application, microencapsulated linseed oil oxidative stability (Rubilar *et al*., 2012). Phenolics also play an important role in plant resistance and protection against microbial infections, which are intimately connected with reactive oxygen species (Emmons and Peterson, 2001; Grassmann *et al*., 2002; Malenčić *et al*., 2008, 2012) and possibly with Se content. Indeed, the concentration of Se water soluble forms was shown to be positively correlated with the resistance of *Brassica chinensis* to bacterial diseases (Golubkina *et al*., 2002).

In conclusion, interesting results have arisen from the research carried out on both *Allium cepa* cultivars and perennial onion species seeds, due to the positive correlations found between oil content, Se and total phenolics concentrations. Notably, Se is supposed to favour an increase in seed oil content and to interact with phenolics, essential for building up protection against oxidation and infections. In this respect, fatty acids composition also exerts a crucial role.

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**References**

ALFTHAN G., 1984 - *A micromethod for the determination of selenium in tissues and biological fluids by single-test-tube fluorimetry*. - Anal. Chim. Acta, 65: 187-194.

AOAC, 1990 - *Official methods of analysis. - 1st ed., Association of Official and Analytical Chemists*. - American Oil Chemists Society, Washington, USA.

AOCS, 1999 - *Fatty acid composition by gas chromatography.*

*official methods and recommended practices of the American Oil Chemists Society* - 5th ed. - American Oil Chemists Society, Champaign Method, Ce: 1-62.

BELLALOUI N., MENGISTU A., ABDELMAJID KASSEM M., 2013 - *Effects of genetics and environment on fatty acid stability in soybean seed*. - Food Nutri. Sci., 4: 165-175.

BYSTRICKÁ J., KAVALCOVÁ P., MUSILOVÁ J., TOMÁŠ J., TÓTH T., ORSÁK M., 2015 - *Selenium and its influence on the content of polyphenol compounds in onion (*Allium cepa *L.).* - J. Microbiol. Biotechnol. Food Sci., 4 (3): 23-26.

DINI I., TENORE G.C., DINI A., 2008 - *Chemical composition, nutritional value and antioxidant properties of* Allium cepa *L. Var. tropeana (red onion) seeds*. - Food Chem., 107: 613-621.

DOBRUTSKAYA H.G., KURBAKOVA O.V., GOLUBKINA N.A., 2010 - *Selenium effect of carrot and dill seeds germination.* - Vestnik of Russian Academy of Agricultural Sciences, 4: 41-43.

DU Q., WANG C.-Q., LI B., LI H.-X., LIU Y., 2009 - *Effects of Se, Zn and their interaction on polyphenol oxidase activity of tea leaves in summer season*. - Acta Metallurgica Sinica, 15(4): 930-935.

EMMONS C.L., PETERSON D.M., 2001 - *Antioxidant activity and phenolic content of oat as affected by cultivar and location*. - Crop Sci., 41: 1676-1681.

FALLEN B.D., PANTALONE V.R.,. SAMS C.E., KOPSELL D.A., VAUGHN S.F., MOSE B.R., 2011 - *Effect of soybean oil fatty acid composition and selenium application on biodiesel properties*. - J. Am. Oil Chem. Soc., 88: 1019-1028.

GERM M., STIHOLJ V., KREFT I., 2007 - *Metabolic importance of selenium for plants*. - Eur. J. Plant Sci. Biotechnol., 1(10): 91-97.

GOLUBKINA N.A., KISELEVA T.V., KROL T.A., STRELETS V.D., 2012 - *Effect of sodium selenate on linseeds oil accumulation and its fatty acids composition*. - Fat and oil industry, 3: 26-28.

GOLUBKINA N.A., KISILEVA T.V., VICTOROVA H.V., KURBAKOVA O.V., FEDOROVA M.I., 2010 - *Fatty acids composition of salary seeds oil*. - Gavrish, 3: 45-48.

GOLUBKINA N.A., PAPAZYAN T.T., 2006 - *Selenium in nutrition: plants, animals, human beings*. - Pechatny Gorod, Moscow, Russian.

GOLUBKINA N.A., STARTSEV V.I., BESPALKO A.V., TEMICHEV A.V., 2002 - *Biological role of several antioxidants in* Brassica chinenses. - Agrarian Sci., 12: 14-15.

GRASSMANN J., HIPPELI S., ELSTNER E.F., 2002 - *Plant's defense and its benefits for animals and medicine: role of phenolics and terpenoids in avoiding oxygen stress*. - Plant Physiol. Biochem., 40: 471-478.

HARMANN G.E., MATTICK R.L., 1976 - *Association of lipid oxidation with seed aging and death.* - Nature, 260: 323-324.

HARTIKAINEN H., XUE T.L., PIIRONEN V., 2000 - *Selenium as an anti-oxidant and pro-oxidant in ryegrass*. - Plant Soil, 225: 193-200.

KAYMAK H.Ç., 2014 - *Potential effect of seed fatty acid profile of pepper (*Capsicum annuum *L.) cultivars on germination at various temperatures*. - Zemdirbyste-Agriculture, 101(3): 321-326.

MALENČIĆ D., CVEJIIC J., MILADINOVIC J., 2012 - *Polyphenol content and antioxidant properties of colored soybean seeds from Central Europe*. - J. Med. Food, 15(1): 89-95.

MALENČIĆ D., MAKSIMOVIĆ Z., POPOVIĆ M., MILADINOVIĆ J., 2008 - *Polyphenol contents and antioxidant activity of soybean seed extracts*. - Bioresource Technol., 99: 6688-6691.

MORELLO J.R., MOTILVA M.J., TOVAR M.J., ROMERO M.P., 2004 - *Changes in commercial virgin olive oil (cv Arbequina) during storage with special emphasis on the phenolic fraction.* - J. Food Chem., 85: 357-364.

NAKAMURA Y.K., FLINTOFF-DYE N., OMAYE S.T., 2008 - *Conjugated linoleic acid modulation of risk factors associated with atherosclerosis*. - Nutr. Metab., 22: 1-20.

NORTH L.S.A., NORTH J.A., KIMINYO K.P., BUETTNER G.R., SPECTOR A.A., 1994 - *Polyunsaturated fatty acids increase lipid radical formation induced by oxidant stress in endothelial cells*. - J. Lipid Res., 35: 1773-1785.

PILON-SMITS E.A.H., 2015 - *Selenium in plants*. - Progress in Botany. Luttge U. and Beyschlag W. Eds., Springer International Publishing Switzerland, 93: 107.

PÕLDMA P., MOOR U., TÕNUTARE T., HERODES K., REBANE R., 2013 - *Selenium treatment under field conditions affects mineral nutrition, yield and antioxidant properties of bulb onion (*Allium cepa *L.).* - Acta Sci. Pol., Hortorum Cultus, 12(6): 167-181.

RUBILAR M., MORALES E., SAEZ R., ACEVEDO F., PALMA B., VILLARROEL M., SHENE C., 2012 - *Polyphenolic fractions improve the oxidative stability of microencapsulated linseed oil.* - Eur. J. Lipid Sci. Technol., 114: 760-771.

SAGDIC O., SILICI S., EKICI L., 2011 - *Evaluation of the phenolic content and antiradical, antioxidant, antimicrobial activities of different floral sources of honey*. - Int. J. Food Prop., 16: 658-666.

SIDLAUSKAS G., BERNOTAS S., 2003 - *Some factors affecting seed yield of spring oilseed rape (*Brassica napus *L.).* - Agronomy Res., 1(2): 229-243.

TERRY N., ZAYED A., DE SOUZA M., TARUN A., 2000 - *Selenium in higher plants*. - Annu. Rev. Plant Physiol., 51: 401-432.

VOLLMANNOVA A., MARGITANOVA E., TOTH T., TIMORACKA M., URMINSKA D., BOJNANSKA T., CICOVA I., 2013 - *Cultivar influence on total polyphenol and rutin contents and total antioxidant activity in buckwheat, amaranth and quinoa seeds*. - Czech J. Food Sci., 31(6): 589-595.

XUE T., HARTIKAINE H., PIIRONEN V., 2001 - *Antioxidative and growth-promoting effect of selenium on senescing lettuce*. - Plant Soil, 237: 55-61.

YALCIN H., KAVUNCUOGLU H., 2014 - *Physical, chemical and bioactive properties of onion (*Allium cepa *L.) seed and seeds oil*. - J. Appl. Botany Food Anal., 87: 87-92.

ŽILIĆ S., MAKSIMOVIĆ DRAGIŠIĆ J., MAKSIMOVIĆ V., MAKSIMOVIĆ M., BASIĆ Z., CREVAR M., STANKOVIĆ G., 2010 - *The content of antioxidants in sunflower seed and kernel*. - Helia, 33(52): 75-84.

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# Effects of extracellular $K^+$ on grapevine membrane potential as influenced by the antiviral mycophenolic acid. An electrophysiological study

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*Key words:* chemotherapy, virus host interaction, *Vitis vinifera*.

