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*Full Length Research Paper*

# Influence of rooting media and number of nodes per stem cutting on nursery performance of vanilla (*Vanilla planifolia* Andr. *syn. Vanilla fragrans*)

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**The effect of rooting media and number of nodes per cutting on nursery performance of vanilla fragrance were evaluated in South-western Ethiopia in 2011/ 2012. Six rooting media (forest soil, decomposed animal manure, fine sand, 1:1 mixture of forest soil: fine sand, 1:1 mixture of decomposed animal manure: fine sand and 1:1:1 mixture of forest soil: decomposed animal manure: fine sand) and four levels of node number (two, three, four, and five node cutting) were used in this experiment. Treatments were arranged in randomized complete block design with three replications. The result revealed that interaction between the two factors was significant (P≤0.05) for all parameters studied except sprouting percentage and root number. The highest shoot length, shoot girth, shoot fresh weight, shoot dry weigh, leaf number, leaf area, leaf fresh weight, leaf dry weight and root to shoot ratio were obtained from four node cuttings grown on a 1:1:1 mixture of forest soil: decomposed animal manure: fine sand rooting media, with the exception of the highest root length and rooting percentage of cuttings obtained from the rooting media containing pure sand. In addition highest root fresh weight, root volume and root dry weight were obtained from five nodal cuttings. Two nodal cutting grown on decomposed animal manure and pure fine sand media showed lower root initiation and shoot growth.**

**Key words:** Vanilla fragrance, propagation, rooting media, node number.

## INTRODUCTION

Vanilla (*Vanilla planifolia* Andr.) belongs to the family Orchidaceae, of which some 110 species have been reported so far (Talubnak and Soytong, 2010). Etymologically, vanilla is derived from the Spanish word "vainilla" meaning *little pod* (James and Ackerman, 2003). Vanilla grows best under hot humid climate from sea level to an elevation of 1500 m. Most of its production is carried out between 10°- 20° above and below the equator. The ideal growing conditions include moderate rainfall of 1500 to 3000 mm evenly distributed through 10 months of the year; temperatures of 15 to 30°C during the day and 15 to 20°C during the night and relative

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humidity of around 80% (Bianchessi, 2004).

Ethiopia has favorable environment for vanilla production and the crop has a huge local and international market (Zerihun et al., 2009). Currently, three accessions of vanilla introduced at different times are under adaptation trial in South-western Ethiopia. The first accession (Van.1/93) was introduced from Bali Island, Indonesia in 2001; the second accession (Van.3/04) was introduced from Uganda in 2004 and the third accession (Van.2/05) was introduced from Mauritius in 2005 (Girma et al., 2008). Currently, all three accessions are very well adapted at Teppi and their seedlings are being multiplied at Teppi National Spices Research Center (TNSRC) for further study.

Predominantly, vanilla is propagated through the vegetative means mainly from stem cuttings collected from a healthy and vigorously growing mother plant (Carlos and Balakrishnan, 1991; KAU, 2002; Purseglove, 1973; Sengupta, 2003). Different experiences are available from various locations about the number of nodes per stem cutting and ideal rooting media for propagation of vanilla. The type of rooting medium used can have a major influence on the rooting capacity of cuttings (Hartman et al., 1990). Vanilla stem cuttings are adversely affected by the availability of water and nutrients in the medium used during propagation. The length of cuttings used for vegetative propagation of vanilla is commonly determined by the availability of mother plants (Palama et al., 2010). Preliminary study in TNSRC indicated that vanilla cuttings with a minimum of a single node, cuttings with two, three and more numbers or more can be used for propagation (Girma et al., 2011). There was also an increasing trend in mean values of most planting materials growth parameters such as numbers of leaves, length of vine and root dry weight as the number of nodes per original stem cutting was increased (Girma, 2012). In addition, the use of short cuttings (30 cm long with 3 to 4 nodes) is becoming widespread due to shortage and unavailability of the preferred planting materials (Namirembe-Ssonkko et al., 2005). Therefore, the objective of this research is to determine influence of different rooting media and number of nodes per stem cutting on nursery performance of vanilla.

## MATERIALS AND METHODS

### Description of the study area

The experiment was conducted in South-western Ethiopia, Teppi National Spices Research Center (TNSRC) at nursery condition in 2011/2012. The site is located within latitude 7°3' N and longitude 35°0' E in an altitude of 1200 m a.s.l (metres above sea level) and receives a mean annual rainfall of 1688 mm. The mean maximum and minimum temperature of the area is 29.5 and 15.3°C, respectively. The soil type of the study area is dystric nitisols, eutric vertisols and vertic gleysols dominated by forest soil (Abayneh and Ashenafi, 2005). Minimum and maximum temperature of the experimental periods is presented in Table 1. In general, the area is characterized by high rainfall, temperature and humidity and representative hot humid low lands (EIAR, 2010).

### Experimental materials

This experiment comprises two factors (rooting media composition and vanilla cuttings length). Semi hard wood cuttings of introduced vanilla accession (Van1. /93) with different node number (2, 3, 4 and 5) and six types of rooting media (fine sand, forest soil, decomposed animal manure, 1:1 mixture of forest soil and fine sand, fine sand: decomposed animal manure and 1:1:1 mixture of forest soil, decomposed animal manure and fine sand) were used for this experiment.

### Preparation of rooting media and cuttings

The materials used for the preparation of the potting mixture were fine sand (2.5 mm), forest soil and decomposed animal manure. Forest soil was collected from the upper 15 cm layer of the soil under a forest canopy at the TNSRC. Decomposed animal manure was also collected from the Centre and fine sand was purchased from the local market. All rooting media were sieved through a 2.5 mm mesh and fine sand thoroughly washed to remove soil particles and other undesirable materials (Plate 2). The pH of each potting mix was determined using JENWAY digital electrode pH meter on a 1:2.5 basis of soil and water (Table 2), respectively at the soil laboratory of the Jimma University College of Agriculture and Veterinary Medicine.

Semi hard wood slant cuttings of vanilla vine with 2, 3, 4 and 5 nodes per stem cutting were taken from healthy mother plants using a pruning shear disinfected with alcohol. All leaves were removed from the vine before planting (Plate 1) and the cuttings were planted in the respective growth media followed by watering. After planting, the necessary nursery management practices such as watering and weeding were applied as per the recommendation of Girma et al. (2011) (Plate 3).

### Experimental design and treatments

The study was conducted as a factorial experiment (6 media types × 4 different node numbers) in a randomized complete block design (RCBD) with three replications. Each treatment consisted of ten cuttings and a total of 720 cuttings were used in the study.

### Data collection and analyses

The parameters evaluated in this experiment were shoot length (cm), shoot girth (mm), shoot fresh and dry mass (g), leaf number, total leaf area ($cm^2 \cdot plant^{-1}$), leaf fresh and dry mass (g), root number, root fresh and dry mass (g), root volume (ml), root length (cm) per rooted cutting, root to shoot ratio in terms of dry mass (g) as well as percentages of rooting and sprouting (out of ten cuttings within a plot). In all cases, roots were gently washed with tap water before measuring root growth parameters. Total leaf area was measured using leaf area meter (ADC Bio scientific Ltd Area Meter AM 200, England) in $cm^2$. Root volume was determined by the displacement method using a graduated cylinder half filled with water. For dry mass determination, both roots and shoots were oven dried at 80°C until the samples attained a constant mass (72 h). Except for percentage of rooted and sprouted cuttings the results are presented for discussion per plant basis.

The collected data on different growth parameters were analysed by Analysis of Variance (ANOVA). The ANOVA was done by using SAS version 9.2 Computer software (SAS Institute Inc., 2008).

**Table 1.** The weekly average minimum and maximum temperature of the nursery.

| | Temperature | |
|---|---|---|
| **Week** | **Min** | **Max** |
| 1 | 14.40 | 24.58 |
| 2 | 12.32 | 23.48 |
| 3 | 12.60 | 23.50 |
| 4 | 14.20 | 23.48 |
| 5 | 14.80 | 24.04 |
| 6 | 15.94 | 25.32 |
| 7 | 13.41 | 24.02 |
| 8 | 13.40 | 22.96 |
| 9 | 12.50 | 22.30 |
| 10 | 12.03 | 23. 30 |
| 11 | 13.00 | 25.50 |
| 12 | 12.70 | 24.90 |
| 13 | 14.50 | 25.30 |
| 14 | 15.89 | 24.10 |
| 15 | 11.60 | 22.85 |
| 16 | 13.20 | 24.90 |
| 17 | 12.32 | 23.60 |
| 18 | 11.40 | 23.48 |
| 19 | 15.20 | 24.80 |
| Mean | 13.44 | 24.06 |

**Table 2.** pH analysis of the media used in potting mix

| Growing media | pH |
|---|---|
| S | 6.48 |
| F | 5.09 |
| M | 7.88 |
| F:S | 6.48 |
| M:S | 7.67 |
| F:M:S | 7.57 |

F -Forest soil, FMS- Forest soil: decomposed animal manure: fine sand, FS- Forest soil: Fine sand, M- Decomposed animal manure:, MS -Decomposed animal manure: fine sand , S- Fine sand.

When ANOVA showed significant differences, mean separation was carried out using Least Significant Difference (LSD) test at 5% level of significance (Gomez and Gomez, 1984). All the figures and tables were generated by Excel computer program.

## RESULTS AND DISCUSSION

All planting materials root and shoot growth parameters were significantly influenced by the type of rooting media and number of node per stem cuttings used ($p \leq 0.05$). However, root numbers were not significant. The highest root length (17.5 cm) (Plate 7) and percentage of rooting (99.3%) were obtained from four nodal cuttings which are grown in fine sand rooting media followed by five nodal cuttings (16.99 cm and 93.33% respectively) which were grown in 1:1:1 mixture of forest soil, decomposed animal manure and fine sand media. This could be due to the fact that low bulk density of fine sand medium invariably allows for greater root penetration leading to formation of longer roots. In accordance with this, Fagge et al. (2011) reported that influence of the medium is felt even before rooting occurs due to water retention and aeration properties and that the percentage and quality of roots in terms of number, length and weight can in many ways be directly linked to the rooting medium itself. Higher rooting percentage observed from longer cuttings could be linked to better initial carbohydrate reserves stored of longer cuttings (Ky-Dembele et al., 2011). In addition fine sand medium provides adequate aeration and drainage leading to increased porosity to promote better root initiation. This is supported by the work of Hans and Gisler (1983) where increasing air content of the rooting media improved rooting in Poinsettia and Hydrangea. While the lowest root length (6.05 cm) (Plate 8) and percentage of rooting (44.33%) were obtained from two nodal cuttings which were grown in decomposed animal manure (Table 3).

Maximum root fresh weight (1.16 g), root dry weight (0.13) and root volume (1.72 mm) was observed from five node cuttings grown on 1:1:1 mixture of forest soil: decomposed animal manure: fine sand. This may reveal that longer cuttings denote longer roots. Longer roots can have an ability of deeper penetration and therefore greater capacity in water and nutrients absorption leading to heavier root fresh weights in this media (1:1:1 mixture of forest soil: decomposed animal manure: fine sand) as compared to the decomposed animal manure medium. The result of the present investigation is in agreement with the work of Girma et al. (2012). Whereas the lowest root fresh weight (0.34 g), root dry weight (0.03) and root volume (0.78 mm) was observed from two nodal cutting grown on decomposed animal manure (Table 3). Similar trends in root fresh and dry weight were reported by Umesha et al. (2011).

The result of this experiment on the percentage of cuttings that develop new shoot (sprouting percentage) showed that there was no significant difference between interaction of factors (rooting media and node number). However, there was significant ($p<0.05$) difference between node number treatments. The early sprouting and highest sprouting percentage of vanilla cuttings was recorded from four nodal cuttings (81.94%) which are statistically at par with five nodal cuttings (78.89%), whereas the lowest sprouting percentage of vanilla was recorded from cutting with two and three nodes (68.06 and 72.5%, respectively) (Figure 1). The current result supported by Umesha et al. (2011) who observed early sprouting and better shoots growth and development from longer vanilla cuttings than single and three nodal cuttings.

**Table 3.** Interaction effect of different rooting media and number of nodes per cutting on the percentage of rooting (PR), root length (RL), root fresh weight (RFW), root dry weight (RDW) and root volume (RV) of *Vanilla fragrans.*

| Number of nodes per stem cutting | Rooting media | Parameter | | | | |
|---|---|---|---|---|---|---|
| | | PR(%) | RL(cm) | RFW(g) | RDW(g) | RV(g) |
| 2 | F | 46.67$^{jk}$ | 10.64$^{ijk}$ | 0.42$^{ghi}$ | 0.057$^{ghijk}$ | 0.94$^{e}$ |
| | FMS | 53.33$^{hijk}$ | 8.67$^{lm}$ | 0.55$^{fg}$ | 0.08$^{cdef}$ | 0.83$^{fg}$ |
| | FS | 50.00$^{ijk}$ | 7.63$^{mn}$ | 0.49$^{fgh}$ | 0.047$^{jkl}$ | 0.83$^{fg}$ |
| | M | 43.33$^{k}$ | 6.05$^{n}$ | 0.34$^{i}$ | 0.03$^{l}$ | 0.78$^{g}$ |
| | MS | 46.67$^{jk}$ | 12.82$^{fgh}$ | 0.54$^{fgh}$ | 0.05$^{hijkl}$ | 1.05$^{cdefg}$ |
| | S | 83.33$^{bcd}$ | 11.98$^{hij}$ | 0.78$^{de}$ | 0.04$^{kl}$ | 1.11$^{cdef}$ |
| 3 | F | 56.67$^{ghij}$ | 11.86$^{hij}$ | 0.42$^{ghi}$ | 0.06f$^{ghijk}$ | 0.96$^{defg}$ |
| | FMS | 66.67$^{efg}$ | 11.47$^{hij}$ | 0.78$^{de}$ | 0.08$^{cdef}$ | 1.31$^{bc}$ |
| | FS | 46.67$^{jk}$ | 12.90$^{efgh}$ | 0.76$^{de}$ | 0.07$^{efghij}$ | 0.87$^{fg}$ |
| | M | 56.67$^{ghij}$ | 11.31$^{hij}$ | 0.61$^{ef}$ | 0.05$^{ijkl}$ | 0.80$^{g}$ |
| | MS | 60.00$^{ghi}$ | 9.34$^{klm}$ | 0.35$^{hi}$ | 0.05$^{hijkl}$ | 1.07$^{cdefg}$ |
| | S | 76.67$^{de}$ | 9.02$^{klm}$ | 1.00$^{abc}$ | 0.08$^{cdef}$ | 1.11$^{cdef}$ |
| 4 | F | 80.00$^{cd}$ | 14.81$^{cde}$ | 1.08$^{ab}$ | 0.09$^{bcde}$ | 1.46$^{ab}$ |
| | FMS | 93.33$^{ab}$ | 10.13$^{jkl}$ | 1.08$^{ab}$ | 0.09$^{cde}$ | 1.48$^{ab}$ |
| | FS | 63.33$^{fgh}$ | 14.81$^{cde}$ | 0.95$^{bcd}$ | 0.08$^{defg}$ | 1.36$^{bc}$ |
| | M | 56.67$^{ghij}$ | 12.24$^{hi}$ | 0.87$^{cd}$ | 0.06$^{fghijk}$ | 1.30$^{bc}$ |
| | MS | 73.33$^{def}$ | 14.51$^{def}$ | 0.78$^{de}$ | 0.08$^{cdef}$ | 1.27$^{bcd}$ |
| | S | 99.30$^{a}$ | 17.50$^{a}$ | 0.62$^{ef}$ | 0.12$^{ab}$ | 0.94$^{efg}$ |
| 5 | F | 66.67$^{efg}$ | 14.22$^{defg}$ | 0.90$^{bcd}$ | 0.06$^{ghijk}$ | 1.06$^{cdefg}$ |
| | FMS | 93.33$^{ab}$ | 16.99$^{ab}$ | 1.16$^{a}$ | 0.13$^{a}$ | 1.72$^{a}$ |
| | FS | 66.67$^{efg}$ | 15.15$^{bcd}$ | 1.08$^{ab}$ | 0.10$^{bcd}$ | 1.33$^{bc}$ |
| | M | 76.67$^{de}$ | 13.11$^{efg}$ | 0.90$^{bcd}$ | 0.11$^{abc}$ | 1.24$^{bcde}$ |
| | MS | 73.33$^{def}$ | 12.33$^{ghi}$ | 0.68$^{ef}$ | 0.07$^{efghi}$ | 1.43$^{ab}$ |
| | S | 90.00$^{abc}$ | 16.58$^{abc}$ | 0.91$^{bcd}$ | 0.08$^{defgh}$ | 1.46$^{ab}$ |
| LSD (5%) | | 13.08 | 1.93 | 0.20 | 0.31 | 0.026 |
| CV (%) | | 11.82 | 9.53 | 15.83 | 15.88 | 21.41 |

Means followed by the same letter(s) are not significantly different at p=0.05, F = forest soil , FMS = forest soil : decomposed animal manure : fin sand , FS = forest soil : fine sand , M = decomposed animal manure , MS = decomposed animal manure : fine sand , S = fine sand.