**Abstract: Mycophenolic acid (MPA) is an effective antiviral drug in plants, and its action in modulating the activity of $K_{ATP}$ channels is already known in animals. In the present work an electrophysiological study was carried out to investigate MPA effects on plant $K^+$ channels, through the measurement of trans-plasma membrane potential in samples of *Vitis vinifera* cv. Sangiovese treated with extracellular $K^+$. Tests confirmed that the administration of MPA (in preincubated samples or in those maintained under chemical treatment) can reduce the membrane depolarization induced by $K^+$. However, MPA-induced alteration in membrane potential was sensitive to the $K_{ATP}$ channel opener diazoxide, as well to treatments with guanosine. This result confirms the effectiveness of MPA in influencing $K_{ATP}$ channel activity as well as inhibiting activity of the inward-rectifier potassium ion channel which could be mediated by guanosine depletion induced by MPA.**

## 1. Introduction

Ion fluxes across cellular membranes are known to play the key role in triggering and mediating defense mechanisms in plants, however little data are currently available on ion signatures generated during plant-virus interactions. Changes in $K^+$ fluxes after virus inoculation may be mediated by depolarization-activated outward-rectifying $K^+$ channels (Shabala *et al*., 2010).

In animals, the immunosuppressant drug mycophenolic acid (MPA) [(4E)-6-(4-Hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydro-2-benzofuran-5-yl)-4-methylhex-4-enoic acid] depletes cellular guanine nucleoside (GN) by inhibition of inosine monophosphate dehydrogenase, modulating the activity of $K_{ATP}$ channels (Li *et al*., 2000). MPA is also an effective antiviral drug in plants such as grapevine (Panattoni *et al*., 2007; Skiada *et al*., 2009; Luvisi *et al*., 2012 a; Skiada *et al*., 2013; Panattoni *et al*., 2014; Guazzelli *et al*., 2015), but no reports about its effect on K-conducting ion channels in plants are available.

In terms of metabolic dependence of MPA action in plant cells, antiviral drug translocation across membranes was linked to the free energy available in a proton electrochemical potential difference (Luvisi *et al*., 2012 b). Nowadays, compounds that influence $K_{ATP}$ channel activity are currently available for clinical use, and include diazoxide (DO) (7-Chloro-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide) (Babenko *et al*., 1998; 2000) whose effectiveness was also confirmed in plants to investigate the inhibition of mitochondrial $K_{ATP}$ channels (Chiandussi *et al*., 2002).

With regard to the potential effect due to GN depletion by MPA, cyclic derivatives of GN can be involved in plant K-conducting ion channels. In fact, inward-rectifier potassium ion channels have been cloned from *Hordeum vulgare*, *Nicotiana tabacum* and *Arabidopsis thaliana* (Leng *et al*., 1999). These channels open in the presence of cyclic nucleotides such as cyclic guanosine monophosphate (cGMP), and are therefore referred to as cyclic nucleotide gated channels (Leng *et al*., 2002). The cGMP is thought to be present in the cytosol of plant cells, and to be involved in signal transduction pathways which regulate many aspects of cellular metabolism (Assmann, 1995).

The activity of antiviral drugs in grapevine cells was recently investigated using electrophysiological methods (Panattoni *et al*., 2013 a). In the present paper we investigate the effects of MPA on depolarization induced by extracellular $K^+$ in foliar samples of *Vitis vinifera* cv. Sangiovese. This grapevine cultivar is affected by various plant viruses (Rizzo *et al*., 2012; Rizzo *et al*., 2015) and the ef-

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fects of antiviral drugs where investigated (Panattoni *et al*., 2011; Luvisi *et al*., 2011; Panattoni *et al.*, 2013 b). In addition, the effects of GN- or DO- treatments were evaluated in order to investigate the effect of GN depletion caused by MPA or its effects on $K_{ATP}$ channels.

## 2. Materials and Methods

*Plant material*

Virus-free (with regard to viruses included in the European Commission directive 2005/43/EC), eight-year-old *V. vinifera* cv. Sangiovese were used for electrophysiological tests. The sanitary condition of each plant was confirmed by RT-PCR (Nakaune and Nakano, 2006; Faggioli *et al*., 2013). Symptomless plants were used. In June 2013, fully expanded leaves were excised and fresh freehand samples (3-5 mm diameter) were cut with an ethanol-cleaned razor blade for testing (Rinaldelli *et al*., 2012; 2014).

*Measurement of membrane potential (Em)*

For chemical assays (Table 1), preincubation for 1 h in basal solution (BS) or chemical-BS solutions (MPA-BS, GN-BS, DO-BS at 0.5, 1.0, 2.0 mM) adjusted to pH 5.6 with TRIS (2-Amino-2-hydroxymethyl-propane-1,3-diol) was followed by preparation of leaf segments according to Luvisi *et al*. (2012 b). Step 1 for Em measurements was conducted perfusing aerated BS or chemical-BS through the chamber that fixes the sample at a flow rate of 10.0 x $^{10\text{-}3}$ L $min^{-1}$. The measuring electrodes used were micropipettes (tip diameter < 1µm) obtained from single-barreled borosilicate capillaries (World Precision Instruments, Sarasota, USA) as described in Rinaldelli *et al.* (2012). Insertion of the microelectrodes took place in the central zone of the mesophyll by way of a micromanipulator (World Precision Instruments, Sarasota, USA). Successful microelectrode impalement was determined by rapid attainment of a steady value without subsequent decay (Ober and Sharp, 2003), followed by a stabilized Em for 5 min. Em after stabilization was recorded to evaluate the effect of chemicals on membrane potential. Membrane signal steadiness was calculated considering the number of successful cell impalements out of those attempted (%). Step 2 began after Em stabilization, using solutions enriched with KCl.

Two procedures were carried out in order to investigate the effects induced by extracellular $K^+$ following MPA treatments. The first procedure was carried out on samples in which treatment involved only the preincubation step (Li *et al*., 2000). Thus, samples were preincubated in MPA-BS, followed by Em measurement under BS (step 1) and K-BS (step 2). In this test, two concentrations of KCl (5, 10 mM) were evaluated. The effect of chemical treatment was calculated considering Em after BS stabilization and maximum Em achieved after K-BS administration, expressed as Δ Em (Table 1). The second procedure was carried out following a conventional electrophysiological approach. In order to evaluate the interference of chemicals (MPA, GN or DO) on effects induced by $K^+$, tests were carried out using chemical-BS solution both in preincubation and in step 1, while in step 2 the solution was enriched with KCl (chemical-K-BS) at 10 mM (concentration chosen considering results of the first procedure). The interference of chemicals on membrane depolarization induced by $K^+$ was calculated considering Em after chemical-BS

Table 1 - Solutions used in membrane potential tests

| | Chemical assay | | |
|---|---|---|---|
| Solution | Abbreviation | Membrane potential (Em) | |
| $CaCl_2$ 5.0x10$^{-4}$ M, $K_2SO_4$ 2.5x10$^{-3}$ M, MES 5.0x10$^{-3}$ M | BS | $Em_{BS}$ | |
| BS with MPA | MPA-BS | $Em_{MPA}$ | |
| BS with Guanosine | GN-BS | $Em_{GN}$ | |
| BS with Diazoxide | DO-BS | $Em_{DO}$ | |
| | Extracellular $K^+$ assay | | |
| Solution | Abbreviation | Membrane potential (Em) | Variation of membrane potential (%) |
| BS with KCl | K-BS | $Em_K$ | $Em_K - Em_{BS} = \Delta Em_K$ |
| MPA-BS with KCl | MPA-K-BS | $Em_{MPA\text{-}K}$ | $Em_{MPA\text{-}K} - Em_{MPA} = \Delta Em_{MPA\text{-}K}$ |
| GN-BS with KCl | GN-K-BS | $Em_{GN\text{-}K}$ | $Em_{GN\text{-}K} - Em_{GN} = \Delta Em_{GN\text{-}K}$ |
| DO-BS with KCl | DO-K-BS | $Em_{DO\text{-}K}$ | $Em_{DO\text{-}K} - Em_{DO} = \Delta Em_{DO\text{-}K}$ |
| MPA-BS with Guanosine | MPA-GN-BS | $Em_{MPA\text{-}GN}$ | |
| MPA-BS with Diazoxide | MPA-DO-BS | $Em_{MPA\text{-}DO}$ | |
| MPA-GN-BS with KCl | MPA-GN-K-BS | $Em_{MPA\text{-}GN\text{-}K}$ | $Em_{MPA\text{-}GN\text{-}K} - Em_{MPA\text{-}GN} = \Delta Em_{MPA\text{-}GN\text{-}K}$ |
| MPA-DO-BS with KCl | MPA-DO-K-BS | $Em_{MPA\text{-}DO\text{-}K}$ | $Em_{MPA\text{-}DO\text{-}K} - Em_{MPA\text{-}DO} = \Delta Em_{MPA\text{-}DO\text{-}K}$ |

stabilization and maximum Em achieved after chemical-K-BS administration, expressed as Δ Em (Table 1).

Preincubation and electrophysiological tests were carried out at 22±0.5°C under light (30 watt $m^{-2}$). All tests were conducted on 15 healthy or infected samples. Plots are representative of 15 equivalent experiments. Measurements were performed under Faraday cage to protect tests from external radio frequency interference.

*Statistical analysis*

The effects of treatments on Δ Em were elaborated using Sigma-Plot software (version 11; Systat Software, San Jose, CA). The software was used to perform one- or two-way analysis of variance (ANOVA) in a random design and pairwise multiple comparisons on significant effects and interactions using the Holm-Sidak method. Data expressed in percent were converted to arcsin values. $P \leq 0.05$ was considered to be significant.

## 3. Results and Discussion

*Em measurement in chemical preincubated samples*

Foliar samples were preincubated in chemical-BS solutions and membrane potential was measured with microelectrodes. Preincubation with MPA-BS solution did not change the resting membrane potential with regard to the chosen concentration (Table 2). However, MPA seems to interfere with the signal steadiness at 1.0 and 2.0 mM, causing a reduction of successful impalement by 37.9 and 64.7 %, respectively. Assays carried out with BS enriched by GN or DO showed no effect on membrane potential and did not interfere with the steadiness of the signal.

With regard to the effects induced by extracellular $K^+$, Em measurement was carried out perfusing aerated BS solution throughout the chamber with the fixed sample (step 1), followed by perfusion of K-BS solution (step 2), and recording the depolarization due to K+ treatment. In MPA-preincubated samples, the antiviral drug can cause a differential response to potassium effects, as shown in Figure 1. The cell response confirms the Nernstian changes in diffusion potential due to increased $K^+$-concentration or activation of $K^+$ channels.

As reported in Table 3, KCl caused different depolarization according to each concentration, but the preincuba-

Table 2 - Effect of sample preincubation with chemical administered at different concentrations (0.5, 1.0, 2.0 mM) on membrane potential (Em, mV) or membrane potential signal steadiness on samples of *Vitis vinifera* cv. Sangiovese, expressed as % of successful microelectrode impalement

| Treatment | Em (mV) | Signal steadiness (%) |
|---|---|---|
| BS | -109.5±6.0 a [z] | 53.6 |
| MPA-BS (mM) | | |
| 0.5 | -110.6±7.2 a | 51.7 |
| 1.0 | -110.0±6.9 a | 33.3 |
| 2.0 | -107.6±5.7 a | 18.8 |
| GN-BS (mM) | | |
| 0.5 | -111.6±8.5 a | 50.0 |
| 1.0 | -112.0±9.5 a | 48.4 |
| 2.0 | -109.0±7.0 a | 53.6 |
| DO-BS (mM) | | |
| 0.5 | -110.9±7.8 a | 45.5 |
| 1.0 | -113.5±8.5 a | 48.4 |
| 2.0 | -108.3±7.1 a | 51.7 |

[z] Values in the same column followed by the same letter do not differ significantly according to Duncan's multiple range test ($P \leq 0.05$).

BS= Basal solution; MPA= Mycophenolic acid; GN= Guanosine; DO= Diazoxide.

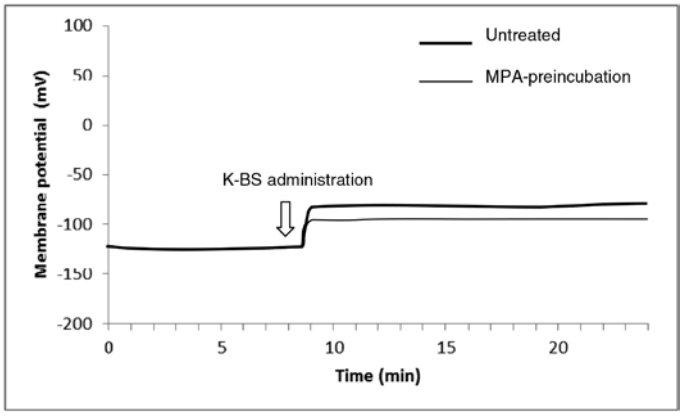


Fig. 1 - Effects induced on samples of *Vitis vinifera* cv. Sangiovese by extracellular $K^+$ (KCl 10 mM) subsequent to MPA 1.0 mM treatment compared to untreated sample. BS = basal solution; MPA = mycophenolic acid. Plot is representative of 15 equivalent experiments.

Table 3 - Depolarization induced in samples of *Vitis vinifera* cv. Sangiovese by extracellular K+ at 5 or 10 mM following MPA-BS preincubation compared to untreated sample

| | Δ Em (%) | |
|---|---|---|
| | K-BS (mM) | |
| | 5 | 10 |
| BS | 18.2±3.5a [z] A [y] | 30.7±3.7 aB |
| MPA-BS (mM) | | |
| 0.5 | 17.9±4.1 aA | 29.8±3.2 aB |
| 1.0 | 12.0±3.2 bA | 23.3±2.0 bB |
| 2.0 | 11.1±2.3 bA | 16.4±4.1 cB |

[z] values in the same column followed by the same letter do not differ significantly according to Duncan's multiple range test ($P \leq 0.05$).

[y] values in the same line followed by the same letter do not differ significantly according to Duncan's multiple range test ($P \leq 0.05$).

The effect was calculated considering Em after BS stabilization and maximum Em achieved after K-BS administration, expressed as Δ Em (%).

BS = basal solution; MPA = mycophenolic acid.

tion with MPA was able to cause a reduction in depolarization. In particular, MPA at 1.0 or 2.0 mM significantly affected KCl at 10 mM, while MPA at 5 mM seemed not to alter the effect induced by extracellular $K^+$. KCl at 10 mM was used for the subsequent test.

*Em measurement in samples maintained under chemical treatment*

Tests carried out while maintaining chemical administration throughout steps 1 (perfusion of chemical-BS solution) and 2 (perfusion of chemical-BS solution enriched by KCl) of Em measurement confirmed the effectiveness of MPA in reducing the effect induced by $K^+$ (Table 4). MPA interference was concentration-dependent and at the lower concentration MPA did not interfere with the effect on membrane potential induced by $K^+$. Conversely, at higher dosages (2:1 MPA:KCl), MPA causes a $\Delta Em_{MPA\text{-}K}$ of 2.5±0.7%, with a reduction of more than 90% of potassium effects. MPA effectiveness was maintained at equal concentration (1:1 MPA:KCl), showing $\Delta Em_{MPA\text{-}K}$ at 9.5±1.1 %, with a reduction of almost 70 % of potassium effects. No effects on trans-plasma membrane depolarization due to $K^+$ were registered by GN or DO.

Simultaneous administration of GN with MPA (2:1) caused complete restoration of external $K^+$ effect on membrane potential, as well as adding DO (1:1) (data not shown).

## 4. Conclusions

Membrane depolarization caused by MPA (Rinaldelli *et al*., 2012) is a temporary effect. In fact, after 1 h of preincubation in MPA-BS solution, the trans-plasma membrane potential of the sample was not altered by the antiviral drug compared to the untreated control. Similarly, GN or DO did not change resting membrane potential. Interference of signal steadiness caused by MPA at a higher concentration may be linked to cell toxicity induced by the antiviral drug (Panattoni *et al*., 2007; Luvisi *et al*., 2012 a). In fact, samples under stress conditions increase the difficulty of cell membrane measurements (Vuletic *et al*., 1987; Rawyler *et al*., 2002).

With regard to effects induced by extracellular $K^+$, preincubation tests showed how MPA at 1.0 mM or higher concentrations can interfere up to 10 mM of KCl. The MPA reduction of membrane depolarization caused by extracellular $K^+$ was confirmed by following tests in which chemical administration was maintained throughout all steps of measurement. The reduction of potassium effects was higher compared to administering MPA only in the preincubation step. This result is probably linked to the variation in MPA intra-/extracellular gradient due to solution changes between preincubation and steps 1-2.

Our results indicate that the antiviral MPA inhibited the effects of extracellular $K^+$ in plants, through specific channels. In fact, the MPA-induced alteration in membrane potential was sensitive to the $K_{ATP}$ channel opener DO, which hyperpolarized resting membrane potential in treated cells to a level similar to that achieved in control cells; results were also similar to those obtained in animal cells (Li *et al*., 2000). Moreover, GN was able to inhibit the MPA action against extracellular $K^+$, suggesting that MPA could act also as an inward-rectifier potassium ion channel inhibitor through the depletion of GN.