The highest leaf number (9.2), leaf fresh weight (7.16 g), leaf dry weight (2.127 g), shoot length (57.60 cm) and shoot fresh weight (33.38 g) was observed from four nodal cutting grown on 1:1:1 mixture of forest soil: decomposed animal manure: fine sand rooting media. Consequently, the highest mean values from four nodal cutting were supportive to a report by Girma et al. (2012). Whereas the lowest leaf number (5.40) was observed from two nodal cuttings which are grown on sole fine sand but statistically on par with three nodal cuttings. In addition the lowest leaf fresh weight (6.34 g), leaf dry weight (2.127 g), shoot length (25.04 cm) and shoot fresh weight (14.53 g) was observed from two nodal cutting grown on sole fine sand (Table 4). Maximum shoot girth (0.43 mm) and shoot dry weight (4.16 g) was recorded from four and five node cutting grown on 1:1:1 mixture of forest soil: decomposed animal manure: fine sand; whereas the lowest shoot girth (0.307 mm) observed from two nodal cutting grown on sole fine sand medium and the lowest shoot dry weight (2.56 g) observed from two nodal cutting grown on sole fine sand medium but statistically on par with three node cutting grown on fine sand medium (Table 4). Generally, there was an increasing trend in shoot growth and development as the node number per cutting increased.

Maximum leaf area (78.85 and 78.51 $cm^2$) was observed from four and five node cuttings grown on 1:1:1 mixture of forest soil: decomposed animal manure: fine

**Table 4.** Interaction effect of different rooting media and number of nodes per cutting on the leaf number (LN), leaf fresh weight (LFW), leaf dry weight (LDW), shoot length (SL) and shoot fresh weight (SFW) of *V. fragrans.*

| Number of nodes per stem cutting | Rooting media | Parameter | | | | |
|---|---|---|---|---|---|---|
| | | LN | LFW(g) | LDW(g) | SL(cm) | SFW(g) |
| 2 | F | 6.27$^{fgh}$ | 6.42$^{ghi}$ | 2.057$^{ghijk}$ | 28.58$^{jkl}$ | 19.98$^{ijkl}$ |
| | FMS | 7.48$^{bcdef}$ | 6.55$^{fg}$ | 2.08$^{cdef}$ | 41.49$^{efg}$ | 19.74$^{jkl}$ |
| | FS | 7.6$^{abcdef}$ | 6.78$^{de}$ | 2.04$^{kl}$ | 26.40$^{k}$l | 16.28$^{mn}$ |
| | M | 6.68$^{dcfgh}$ | 6.49$^{fghi}$ | 2.053$^{hijkl}$ | 35.92$^{ghij}$ | 18.52$^{lm}$ |
| | MS | 5.63$^{gh}$ | 6.54$^{fgh}$ | 2.053$^{hijkl}$ | 30.79$^{ijkl}$ | 19.69$^{jkl}$ |
| | S | 5.40$^{h}$ | 6.34$^{i}$ | 2.03$^{l}$ | 25.04$^{l}$ | 14.53$^{n}$ |
| 3 | F | 7.80$^{abcdef}$ | 6.42$^{ghi}$ | 2.06$^{fghijk}$ | 33.96$^{hij}$ | 26.84$^{cde}$ |
| | FMS | 8.18$^{abcde}$ | 6.78$^{de}$ | 2.08$^{cdef}$ | 44.69$^{cde}$ | 20.27$^{ijkl}$ |
| | FS | 7.48$^{bcdef}$ | 6.76$^{de}$ | 2.07$^{efghij}$ | 43.86$^{cde}$ | 23.91$^{fgh}$ |
| | M | 7.25$^{cdefg}$ | 6.61$^{ef}$ | 2.05$^{ijkl}$ | 33.07$^{hijk}$ | 21.50$^{hij}$ |
| | MS | 8.45$^{abcd}$ | 6.35$^{hi}$ | 2.047$^{jkl}$ | 33.09$^{hijk}$ | 22.75$^{jkl}$ |
| | S | 5.48$^{h}$ | 6.00$^{abc}$ | 2.083$^{cdef}$ | 41.40$^{efg}$ | 19.98$^{ijkl}$ |
| 4 | F | 7.93$^{abcdef}$ | 7.08$^{ab}$ | 2.09$^{bcde}$ | 42.63$^{def}$ | 28.81$^{bcd}$ |
| | FMS | 9.20$^{a}$ | 7.16$^{a}$ | 2.127$^{a}$ | 57.60$^{a}$ | 33.38$^{a}$ |
| | FS | 7.45$^{bcdef}$ | 6.95$^{bcd}$ | 2.08$^{defg}$ | 52.07$^{ab}$ | 26.02$^{def}$ |
| | M | 8.80$^{abc}$ | 6.87$^{cd}$ | 2.063$^{fghijk}$ | 53.07$^{ab}$ | 26.84$^{cde}$ |
| | MS | 8.45$^{abcd}$ | 6.78$^{de}$ | 2.083$^{cdef}$ | 55.79$^{ab}$ | 29.00$^{bc}$ |
| | S | 8.45$^{abcd}$ | 6.62$^{ef}$ | 2.09$^{cde}$ | 40.19$^{efgh}$ | 24.94$^{efg}$ |
| 5 | F | 8.93$^{ab}$ | 6.90$^{bcd}$ | 2.057$^{ghijk}$ | 42.63$^{def}$ | 22.74$^{ghi}$ |
| | FMS | 9.11$^{ab}$ | 6.90$^{bcd}$ | 2.10$^{bcd}$ | 53.90$^{ab}$ | 30.66$^{ab}$ |
| | FS | 9.00$^{ab}$ | 7.08$^{ab}$ | 2.12$^{ab}$ | 49.90$^{bc}$ | 24.71$^{efg}$ |
| | M | 6.68$^{fgh}$ | 7.08$^{ab}$ | 2.107$^{abc}$ | 52.83$^{ab}$ | 18.69$^{klm}$ |
| | MS | 8.60$^{abc}$ | 6.68$^{ef}$ | 2.073$^{efghi}$ | 28.323$^{jkl}$ | 21.50$^{hijk}$ |
| | S | 6.53$^{fgh}$ | 6.91$^{bcd}$ | 2.077$^{defgh}$ | 35.92$^{fghi}$ | 26.26$^{cdef}$ |
| LSD (5 %) | | 1.67 | 0.196 | 0.026 | 7.25 | 2.80 |
| CV (%) | | 13.34 | 15.826 | 21.411 | 10.71 | 10.03 |

Means followed by the same letter(s) are not significantly different at P=0.05 level significance , F = forest soil , FMS = forest soil : decomposed animal manure : fin sand , FS = forest soil : fine sand , M = decomposed animal manure , MS = decomposed animal manure : fine sand , S = fine sand.

sand and 1:1 mixture of forest soil: fine sand rooting media, respectively; whereas the lowest leaf area (46.98 $cm^2$) was observed from two node cuttings raised on pure fine sand. However, root number showed no significant difference ($p > 0.05$) between interaction of rooting media and number of nodes per stem cutting. Highest root to shoot dry weight ratio (0.133 g) was recorded from four node cutting grown on 1:1:1 mixture of forest soil: decomposed animal manure: fine sand followed by five nodal cutting (0.103) grown on 1:1:1 mixture of forest soil: fine sand: decomposed animal manure and 1:1 mixture of forest soil: fine sand whereas the lowest root to shoot dry weight (0.04 g) ratio is recorded from two and three node cutting grown on sole fine sand rooting media (Table 5). On the other hand, an increasing pattern of root to shoot ratio was obtained from two nodes to four nodes cutting while a decreasing order was recorded from four nodes to five node cutting.