Inhibition of the activity of $K^+$ channels caused by MPA may be involved in programmed cell death (PCD) in

Table 4 - Two-way factorial analysis of variance of Δ Em caused by chemical-K-BS (MPA-K-BS, GN-K-BS, DO-K-BS, KCl at 10 mM) on samples of *Vitis vinifera* cv. Sangiovese treaded chemical-BS (MPA-BS, GN-BS, DO-BS). Pairwise multiple comparison analysis with Holm-Sidak test was performed

| Source of Variation | DF | SS | P |
|---|---|---|---|
| Treatment (A) | 2 | 0.836 | <0.001 |
| Concentration (B) | 3 | 0.228 | <0.001 |
| A x B | 6 | 0.696 | <0.001 |
| Residual | 170 | 0.207 | |
| Total | 181 | 1.961 | |
| Comparison for factor | DM | t | P |
| Comparison for A | | | |
| DO vs. MPA | 0.149 | 23.461 | <0.001 |
| GN vs. MPA | 0.139 | 21.880 | <0.001 |
| DO vs. GN | 0.010 | 1.5888 | NS |
| Comparison for B within MPA | | | |
| 0.0 mM vs. 2.0 mM | 0.284 | 22.276 | <0.001 |
| 0.5 mM vs. 2.0 mM | 0.250 | 19.586 | <0.001 |
| 0.0 mM vs. 1.0 mM | 0.231 | 18.160 | <0.001 |
| 0.5 mM vs. 1.0 mM | 0.197 | 15.470 | <0.001 |
| 1.0 mM vs. 2.0 mM | 0.052 | 4.116 | <0.001 |
| 0.0 mM vs. 0.5 mM | 0.034 | 2.690 | NS |
| Comparison for B within GN | | | |
| 2.0 mM vs. 0.5 mM | 0.00543 | 0.433 | NS |
| 2.0 mM vs. 0.0 mM | 0.00467 | 0.366 | NS |
| 1.0 mM vs. 0.5 mM | 0.00276 | 0.220 | NS |
| 2.0 mM vs. 1.0 mM | 0.00267 | 0.209 | NS |
| 1.0 mM vs. 0.0 mM | 0.00200 | 0.157 | NS |
| 0.0 mM vs. 0.5 mM | 0.00076 | 0.061 | NS |
| Comparison for B within DO | | | |
| 2.0 mM vs. 0.5 mM | 0.0473 | 3.771 | NS |
| 2.0 mM vs. 0.0 mM | 0.0447 | 3.505 | NS |
| 2.0 mM vs. 1.0 mM | 0.0406 | 3.189 | NS |
| 1.0 mM vs. 0.5 mM | 0.0067 | 0.531 | NS |
| 1.0 mM vs. 0.0 mM | 0.0040 | 0.316 | NS |
| 0.0 mM vs. 0.5 mM | 0.0026 | 0.210 | NS |

BS= Basal solution; MPA = Mycophenolic acid; GN = Guanosine; DO = Diazoxide.
SS= sum of square, DF = degrees of freedom, DM = difference of means, t = t-value.
NS= Non significant at P> 0.05.

response to viral infection. Excessive $K^+$ efflux and intracellular $K^+$ depletion are the key early steps in apoptosis (one form of the PCD) in mammalian systems. A critical role for potassium homeostasis in the apoptotic process has also been endorsed for plants (Huh *et al*., 2002; Shabala *et al*., 2007; Shabala, 2009) even if it has not yet been linked to the mechanism of action of MPA in plants.

## References


ASSMANN S.M., 1995 - *Cyclic AMP as a second messenger in higher plants. Status and future prospects*. - Plant Physiol., 108: 885-889.

BABENKO A.P., AGUILAR-BRYAN L., BRYAN J., 1998 - *A view of sur/KIR6.X, KATP channels*. - Annu. Rev. Physiol., 60: 667-687.

BABENKO A.P., GONZALEZ G., BRYAN J., 2000 - *Pharmaco-topology of sulfonylurea receptors. Separate domains of the regulatory subunits of K(ATP) channel isoforms are required for selective interaction with K(+) channel openers*. - J. Biol. Chem., 275: 717-720.

CHIANDUSSI E., PETRUSSA E., MACRÌ F., VIANELLO A., 2002 - *Modulation of a plant mitochondrial $K^+_{ATP}$ channel and its involvement in cytochrome c release*. - J. Bioenerg. Biomembr., 34: 177-184.

FAGGIOLI F., ANACLERIO F., ANGELINI E., BERTAZZON N., ANTONELLI M.G., BIANCHI G., BIANCHEDI P., BIANCO P.A., BOTTI S., BRAGAGNA P., CARDONI M., CASATI P., CREDI R., DE LUCA E., DURANTE G., GIANINAZZI C., GAMBINO G., GUALANDRI V., LUISON D., LUVISI A., MALOSSINI U., MANNINI F., SALDARELLI P., TERLIZZI F., TRIOLO E., TRISCIUZZI N., BARBA M., 2013 - *Harmonization and validation of diagnostic protocols for the detection of grapevine viruses covered by phytosanitary rules*. - Adv. Hort. Sci., 27(3): 107-108.

GUAZZELLI L., D'ANDREA F., GIORGELLI F., CATELANI G., PANATTONI A., LUVISI A., 2015 - *Synthesis of PAMAM dendrimers loaded with mycophenolic acid to be studied as new potential immunosuppressants*. - Journal of Chemistry, 2015: 263072.

HUH G.H., DAMSZ B., MATSUMOTO T.K., REDDY M.P., RUS A.M., IBEAS J.I., NARASIMHAN M.L., BRESSAN R.A., HASEGAWA P.M., 2002 - *Salt causes ion disequilibrium-induced programmed cell death in yeast and plants*. - Plant J., 29: 649-659.

LENG Q., MERCIER R.W., HUA B.G., FROMM H., BERKOWITZ G.A., 2002 - *Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels*. - Plant Physiol., 128: 400-410.

LENG Q., MERCIER R.W., YAO W., BERKOWITZ G.A., 1999 - *Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel*. - Plant Physiol., 121: 753-761.

LI G.D., LUO R.H., METZ S.A., 2000 - *Effects of inhibitors of guanine nucleotide synthesis on membrane potential and cytosolic free Ca21 Levels in insulin-secreting cells*. - Biochem. Pharmacol., 59: 545-556.

LUVISI A., PANATTONI A., TRIOLO E., 2011 - *Thiopurine prodrugs for plant chemotherapy purposes*. - J. Phytopathol., 159(5): 390-392.

LUVISI A., PANATTONI A., TRIOLO E., 2012 a - *Eradication trials of tobacco mosaic virus using chemical drugs*. - Acta Virol., 56: 155-158.

LUVISI A., RINALDELLI E., PANATTONI A., TRIOLO E., 2012 b - *Membrane transport of antiviral drugs in plants: an electrophysiological study in grapevine explants infected by Grapevine leafroll associated virus 1*. - Acta Physiol. Plant., 34: 2115-2123.

NAKAUNE R., NAKANO M., 2006 - *Efficient methods for sample processing and cDNA synthesis by RT-PCR for the detection of grapevine viruses and viroids*. - J. Virol. Methods, 134: 244-249.

OBER E.S., SHARP R.E., 2003 - *Electrophysiological responses of maize roots to low water potential: relationship to growth and ABA accumulation*. - J. Exp. Bot., 54: 813-824.

PANATTONI A., D'ANNA F., TRIOLO E., 2007 - *Antiviral activity of tiazofurin and mycophenolic acid against Grapevine Leafroll-associated Virus 3 in* Vitis vinifera *explants*. - Antivir. Res., 73: 206-211.

PANATTONI A., LUVISI A., FUSELLI S., D'ANDREA F., GIORGELLI F., GUAZZELLI L., CATELANI G., TRIOLO E., 2014 - *Antiviral activity of mycophenolic acid derivatives in plants*. - Acta Virol., 58: 99-102.

PANATTONI A., LUVISI A., TRIOLO E 2013 b - *Elimination of viruses in plants: twenty years of progress*. - Span. J. Agric. Res., 11(1): 173-188.

PANATTONI A., LUVISI A., TRIOLO E., 2011 - *Selective chemotherapy on Grapevine Leafroll-associated viruses*. - Phytoparasitica, 39: 503-508.

PANATTONI A., RINALDELLI E., TRIOLO E., LUVISI A., 2013 a - In vivo *inhibition of trans-plasma membrane electron transport by antiviral drugs in grapevine*. - J. Membrane Biol., 246: 513-518.

RAWYLER A., ARPAGAUS S., BRAENDLE R., 2002 - *Impact of oxygen stress and energy availability on membrane stability of plant cells*. - Ann. Bot., 90: 499-507.

RINALDELLI E., LUVISI A., PANATTONI A., 2014 - *Virus interference with trans-plasma membrane activity in infected grapevine leaves*. - Acta Physiol. Plant., 36(12): 3345-3349.

RINALDELLI E., PANATTONI A., LUVISI A., TRIOLO E., 2012 - *Effect of mycophenolic acid on trans-plasma membrane electron transport and electric potential in virus-infected plant tissue*. - Plant Physiol. Bioch., 60: 137-140.

RIZZO D., MATERAZZI A., STEFANI L., FARINA P., VANARELLI S., PANATTONI A., LUVISI A., 2015 - *Distribution of regulated viruses in cv. Sangiovese vineyards in Tuscany*. - J. Plant Pathol., 97(2): 131-135.

RIZZO D., STEFANI L., PAOLI M., TRIOLO E., PANATTONI A., LUVISI A., 2012 - *The sustainability of old grapevine mother plants in relation to new mandatory diagnostic tests for virus control*. - Adv. Hort. Sci., 26(3-4): 148-150.

SHABALA S., 2009 - *Salinity and programmed cell death: unraveling mechanisms for ion specific signaling*. - J. Exp. Bot., 60: 709-711.

SHABALA S., BABOURINA O., RENGEL Z., NEMCHINOV L.G., 2010 - *Non-invasive microelectrode potassium flux*

*measurements as a potential tool for early recognition of virus-host compatibility in plants*. - Planta, 232: 807-815.

SHABALA S., CUIN T.A., PRISMALL L., NEMCHINOV L.G., 2007 - *Expression of animal CED-9 anti-apoptotic gene in tobacco modifies plasma membrane ion fluxes in response to salinity and oxidative stress*. - Planta, 227: 189-197.

SKIADA F., MALIOGKA V.I., ELEFTHERIOU E.P., KATIS N.I., 2009 - *Advances on the eradication of Grapevine rupestrist stem pitting associated virus (GRSPaV) from* Vitis vinifera *explants*. - Le Progrès Agricole et Viticole, 2009: 262-263.

SKIADA F.G., MALIOGKA V.I., KATIS N.I., ELEFTHERIOU E.P., 2013 - *Elimination of Grapevine rupestris stem pitting-associated virus (GRSPaV) from two* Vitis vinifera *cultivars by* in vitro *chemotherapy*. - Eur. J. Plant. Pathol., 135: 407-414.

VULETIC M., RADENOVIC C., VUCINIC Z., 1987 - *The role of calcium in the generation of membrane potential oscillations in Nitella cells*. - Gen. Physiol. Biophys., 6: 203-207.

Adv. Hort. Sci., 2015 29(4): 213-219

# Alternate bearing in olive initiated by abiotic induction leading to biotic responses

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*Key words:* alternate bearing, environmental control, fruiting metabolism, olive.

**Abstract: Alternate bearing of olive trees is one of the most troublesome characteristics of this commodity, impacting its economy due to labor distribution, fruit and oil availability, oil mill capacity and marketing. The metabolic changes leading to alteration in fruit production are generally considered of direct genetic nature. In the present review this approach is challenged, showing that all the biotic-metabolic changes in olive leading to 'on' and 'off' years are the results of initial abiotic effects on the trees. The nature of the metabolic changes induced by the abiotic regional and annual conditions described are, no doubt, genetically controlled but initiated only as a result of adverse environmental abiotic conditions such as seasonal temperatures, water stress, and soil nutrition conditions.**

Olive is one of the most alternating tree species among the commercially grown fruit trees and it is known as such worldwide. Fruiting alternation of olive is considered to be dependent both on environmental abiotic and endogenous biotic genetic factors. The degree of orchard fruiting alternation, even of the same cultivar, differs considerably between different areas and regions, thus an initial or independent genetic involvement is questionable. Fruit bearing alternation is particularly recognized in regions with climatic conditions that vary annually, particularly winter temperatures such as in the eastern Mediterranean basin (Fig. 1). In such regions alternate bearing is usually expressed and synchronized within the entire orchard, area or even region. Still, alternate bearing, although to a lesser degree, develops also in regions with a stable annual climate favorable for the olive tree's developmental cycle. The requirement of low, particularly varying temperatures between day and night in the winter to induce reproductive bud differentiation was established many years ago (Hartmann and Prolingis, 1957; Hartmann and Whisler, 1975). The buds of *Olea europaea* develop uniformly as they are of undefined nature and need to be induced to become either vegetative or reproductive (Fig. 2). Various studies describing the anatomical changes occurring in buds have been published and have mainly emphasized the changes leading to the reproductive state, though in some studies also leaf buds (Fabbri and Alerci, 1999). It is still controversial whether low temperature is required for the induction leading to differentiation, as during the process of vernalization (Lavee, 1989; 1996; Troncoso

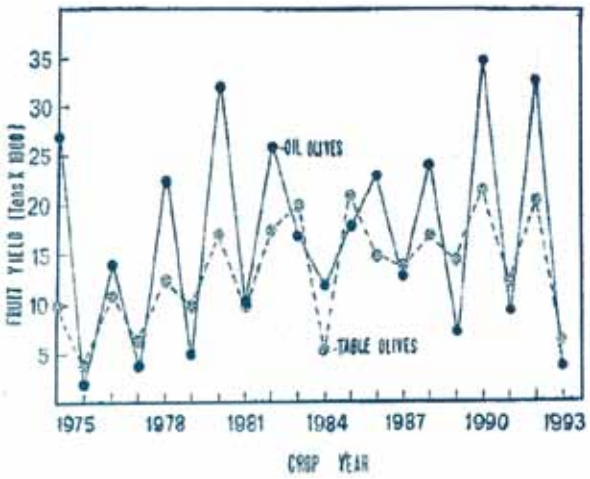


Fig. 1 - Alternate bearing over a period of six years in rain-fed and irrigated olive orchards (Lavee, 2006).

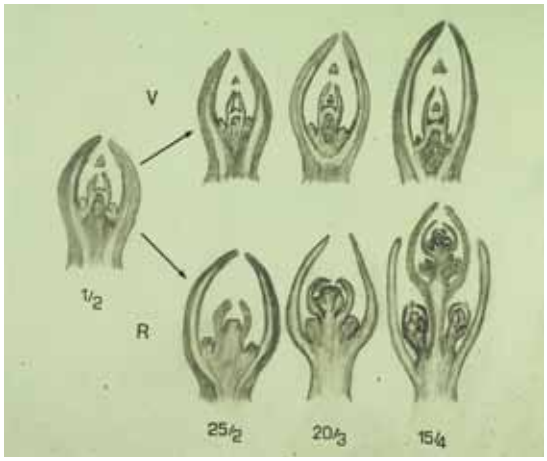


Fig. 2 - Differentiation of an undefined olive bud to a vegetative and reproductive state (Lavee, 1996).

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*et al*., 2012). On the other hand, it was suggested that low temperature is required mainly for predetermined reproductive bud opening, similar to the dormancy process in deciduous trees (Rallo and Martin, 1991; Rallo *et al*., 1994). Some bridging ideas related to that gap of the two approaches have recently been considered, though the need for a chilling period, or comparable conditions causing temporarily seasonal growth retardation for reproductive bud differentiation during the winter, has been well established.

Various growth and metabolic changes between fruiting ('on') trees and low or non fruiting ('off') olive trees have been described, as reviewed some years ago for "Olea" (Lavee, 2006) and fruit crops in general (Goldschmidt, 2005). The overall affect of olive alternate bearing is expressed by antagonism between the developing fruit and vegetative growth (Fig. 3). As olive fruit is initiated and develops from buds on shoots grown during the previous year, a reduction of vegetative shoot elongation and inhibition of lateral shoot out growth due to fruit development has a major effect on the general number of buds and thus on the potential reproductive buds in particular. As olive is a sectorial tree, the antagonism between developing fruit and vegetative growth might appear on a single scaffold, a tree section or a whole tree depending on the amount and distribution of the fruit in the 'on' year. It should be noted that the greater the amount of fruit in the 'on' year, the greater the chilling required for a return crop in the following year. Still, once the yield in the 'on' year exceeded a level specific for the growing conditions and the cultivar additional chilling will not be effective any more (Fig. 4A). Furthermore, if inflorescences are formed, the amount of male flowers (Fig. 5) usually increases and the fruit set potential of complete flowers is markedly reduced (Cuevas *et al*., 1994). A similar effect is induced by harvest time during current yield. Harvesting the current fruit late in the season will significantly reduce the level of return flowering in the following year with the degree of chilling having only a minor effect (Fig. 4B). All these phenomena are accompanied, and probably controlled by basic metabolic changes within the different organs of the tree. While in leaves during the 'off' year the level of proteins was considerable lower than in leaves during the 'on' year, the opposite trend was found in the bark of young shoots in which the protein level was significantly higher than in the 'on' year (Table 1). Recently, the molecular origin of some of those proteins, such as Ft which is involved in flower induction in Arabidopsis, were identified and their possible function in the process leading to flowering of olive was determined (Haberman, 2012; Samach and Smith, 2013).