In general, longer cuttings grown on 1:1:1 mixture of forest soil: fine sand: decomposed animal manure resulted in highest root and shoot growth. However, cuttings from the medium composed of pure sand showed poor shoot growth and development and cuttings grown in pure decomposed animal manure showed lowest root growth (Plates 4, 5 and 6). The current findings in vanilla fragrance are also supported by the

**Table 5.** Interaction effect of different rooting media and number of nodes per cutting on the leaf area (LA), shoot girth (SG), shoot dry weight (SDW) and root to shoot ratio (RS) of *V. fragrans*.

| Number of nodes per stem cutting | Rooting media | Parameter | | | |
|---|---|---|---|---|---|
| | | LA($cm^2$/plant) | SG | SDW(g) | RS |
| 2 | F | $51.20^{lm}$ | $0.4^{abcde}$ | $2.81^{i}$ | $0.073^{defgh}$ |
| | FMS | $53.55^{kl}$ | $0.317^{hi}$ | $2.83^{i}$ | $0.047^{ij}$ |
| | FS | $57.19^{ijkl}$ | $0.36^{efghi}$ | $2.89^{ghi}$ | $0.067^{efghi}$ |
| | M | $66.42^{cdefg}$ | $0.40^{abcde}$ | $2.88^{hi}$ | $0.060^{ghij}$ |
| | MS | $61.11^{ghij}$ | $0.34^{fghi}$ | $2.81^{i}$ | $0.067^{efghi}$ |
| | S | $46.98^{m}$ | $0.307^{i}$ | $2.56^{j}$ | $0.040^{j}$ |
| 3 | F | $60.88^{ghij}$ | $0.387^{abcdef}$ | $2.82^{i}$ | $0.0767^{cedfg}$ |
| | FMS | $68.75^{bcdef}$ | $0.37^{bcdefg}$ | 2.83i | $0.10b^{c}$ |
| | FS | $62.86^{fghi}$ | $0.40^{abcde}$ | $3.05^{efg}$ | $0.067^{efghi}$ |
| | M | $54.91^{jkl}$ | $0.35^{efghi}$ | $2.81^{i}$ | $0.067^{efghi}$ |
| | MS | $65.62^{cdefg}$ | $0.33^{ghi}$ | $3.03^{fg}$ | $0.053^{ghij}$ |
| | S | $57.78^{ijk}$ | $0.417^{abcd}$ | $2.603^{j}$ | $0.040^{j}$ |
| 4 | F | $69.71^{bcd}$ | $0.36^{defgh}$ | $2.82^{i}$ | $0.043i^{j}$ |
| | FMS | $78.85^{a}$ | $0.43^{a}$ | $3.14^{ef}$ | $0.133^{a}$ |
| | FS | $63.41^{efghi}$ | $0.40^{abcde}$ | $4.16^{a}$ | $0.067^{efghi}$ |
| | M | $66.07^{cdefg}$ | $0.35^{efghi}$ | $3.15^{ef}$ | $0.093^{bcd}$ |
| | MS | $69.27^{bcde}$ | $0.37^{cdefgh}$ | $3.56^{b}$ | $0.06^{ghij}$ |
| | S | $63.11^{efghi}$ | $0.38^{abcdefg}$ | $3.41^{bc}$ | $0.067^{efghi}$ |
| 5 | F | $71.24^{ab}$ | $0.42^{abc}$ | $3.14^{ef}$ | $0.05^{hij}$ |
| | FMS | $73.75^{ab}$ | $0.43^{a}$ | $4.16^{a}$ | $0.103^{b}$ |
| | FS | $78.51^{a}$ | $0.40^{abcde}$ | $3.4^{c}$ | $0.103^{b}$ |
| | M | $60.28^{ghij}$ | $0.40^{abcde}$ | $2.82^{i}$ | $0.090^{bcde}$ |
| | MS | $64.27^{defgh}$ | $0.37^{cdefgh}$ | $2.88^{hi}$ | $0.087^{bcdef}$ |
| | S | $58.09^{hijk}$ | $0.387^{abcdef}$ | $3.27^{de}$ | $0.06^{fghij}$ |
| LSD (5%) | | 6.237 | 0.06 | 0.16 | 0.0257 |
| CV (%) | | 5.977 | 8.62 | 8.95 | 21.83664 |

Means followed by the same letter(s) are not significantly different at p=0.05 level significance, F = forest soil, FMS = forest soil: decomposed animal manure: fin sand, FS = forest soil: fine sand, M = decomposed animal manure, MS = decomposed animal manure: fine sand, S = fine sand.

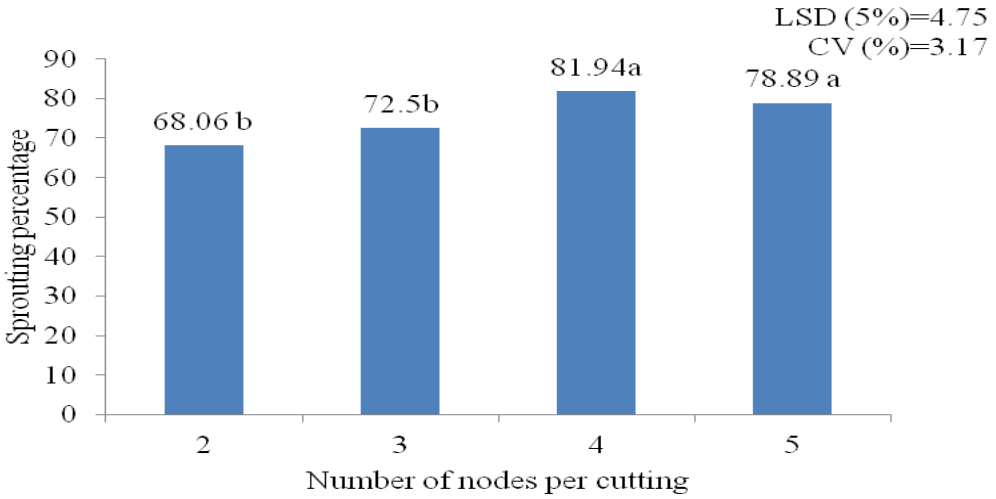


**Figure 1.** Effect of different number of nodes per cutting on the sprouting percentage of *Vanilla fragrans* Means followed by the same letter are not significantly different at p= 0.05.

**Plate 1.** Pots ready for cutting insertion.

**Plate 2.** Cuttings of introduced vanilla accession (Van1. /9) with different node number.

**Plate 3.** Overview of cuttings on the rooting bed.

**Plate 4.** Overview of cuttings after two month.

reports of Digafie (2006) who observed higher shoot growth response in black pepper cuttings raised on a multi-component media than those grown on single component. The results of using four node numbers for vanilla planting material preparation was also supporting to a recommendation by Girma et al. (2012). The authors stated that size and uniformity of planting materials from four node cutting were very promising. They also recommended using four node vanilla (*V. fragrans*) cutting is economical as well as environmental to raise nursery rooted planting materials in South western Ethiopia. According to Namirembe -Ssonkko et al. (2005) *Vanilla fragrans* can be easily and successfully propagated in the nursery using short stem cuttings (30cm, approximately 4 nodes) inserted in a medium with a high OM content and a good water holding capacity. Umesha *et al*. (2011) reported that root initiation is faster and vigorous in three to five node cuttings.

**Plate 5.** Overview of cuttings after four month.

**Plate 6.** Overview of cuttings at the end of nursery work.

**Plate 7.** Root of four node cutting grown on fine sand rooting media.

**Plate 8.** Root of two node cutting grown on decomposed animal manure rooting media.

## CONCLUSION AND RECOMMENDATION

Rooting media type showed considerable effect on all root and shoot development and growth parameters (except sprouting percentage, shoot girth and root number). In most of the case leaf number (9.2), leaf fresh (7.157 g) and dry weight (2.127 g), shoot length (57.60 cm) , shoot fresh weight (33.383 g) and root to shoot ratio (0.133 g) characters evaluated, better results were obtained from four node vanilla cuttings grown on a 1:1:1 mixture of forest soil: decomposed animal manure : fine sand, with the exception of the biggest root length (17.5 cm) and rooting percentage (99.3%) of cuttings obtained from the medium containing pure sand. cuttings obtained from the medium containing pure sand. 1:1:1 mixture of forest soil: decomposed animal manure: fine sand rooting media give rise to branched roots having better chance of adaptation after transplanting, but those grown from the medium composed of pure sand resulted in long, coarse and brittle roots that were not preferable. The highest value for some root parameters (root volume, root fresh and dry weight) was obtained from five node cuttings grown on a 1:1:1 mixture of forest soil: decomposed animal manure: fine sand. In addition early and highest sprouting percentage were obtained from longer (four and five node) cuttings. Higher mean values of leaf area, shoot girth and shoot dry weight were obtained from four and five node cuttings grown on a 1:1:1 mixture of forest soil: decomposed animal manure: fine sand. Therefore, the study reveals that vegetative propagation of vanilla could be successfully attained by raising four node

cuttings on 1:1:1 mixture of forest soil: decomposed animal manure: fine sand rooting media.