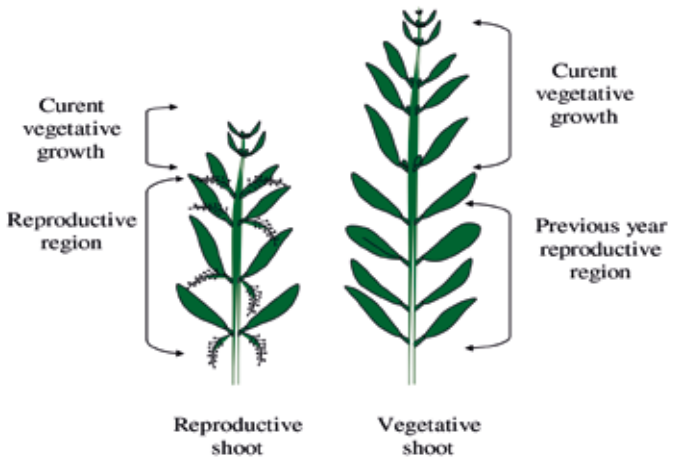


Fig. 3 - Comparison of the annual vegetative shoot growth of an 'on' year (left) and 'off' year (right) branch.

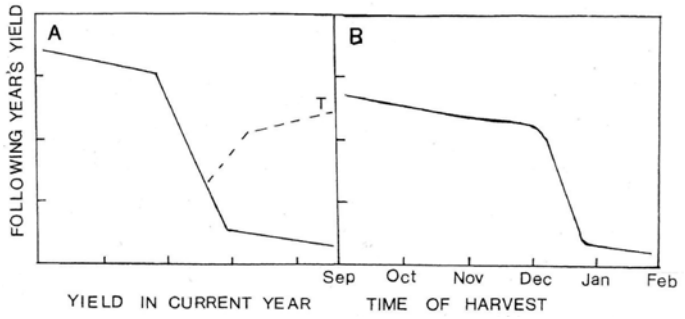


Fig. 4 - The effect of olive fruit yield level (A) and harvest time (B) in a current year on their fruiting potential in the following year (Lavee, 1989).

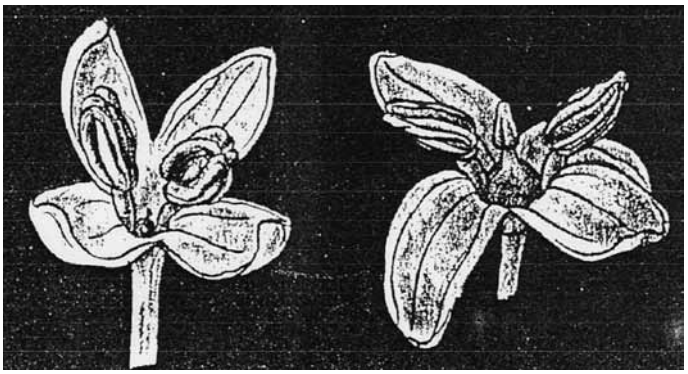

Fig. 5 - Open olive flower types. On the left a male flower, on the right a complete normal (open perfect) androgynous flower (Goor *et al*., 1960).

Table 1 - The protein content (mg/fw) of mature olive leaves of 4 cultivars in "on" and "off" years. Sampled in late summer

| Koroneiki | Uovo di Piccione | Barnea | Manzanillo | Tree phase |
|---|---|---|---|---|
| | | Leaves | | |
| 475 bc | 370 a | 310 a | 295 a | "off" |
| 530 b | 510 b | 405 c | 510 b | "on" |
| | | Bark | | |
| 490 b | 490 b | 500 b | 440 b | "off" |
| 370 a | 360 a | 370 a | 360 a | "on" |

From Lavee and Avidan, 1994.

Another major metabolic change in the leaves of olive trees in their 'on' and 'off' cycle was identified as a dynamic change of some phenolic compounds, particularly chlorogenic acid (CHA). This acid, which olive tissue responds to as a growth promoting auxin (Fig. 6), increases in the leaves during fruit-set and remains high throughout the 'on' year, inhibiting the differentiation of flower buds for the following year. The resulting low fruit set causes the level of CHA to drop again and remains low during the whole 'off' year (Fig. 7). The negating effect of CHA on reproductive bud differentiation was directly demonstrated by injecting CHA into scaffolds of cv. Manzanillo trees during the winter (Fig. 8) which resulted in a reduction of flower bud development for the following spring (Lavee *et al*., 1986). The amount of CHA developing in the leaves during the 'on' year fruit set is proportional to the amount of fruit developing on the trees (Fig. 9). Other biotic changes in the metabolism of olive trees leading to alternate bearing are the level or depletion of minerals, activity of endogenous and exogenous gibberellins, and the level of carbohydrates which, although controversial, were also reported to be involved in controlling alternate bearing.

Various schemes of biotic metabolic changes which lead to or inhibit floral induction were suggested and that indicate the sequences of events controlling vegetative or

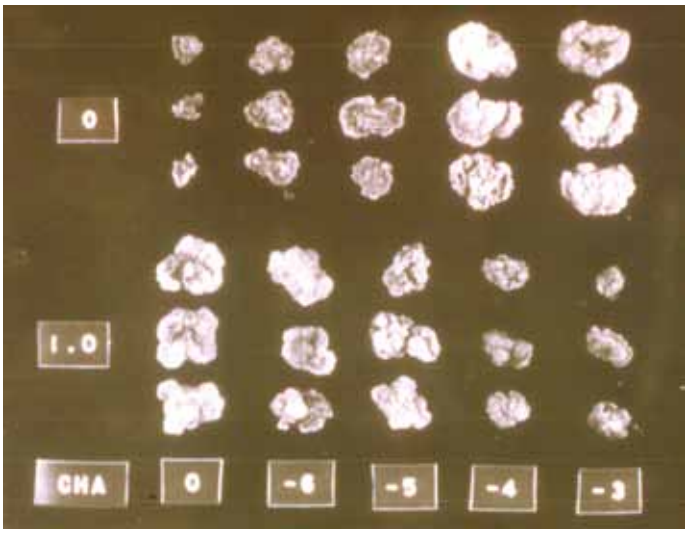


Fig. 6 - The effect of auxin (NAA) and chlorogenic acid (CHA) on the growth of olive callus tissue *in vitro* (Lavee, 1996).

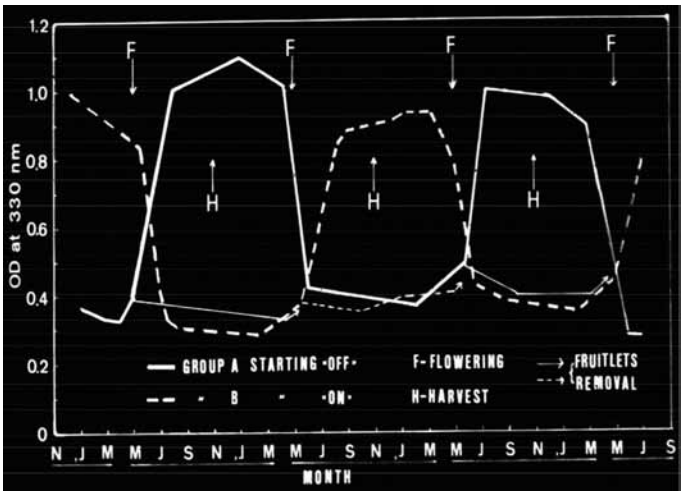


Fig. 7 - The change in content of CHA during the year in the leaves of 'on' and 'off' olive scaffolds and after inflorescences removal of cv. Manzanillo olive trees (Lavee and Avidan, 1994).

Fig. 8 - Pressurized winter injection of CHA in a scaffold of cv. Manzanillo causing a reduction in reproductive differentiation of buds.

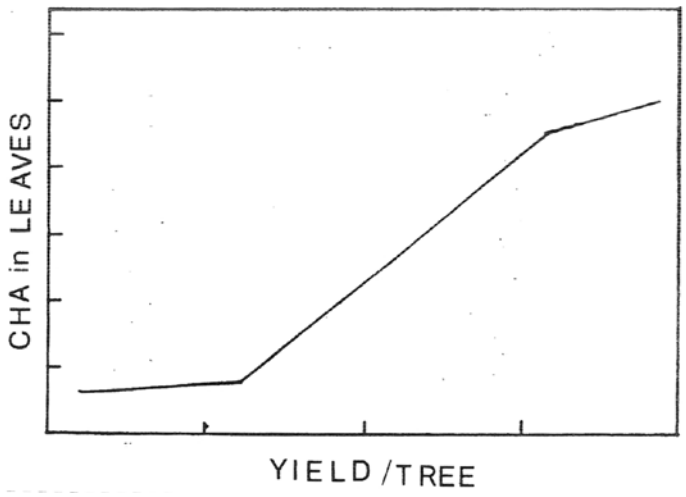


Fig. 9 - The relationship between the CHA level in leaves and fruit yield per tree of cv. Manzanillo (Lavee, 1989).