## Conflict of Interest

The authors have not declared any conflict of interest.

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*Full Length Research Paper*

# Effect of media and regulators of plant growth on micro propagation of Myrobalan 29C rootstock

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**Myrobalan 29C is one of the most important rootstocks that is widely used for plum and apricot trees. This study was conducted to determine the most suitable media culture and regulators of plant growth for micropropagation of Myrobalan 29C in Khorasan Natural Resource and Agricultural Research Centre Mashhad, Iran. 10 explants treated were sterilized in 70% Ethanol for 1 min, Mercuric chloride (0.1%) for 1, 2 and 3 min and sodium hypochlorite (3 and 10%) for 10, 20 and 30 min. Results showed that 10% sodium hypochlorite (30 min) with 2% decay was the best treatment. In this experiment, proliferation and rooting were performed in three kinds of culture media: Murashige and Skoog (MS), McCown and Lioyd (WPM) and Driver and Kuniyuki (DKW). They were supplemented with plant growth regulators (benzyl amino purine (BAP) and thiadizuron (TDZ)) of 0, 1, 2, 3, 4 mg $l^{-1}$ in all treatments of the proliferation; and with indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) of 0, 1, 2, 3 mg $l^{-1}$ in the rooting step. Results showed that the highest number and length of shoot respectively were 5.58 and 2.50 cm in MS medium with 2 mg $l^{-1}$ BAP concentrations. The DKW medium in 1 mg $l^{-1}$ of NAA, the highest percent of rooting (100%) and root length were about 14.5 cm in MS medium with 2 mg $l^{-1}$ of NAA respectively. The acclimatization of plantlets was successful in greenhouse conditions. The survival percent in substrateperlite (100% V) was about 80%.**

**Key words:** Tissue culture, disinfection, micropropagation, acclimatization.

## INTRODUCTION

Myrobalan 29C (*Prunus cerasifera* L.) has been widely used as a fruit tree rootstock for plum and apricot because of its rusticity and adaptability to several soil conditions (Plopa et al., 2012). This rootstock is suitable for high density plum orchard. In addition, Myrobalan has also been used as a parent in several rootstock and edible plum breeding programs (Arbeloa et al., 2006). It has also a good grafting compatibility where majority of the new Romanian plum varieties resulted from. When propagated by softwood cuttings, the results are very good in respect to rooting ability (75 to 95% rooted cuttings); but a lot of cuttings on the rooting beds blossom.

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This finally results in very weak rooted cuttings, not suitable for grafting in nursery field. That is the reason why this valuable rootstock might be micropropagated on commercial scale. The use of *in vitro* propagation technique allows for the efficiency of propagation, but for some species the propagation efficiency depends on some factors. Micropropagation is used as a useful method for propagation of clonal rootstocks (Hossini et al., 2010).

Movsiuw (2011) has reported the high multiplication rate of myrobalan 29C rootstocks in MS culture media containing 0.5 $mgl^{-1}$ benzyl adenine (BA). Ruzick (2013) and Plopa (2012) investigated Myrobalan *in vitro* as having the highest proliferation with 1 $mgl^{-1}$ BA concentration and rooting with 0.1 $mgl^{-1}$ IBA concentration. In another report, Myrobalan MS medium showed the highest percentage rooting. Dardi et al. (2011) reported that Mahaleb rootstock in MS medium containing BAP and NAA has the highest proliferation and rooting. Bonjak (2012) investigated the effect of MS media culture and different types of cytokinin concentrations on the proliferation of Gisela5. BA and TDZ concentration significantly affected the proliferation rate. In a study on micropropagation of *Prunus avium*, the combination of 0.5 $mgl^{-1}$ BAP and 0.05 $mgl^{-1}$ TDZ is suitable for proliferation and a medium culture containing 0.3 mg $l^{-1}$ IBA is desirable for the rooting (Hossini et al., 2010; Nazeri et al., 2010). Ying-Ning (2010) investigated Chinese plum *in vitro* micropropagation that 1/2 MS media had the highest percentage rooting, and rooted plantlets acclimatized to greenhouse conditions had the most successfully system. Medium composition was a decisive factor for plant regeneration.

In previous reports, MS or WPM was used frequently as basal medium for the regeneration of *Prunus* species; the most used cytokinins were TDZ or BA used as auxin in this study, because IBA easily induces the differentiation of root of plum (Yao et al., 2011). Plum regeneration and transformation has been mainly conducted by using cytokinins, such as benzylaminopurine (BA) and thidiazuron (TDZ) to induce cell differentiation (Mikhilov et al., 2008; Tian et al., 2007). Shekafandeh and Qasemi (2008) reported that, higher multiplication rate in embryo-genesis with 4 $mgl^{-1}$ TDZ was obtained. *In vitro* culture on the natural hybrid of *Prunus armanica* × *Prunus cerasus* showed that the best acclimatized rooted plantlets to greenhouse conditions used the system successfully (64.4%). Tatari (2013) and Xiaomei (2008) reported that rooted plantlet were acclimatized after 6 weeks of growth in the lab and 76 to 84% of rooted plantlets survived after acclimatizing with the greenhouse. Due to the importance of achieving an efficient protocol for the mass propaga-tion of Myrobalan 29C, this study was conducted with the purpose of evaluating the most suitable media culture and plant growth regulators for the micropropagation of Myrobalan 29C.

## MATERIALS AND METHODS

### Plant material

The explants were collected from shoots of Myrobalan 29C rootstock maintained in the experimental greenhouse of Khorasan Razavi Agricultural and Natural Resources Research Centre (Mashhad, Iran), on 25 June, 2013. The shoots were transferred to the laboratory where the tips and axillary buds were dissected with a scalpel and used as a source for explants. The explants were washed with water and dishwashing liquid to remove surface contamination and then divided into parts containing one bud. Then they were sterilized with 70% ethanol for 1 min, Mercuric chloride (0.1%) for 1, 2 and 3 min, sodium hypochlorite (3 and 10%) for 10, 20 and 30 min; and then washed 3 times with sterile distilled water and cultured in a medium. Each treatment disinfection consisted of three replications and each replicate had five samples.

### Proliferation

Proliferation was performed in three kinds of culture media: MS (Murashige and Skooge, 1962), WPM (Lloyd and McCown, 1980) and DKW (Driver and Kuniyuki, 1984). They were supplemented with plant growth regulators (benzyl amino purine (BAP) and thiadiazuron) (TDZ) of 0, 1, 2, 3 and 4 mg $l^{-1}$. In the rooting step after three subcultures (21 days between each subculture), the numbers, length and quality of the shoots were measured. This stage was carried with four replications and each replicate had five samples.

### Rooting

For rooting, *in vitro* developed shoots (2 to 3 cm long) were placed on three culture media (MS, DKW and WPM) supplemented with indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) at four levels (0, 1, 2 and 3 $mgl^{-1}$). The explants were transferred to the culture growth chamber at a temperature of 22°C and night temperatures of 16°C with a photoperiod of 16 h light and 8 h dark with light from 2000 to 2500 Lux. This stage was carried out in four replications and each replicate had three samples. After rooted explants were determined as the best cultured media and combination of rooting growth regulators, number and root length, leaf number and stem length and quality of explants were recorded.

### Acclimatization

All plants with properly developed roots were transferred into the growth substrate coco peat and perlite (50%: 50% V) and perlite (100% V). The transferred roots were carefully washed under tap water to remove all remnants of agar. The acclimatization of the transferred plants was conducted in a mist chamber inside the greenhouse. The experiment was carried out based on completely randomized design (CRD) with four replications and each replicates had five samples. Statistical analysis of the data was carried out by using JMP software and difference among treatment means was compared by using least significance difference test (LSD).

## RESULTS

### Surface sterilization

The best result was achieved from the sterilization of the

**Table 1.** The effects of surface sterilization treatments on contamination of explants Myrobalan 29C.

| Treatment | Active explants (%) | Contamination (%) |
|---|---|---|
| Ethanol, 70%, 1 min. | 8.5$^{c*}$ | 80.0$^{ab}$ |
| Mercuric chloride, 0.1%, 1 min. | 4.5$^{c}$ | 5.5$^{c}$ |
| Mercuric chloride, 0.1%, 2 min. | 5.5$^{c}$ | 5.5$^{c}$ |
| Mercuric chloride, 0.1%, 3 min. | 6.5$^{c}$ | 5.5$^{c}$ |
| Sodium hypochlorite, 3%, 10 min. | 7.5$^{c}$ | 100.0$^{a}$ |
| Sodium hypochlorite, 3%, 20 min. | 8.1$^{c}$ | 100.0$^{a}$ |
| Sodium hypochlorite, 3%, 30 min. | 1.2$^{c}$ | 100.0$^{a}$ |
| Sodium hypochlorite, 10%, 10 min. | 66.6$^{ab}$ | 4.5$^{c}$ |
| Sodium hypochlorite, 10%, 20 min. | 66.6a$^{b}$ | 4.5$^{c}$ |
| Sodium hypochlorite, 10%, 30 min. | 100.0$^{a}$ | 2.0$^{c}$ |

* Mean values followed by the same letters within a column are not significantly different according to least significance difference test (LSD) at 5% level.

explants in 10% sodium hypochlorite for 30 min. The results showed that among the sterilization treatments, 3% sodium hypochlorite for 10, 20 and 30 min had the highest contamination, and among other treatments, the percentage of the contamination was 2%; sodium hypochlorite (10%) for 30 min had the least explants of active sterile (Table 1).

### Proliferation

The proliferation results showed that the MS media culture supplemented with 2 mg $l^{-1}$ BAP with mean of 5.58 numbers of shoot, 2.5 cm shoot length and 11.33 numbers of leaf is the most suitable treatment (Figures 1, 2 and 3). There was no significant difference in the quality of plantlet in all three media; however, best quality was observed in the media MS. Moreover, the considerations showed that DKW with 2 mg $l^{-1}$ TDZ has the least numbers and length of shoot (Figures 2 and 3).

### Rooting

The best root formation was observed in DKW media containing 1 mg $l^{-1}$ NAA, which produced roots readily with 100% efficiency compared to other rooting media cultures (Table 2). The mean of numbers and length of roots in this culture media were 6.75 and 14.3 cm, respectively (Table 2). The results showed that DKW culture media without growth regulators having mean of 0.3 root numbers and the MS culture media without growth regulators having mean of 1 cm root length had the least number and length of root, respectively. However, the highest length of roots, length plant, number of leaf and plantlet quality were observed in the MS media (Table 2 and Figure 4).

## DISCUSSION

In the present study, sampling date, genotype and different concentrations of sodium hypochlorite affected surface sterilization. Vujovic (2012) has reported sterilization of Gisela5, apricot and plum buds with 10% sodium hypochlorite for 15 min to be more effective than other treatments. Sulusoglu and Covusoglu (2013) investigated that rootstock micropropagation (*Prunus laurocerasus*) with 5% sodium hypochlorite for 14 min disinfection of treated explants was considered. Nacheva and Gercheva (2009) obtained 85 to 100% sterile explants after sterilization with calcium hypochlorite. For surface sterilization of explants, 70% ethanol, sodium hypochlorite (10 to 50%) and mercuric chloride were used (Asadi et al., 2009; Sana et al., 2006; Jang et al., 2008). Sodium hypochlorite causes the oxidation of cells, microorganisms and affects major components of cells, such as lipids, proteins and DNA. Ethanol damages cell membranes, proteins and causes more rapid disintergration of cell metabolism (George, 2008). Therefore, one can conclude that alternating the use of these two substances that deplete cells and bacterial pathogens is better; and the higher the concentration and time of treatment, the more its effectiveness improved. The use of mercuric chloride with minimum contamination, compared to sodium hypo-chlorite has 0% active buds. Perhaps due to the toxicity and lethality of mercuric chloride, it penetrates into plant tissue and destroys the buds in active meristem (Ruzic and Vujovic, 2013).

A significant interaction between media and growth regulators regarding shoot regeneration percentage was found at $p<0.05$. This study showed that number of shoot was increased as concentration of BAP increased to certain amounts. There is a positive correlation between BAP concentration (2 mgl) and number of shoots. The increasing concentrations of BAP inhibited shoot proliferation (Sulusoglu and Cavusoglu, 2013). The highest

**Table 2.** The effects different media, IBA and NAA concentrations on rooting parameters of Myrobalan 29C.

| Culture media | Growth regulators (mg $l^{-1}$) | Rooting (%) | Root length (cm) | Number of root | Plantlet length (cm) | Number leaf | Plantlet quality |
|---|---|---|---|---|---|---|---|
| | IBA(0) | 3.5$^{c}$ | 0.26$^{f}$ | 0.4$^{de}$ | 3.00$^{defghi}$ | 10.00$^{abc}$ | 1.66$^{ab}$ |
| | IBA(1) | 50.00$^{ab}$ | 11$^{ab}$ | 2.25$^{abcde}$ | 5.5$^{bcdefg}$ | 13.00$^{a}$ | 1.25$^{ab}$ |
| | IBA(2) | 72.08$^{ab}$ | 10.50$^{abc}$ | 3.25 $^{ab}$ | 3.87$^{cdefghi}$ | 9.25$^{abc}$ | 2.00$^{a}$ |
| | IBA(3) | 68.75$^{ab}$ | 13.75$^{a}$ | 2.50$^{abcde}$ | 6.75$^{abc}$ | 13.25$^{a}$ | 2.00$^{a}$ |
| MS | NAA(0) | 50.00$^{ab}$ | 8.50$^{bcd}$ | 1.75$^{abcde}$ | 2.75$^{fghi}$ | 10.00$^{abc}$ | 2.00$^{a}$ |
| | NAA(1) | 91.66$^{a}$ | 10.50$^{abc}$ | 3.25$^{ab}$ | 9.75$^{a}$ | 12.75$^{a}$ | 2.00$^{a}$ |
| | NAA(2) | 75.00$^{ab}$ | 14.50$^{a}$ | 3.25$^{ab}$ | 9.75$^{a}$ | 12.5$^{ab}$ | 2.00$^{a}$ |
| | NAA(3) | 87.50$^{a}$ | 12$^{ab}$ | 3.75$^{a}$ | 8.75$^{ab}$ | 12.75$^{a}$ | 2.00$^{a}$ |
| | IBA(0) | 14$^{c}$ | 0.37$^{f}$ | 0.3$^{e}$ | 2.5$^{fghi}$ | 10.00$^{abc}$ | 1.00$^{b}$ |
| | IBA(1) | 66.25$^{ab}$ | 2.87$^{ef}$ | 3.00$^{abc}$ | 4.75$^{cdefghi}$ | 10.25$^{abc}$ | 1.75$^{ab}$ |
| | IBA(2) | 77.08$^{ab}$ | 4.50$^{def}$ | 2.25$^{abcde}$ | 6.25$^{bcde}$ | 9.75$^{abc}$ | 1.00$^{b}$ |
| | IBA(3) | 52.94$^{ab}$ | 3.75$^{ef}$ | 1.75$^{abcde}$ | 4.5$^{cdefghi}$ | 10.5$^{abc}$ | 1.00$^{b}$ |
| DKW | NAA(0) | 66.50$^{ab}$ | 2.12$^{ef}$ | 1.50$^{abcde}$ | 2.37$^{fghi}$ | 9.50$^{abc}$ | 1.00$^{b}$ |
| | NAA(1) | 100$^{a}$ | 6.00$^{cde}$ | 4.00$^{a}$ | 6.25$^{abcd}$ | 12.75$^{a}$ | 1.00$^{b}$ |
| | NAA(2) | 68.75$^{ab}$ | 2.25 $^{ef}$ | 2.25$^{abcde}$ | 3.87$^{cdefghi}$ | 9.50$^{abc}$ | 1.00$^{b}$ |
| | NAA(3) | 81.25$^{ab}$ | 3.62 $^{ef}$ | 2.75$^{abcd}$ | 5.75$^{bcdef}$ | 10.50$^{abc}$ | 1.00$^{b}$ |
| | IBA(0) | 62.5 | 1.00 $^{f}$ | 0.75$^{bcde}$ | 1.87$^{hi}$ | 6.00$^{cd}$ | 2.00$^{a}$ |
| | IBA(1) | 25.00$^{ab}$ | 1.12$^{f}$ | 1.00$^{bcde}$ | 1.75$^{hi}$ | 6.00$^{cd}$ | 2.00$^{a}$ |
| | IBA(2) | 50.00$^{ab}$ | 2.00$^{ef}$ | 1.75$^{abcde}$ | 2.37$^{fghi}$ | 6.00$^{cd}$ | 2.00$^{a}$ |
| | IBA(3) | 43.75$^{ab}$ | 2.12$^{ef}$ | 1.75$^{abcde}$ | 2.12$^{ghi}$ | 7.25$^{bcd}$ | 1.50$^{ab}$ |
| WPM | NAA(0) | 25.00$^{ab}$ | 1.5$^{ef}$ | 0.50$^{cde}$ | 1.87$^{hi}$ | 5.25$^{cd}$ | 1.50$^{ab}$ |
| | NAA(1) | 93.75$^{a}$ | 3.20$^{ef}$ | 3.75$^{a}$ | 3.00$^{efghi}$ | 6.75$^{cd}$ | 2.00$^{a}$ |
| | NAA(2) | 68.75$^{ab}$ | 2.62$^{ef}$ | 2.75$^{abcd}$ | 2.5$^{fghi}$ | 6.00$^{cd}$ | 2.00$^{a}$ |
| | NAA(3) | 54.16$^{ab}$ | 1.25 $^{f}$ | 2.00$^{abcde}$ | 1.25$^{i}$ | 3.50$^{d}$ | 1.25$^{ab}$ |