reproductive development during the tree growth cycle (Fig. 10). The thermal effect on the biotic processes at the different developmental stages was schematically presented (Fig. 11). Extreme abiotic conditions, particularly temperature, might change the developmental pattern of the reproductive bud, leading to abnormal organs. Juvenal seedlings one to two years old, when submitted to relatively extreme low temperatures, will induce metabolic changes that lead to semi differentiated abnormal reproductive buds (Fig. 12). A one- to two-day period of high temperature in mid winter, after the induction of the biotic metabolism leading to flower bud differentiation but prior to initial morphological changes of the buds, will lead to vegetative opening of all the buds along the shoots similar to that of the reproductive fully differentiated ones (Fig. 13). Thus, the abiotic pulse stopped and changed part of the normal biotic pathway of bud development. Long winter periods with insufficient chilling temperature to induce the biotic processes leading to bud differentiation will result in a lack of inflorescence development and therefore cause a fully synchronized alternant bearing, as is common in relatively warmer climates with variable winter temperatures (Fig. 14). However, in extreme high winter temperatures, rather uniform day length and no other abiotic factors to induce a winter period with growth cessation the abiotic environment will induce biotic conditions changing the entire reproductive development of the olive by developing single terminal flowers instead of the normal lateral inflorescences (Fig. 15). Such flowers are usu-

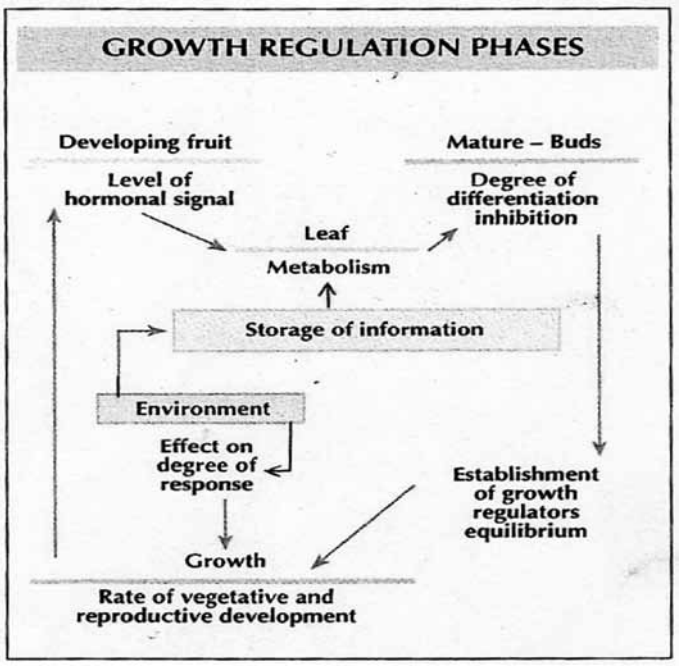


Fig. 10 - A short schematic presentation of the vegetative-reproductive growth cycle of olive (Lavee, 2007).

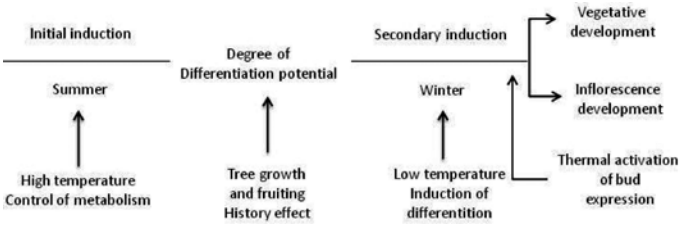


Fig. 11 - Description of temperature involvement in significant metabolic stages during olive vegetative and reproductive development (Lavee, 2006).

Fig. 12 - Abnormal semi-reproductive development of juvenile buds due to unusual strong abiotic thermal induction.

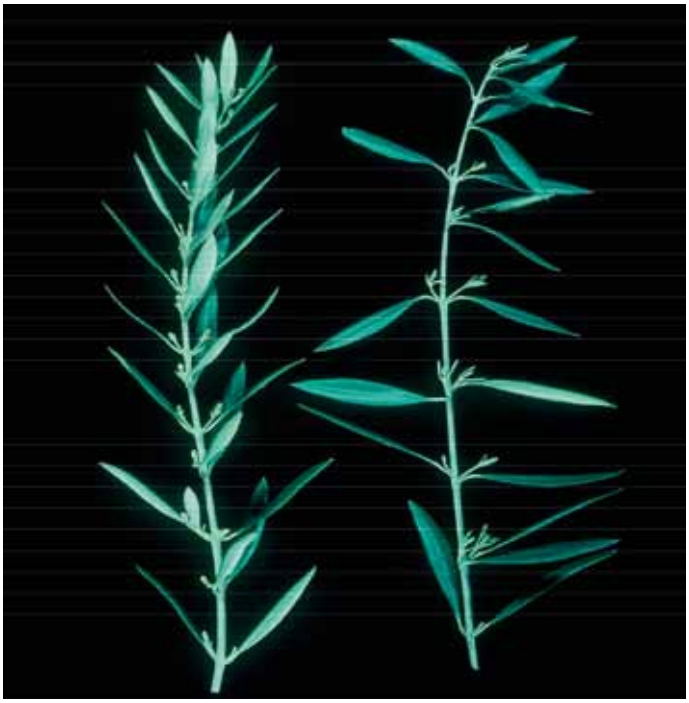

Fig. 13 - Illustration of a fully reproductive olive shoot (left) and a reproductive shoot reversed to vegetative development of all buds due to a period of high temperature during an early stage of winter reproductive differentiation (right) (Lavee, 1996).

ally malformed and those which set fruit, found to present, were all parthenocarpic.

Still, alternate bearing develops in olive also under the most suitable and annually repetitive climates. The alternate bearing under such climatic conditions is less spectacular as it is based on non synchronized alteration of each individual tree. This slowly developing alteration in fruiting is also not due to a genetic property. Various slight abiotic stimuli cause the initiation of the biotic processes leading in receptive buds to differentiation and small changes in fruit load which, accordingly, gradually amplify (Fig. 16). Various abiotic environmental events such as rain during the flowering period wash off the pollen and receptive compounds from the stigma, hot dry winds reduce the respectability of the stigma by drying it, lack of suitable pollen for the required cross pollination, as well as insufficient illumination might lead to the development of shot berries (Fig. 17), which are also, in part, instrumental in inducing alternate bearing in olive (Stutte and Martin, 1986 a, b). However these factors leading to alternate bearing cannot be considered genetic control of alternate bearing as they are all governed by abiotic environmental conditions inducing the onset of specific biotic activities. Furthermore, suitable exposure of the trees to light and radiation might create a period of retarded growth compensating for insufficient chilling starting the biotic endogenous processes which lead to fruit development as occurring in some semi tropical regions.

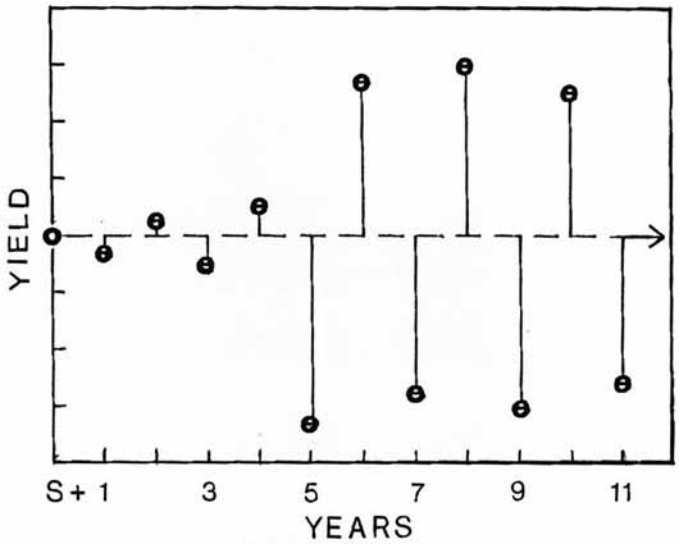


Fig. 14 - Sequence of synchronized alternate bearing development due to extreme high or low winter thermal conditions occurring particularly in regions with varying winter temperatures (Lavee, 1989).

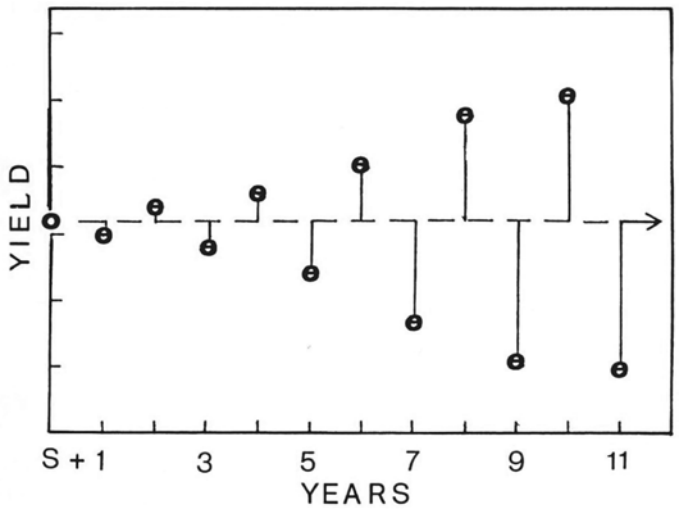


Fig. 16 - Scheme of alternate bearing development in individual trees under relatively stable low annual winter thermal conditions (Lavee, 1989).