$^{a}$– explants strong growth, with no signs of verification, necrosis of leaf are yellowing terminal meristem, $^{b}$– less than15% have the symptoms of verification, necrosis of leaf are yellowing terminal meristem, and $^{c}$– explant weak, 15-30% have the symptoms of verification, necrosis of leaf are yellowing terminal meristem.

shoot multiplication was obtained in 2 mg $l^{-1}$ BAP added to MS medium. Erbenova (2009) and Sulusoglu (2012) reported 50% increase in multiplication rate in the dwarf rootstocks of *Prunus* in MS media culture containing 1.5 mg $l^{-1}$ BAP. George (2008) reported that cytokinins when added to media promote cell division. Nordstrom et al. (2004) suggested that auxins may also play a direct regulatory role in the balance of cytokinins levels by suppressing both the synthesis rate and pool size of cytokinins. These results can be related to the manipulation of exogenous cytokinins concentrations that may cause an increase in endogenous auxin concentrations. This is probably induced by an inhibition of free IAA conjugates due to the presence of exogenous cytokinins (Jaramillo et al., 2008; George (2008).

It is observed that the auxin to cytokinins ratio represents an important signal in the formation of cell phenotype and also in the onset and

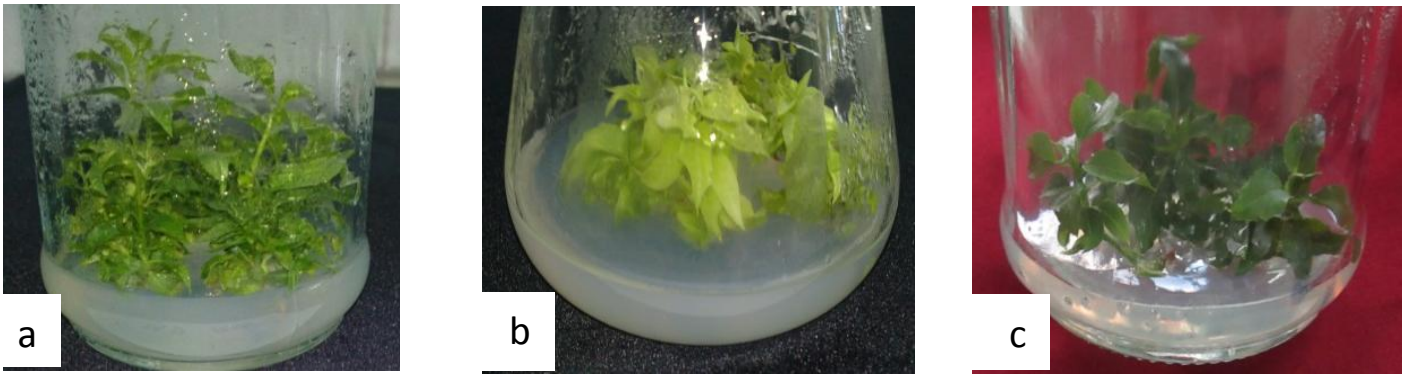


**Figure 1.** Effects of media and plant growth regulator on proliferation of Myrobalan29C (a) (MS), (b) (DKW) and (c ) (WPM).

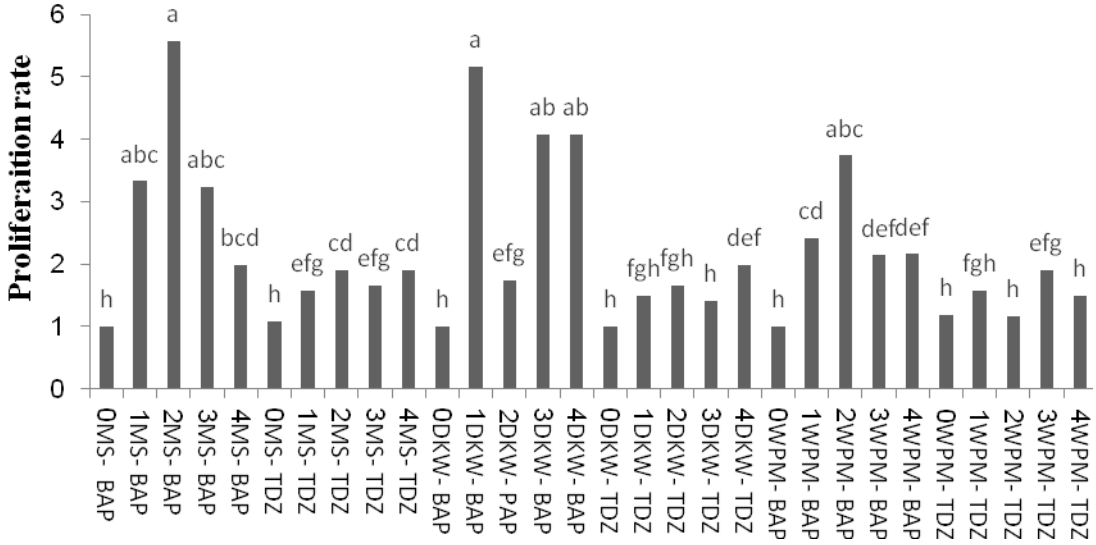


**Figure 2.** The effects of different media, BAP and TDZ concentrations on propagation coefficient of Myrobalan 29C.

maintenance of the process of cell division. Since auxins are capable of imitating cell division involved in the formation of meristems, giving rise to either unorganized tissue or defined organs.

Tatari and Mosavi (2013) reported that low shoot growth with necrotic leaf could be due to the effects of high concentrations of BAP and hormonal imbalances in the explants. Considering the MS medium contrast, WPM and DKW media have no $Cl^-$ in the culture media while chlorotic plants increased stability. The medium increases the amount of $Cl^-$ as plants balance is disturbed at different nutrient absorption. The MS media culture contains more calcium than the two other media which play an important role in the stability and strength of the plantlet. Nutrient concentration of N, K, Mg and macro nutrients is lower in WPM and DKW media than in MS media which is effective in reducing the proliferation of the media culture.