Fig. 15 - Abnormal terminal bud differentiation developing a single flower due to high temperatures under semitropical environments.

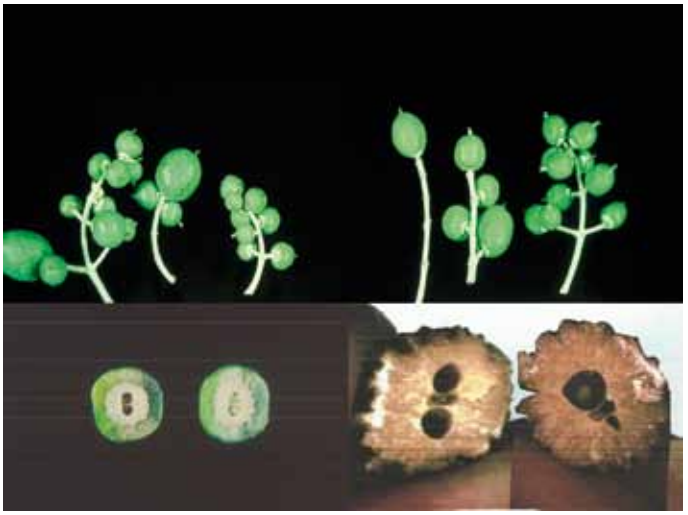

Fig. 17 - Parthenocarpic fruit (shot berries) development with aborted embryos due to unfavorable reproductive differentiation or mal conditions effecting fruit set in the spring.

To eliminate alternate bearing in olive, or at least reduce it, various horticultural techniques are applied such as fruit thinning, girdling, control of harvest time and, to a lesser extent, pruning. These interventions are selectively used for table olives, but in part also in olives for oil extraction. Controlled irrigation and mineral nutrition are helpful tools as well to reduce the biannual bearing, although it does not eliminate it.

Significant metabolic changes were found in various tree parts between 'on' and 'off' years during the development of the annual olive life cycle. These biotic changes, and their degree, are strongly affected by the environmental abiotic conditions. This close interaction between the endogenous metabolic processes and the environmental conditions at various olive growing sites led to the assumption that alternate bearing of olive is a basic genetic characteristic of this commodity which exists in close interaction with the surrounding abiotic conditions. There is no doubt that the biotic endogenous processes leading to alternate bearing are strongly affected, and in many cases even controlled, by local abiotic environmental conditions of olive growing regions. Thus, all the schemes describing alternate bearing clearly indicate the biotic-abiotic interaction (Fig. 18). This can be rather misleading as it is based on the nature and degree of the processes involved during the 'on' or 'off' phase of an already induced developmental cycle. However this approach deals with the level of alternate bearing and does not take into account its initial induction. Based on the various studies dealing with alternate bearing of olive, it should be concluded that the initiation of it is solely abiotic. Without an external, environmental and thus abiotic stimulus, alternate bearing of the olive tree is not initiated. Analysis of the currently available data clearly indicates that without an abiotic induction alternate fruiting of the olive tree would not occur.

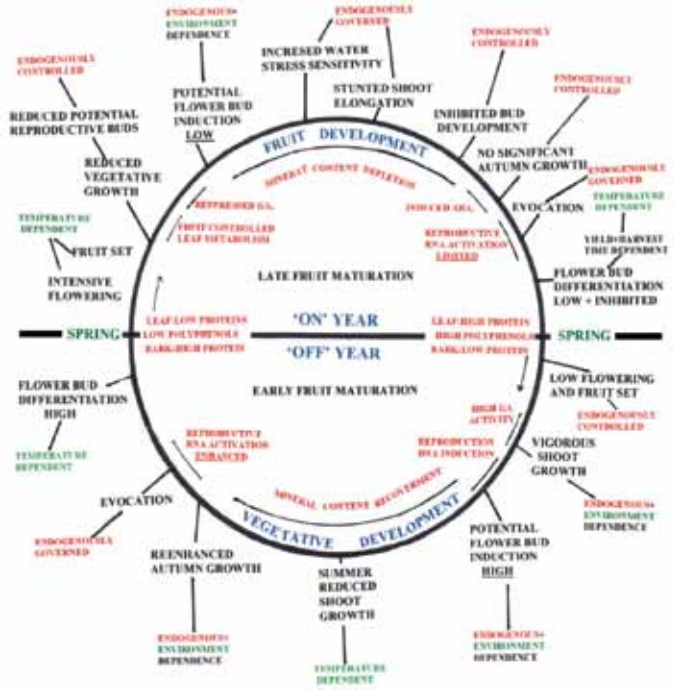


Fig. 18 - A general scheme showing initiation of the abiotic conditions on fruiting induction and subsequent biotic-abiotic interactions on the level of fruit differentiation and development in sequential 'on' and 'off' years (Lavee, 2007).

## Conclusions

Alternate bearing in olive could result from an array of metabolic changes involving tree growth, fruit load, flower and pollen viability, etc., however these biotic changes were shown to occur only as a result of abiotic inductions. Thus, biannual bearing, at least in the case of olive, should not be considered a genetic trait developing as a stage of the growth cycle of the tree. On the other hand, the nature and degree of reproductive alternation and the metabolic changes involved are clearly based on an abiotic-biotic interaction.

## References

CUEVAS J., RALLO L., RAPOPORT H.F., 1994 - *Crop load effects on floral quality in olive*. - Scientia Hortic., 59: 123-130.

FABBRI A., ALERCI L., 1999 - *Anatomical aspects of flower and leaf bud differentiation in olive* Olea europaea *L.* - Acta Horticulturae, 474: 245-249.

GOLDSCHMIDT E.E., 2005 - *Regulatory aspects of alternate bearing in fruit trees*. - Italus Hortus, 12: 11-17.

GOOR A., SPIEGEL P., GRATCH H., 1960 - *The olive*. - State of Israel Ministry of Agriculture Ministry of Education, Tel Aviv, Israel, p. 163

HABERMAN A., 2012 - *Study of the endogenic system for flowering control in olive (*Olea europaea*) and characterization of the effect of fruit load on the transition to flowering*. - MSc Thesis sub. to the Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel.

HARTMANN H.T., PROLINGIS I., 1957 - *Effect of different amount of winter-chilling on fruitfulness of several olive varieties*. - Bot. Gaz., 119: 102-104.

HARTMANN H.T., WHISLER J.E., 1975 - *Flower production in olive as influenced by various temperature regimes*. - J. Amer. Soc. Hort. Sci., 100: 670-674.

LAVEE S., 1989 - *Involvement of growth regulators and endogenous substances in the control of alternate bearing*. - Acta Horticulturae, 239: 311-322.

LAVEE S., 1996 - *Biology and physiology of the olive,* pp. 59-106. - In: CONSEJO OLEICOLO INTERNATIONALE (COI) *World Olive Encyclopaedia*. Plazay Jane S.A. Barcelona, Spain.

LAVEE S., 2006 - *Biennial bearing in olive (*Olea europaea *L).* - Olea, 25: 5-13.

LAVEE S., 2007 - *Biennial bearing in olive (*Olea europaea*).* - Annales: series Historia Naturalis, 17: 101-112.

LAVEE S., AVIDAN N., 1994 - *Protein content and composition of leaves and shoot bark in relation to alternate bearing of olive trees (*Olea europaea *L.).* - Acta Horticulturae, 356: 143-147.

LAVEE S., HARSHEMESH H., AVIDAN N., 1986 - *Phenolic acid - possible involvement in regulating growth and alternate fruiting in olive trees*. - Acta Horticulturae, 179: 317-328.

RALLO L., MARTIN G.C., 1991 - *The role of chilling in releasing olive floral buds from dormancy.* - J. Am. Soc. Hort. Sci., 116(6): 1058-1062.

RALLO L., TORRENO P., VARGAS A., ALVARADO J., 1994 - *Dormancy and alternate bearing in olive*. - Acta Horticulturae, 356: 209-210.

SAMACH A., SMITH H.M., 2013 - *Constraints to obtaining consistent annual yields in perennials. II. Environment and fruit load affect induction of flowering*. - Plant Sci., 207: 168-176.

STUTTE G., MARTIN G.C., 1986 a - *Effect of light intensity and carbohydrate reserves on flowering in olive*. - J. Am. Soc. Hort. Sci., 111: 27-31.

STUTTE G., MARTIN G.C., 1986 b - *Effect of killing seeds on return bloom of olives*. - Scientia Hortic., 29: 107-113.

TRONCOSO A., GARCIA J-l., LAVEE S., 2012 - *Evaluation of the present information on the mechanisms leading to flower bud induction and differentiation in* Olea europaea. - Acta Horticulturae, 949: 93-98.

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AUTHOR INDEX

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