The best root formation was observed in DKW media containing 1 mg $l^{-1}$ NAA. Roots formation in tissue culture can be induced by exogenous auxins such as IBA, NAA and IAA and their interaction with endogenous auxins (Thorpe et al., 2008). Among the three auxins, NAA was found superior to IAA and IBA, though thick and callused rooted were obtained in one- step procedure. Low levels of NAA resulted in the highest rooting, whereas high concentration gave rise to more root initials, which eventually developed into callus rather than rootlets. They are only required at an early stage to emerge new formed roots. The MS culture media with reduced concentration of macro and microelements by ½ gave a maximum rooting percentage of 100% (Plopa et al., 2012). Vujovic et al. (2012) reported that the rooting rates in media containing 1 mg/L IBA or NAA (65 and 70%,

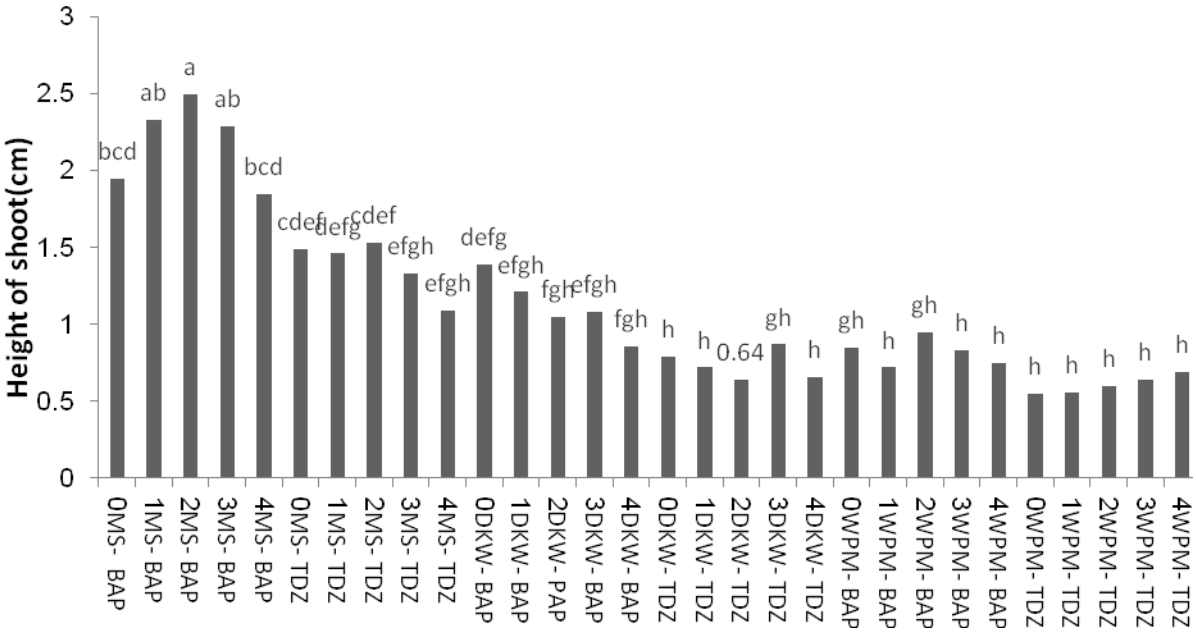


**Figure 3.** Effects BAP and TDZ concentrations on shoot proliferation and elongation of Myrobalan 29C.

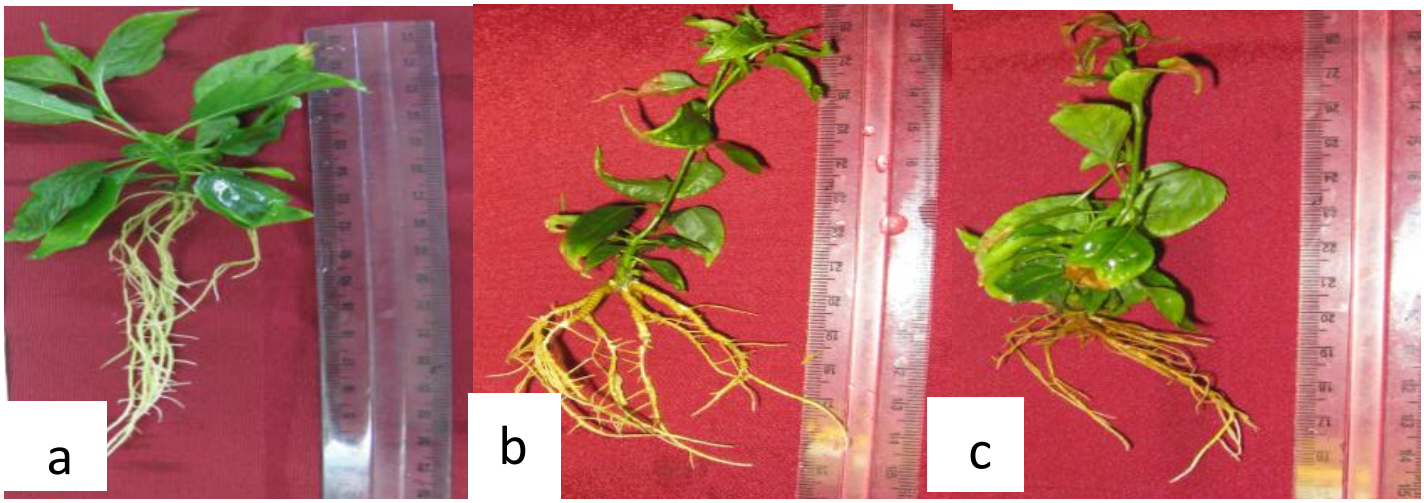


**Figure 4.** Root formation of Myrobalan29c. (a) (MS), (b) (DKW) and (c) (WPM).

respectively); although number of roots and plant height was significantly higher when IBA was supplemented. Hossini et al. (2010) reported that IBA concentration increase caused shortening of the root; 80% rooting and 2.9 cm root length were obtained in culture medium with the addition of 0.5 mg $l^{-1}$ IBA and greater percentage of rooting (88.6%) was attained when rooting medium was supplemented with 1.0 mg $l^{-1}$ NAA. Root percentage of the regenerated shoots was 38.2% by addition of 10.74 µM NAA in the medium (Demiral and Ulger, 2008; Mohamed, 2012).

Balla and Kirilla (2006) reported that following a nice growth during the multiplication and elongation phases, difficulties arose during the rooting phase and large differences were found in the nutrient demand of the rooting phase. In spite of the high percentage of rooting, widespread shoot tip necrosis was detected and optimal levels of different macro elements, iron and sugar had to be determined in a series of experiments for the clonal rootstock cultivars.

**Figure 5.** Acclimatization of Myrobalan29c rootstock on *in vivo* condition.

Acclimatization was affected directly by rooting conditions. Survival was best when plantlets were transferred to pots after a short period of root emergence on rooting media. The plants with older roots and brown colour were better adapted than new plantlets with white roots. The results showed that after 2 months of acclimatization, the best substrates, perlite (100%V) was about 80% (Figure 5). Therefore, acclimatization directly affected rooting plants that have high quality; best rooting had rate induction (Mahdaviyan et al., 2010). Rooted plantlets survived Gisela 6 after acclimatization with the greenhouse was successful (80 and 79.2%) (Hossini et al., 2010; Sulusoglu and Cavusoglu, 2013).

## Conclusion

The results of this research showed that Myrobalan 29C rootstock can be reproduced *in vitro*. According to this research, MS and DKW media including BAP with 2 mg $l^{-1}$ and NAA with 1 mg $l^{-1}$ growth regulators are most suitable for micro propagation.

## Conflict of Interest

The authors have not declared any conflict of interest.

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# Lettuce growth characteristics as affected by fertilizers, liming, and a soil conditioner

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**The aim of this work was to test fertilizers, liming, and levels of a soil conditioner produced from leonardite, composed by humic and fulvic acids on "iceberg" lettuce growth characteristics. The experimental design was completely randomized in a 5×3×2 factorial scheme, with 5 levels of soil conditioner (0, 20, 40, 100 and 200 L ha$^{-1}$), 3 fertilizers [chicken manure, plant compost (plant residues and cattle manure) and mineral], in limed and unlimed soil, with five replicates. The experiment was carried out in a greenhouse. Total shoot fresh and dry weight, shoot commercial fresh and dry weight, plants height, circumference, and number of leaves were evaluated as well as final soil pH and nutrient levels in leaves. Chicken manure rendered the greater circumference and shoot fresh commercial weight. Lettuce produced with chicken manure presented higher content of P, Ca, and Mg in leaves. Soil conditioner, in general, did not influence plant growth, except in its height, in the highest applied dose. With absence of lime, soil conditioner caused an increase of lettuce height with mineral fertilizers, and a decrease with plant compost.**

**Key words:** Humic substances, *Lactuca sativa* L., chicken manure, compost.

## INTRODUCTION

Nowadays, studies on the use of organic materials are very important as the price of fertilizers is increasing. Benefits of organic fertilizers for soil quality and lettuce production were published before. Some publications reported effects of organic wastes (Costa et al., 1994), compost (Silva et al,2010),vermicompost (Ali et al.,2007).

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chicken manure (Peixoto Filho et al., 2013), and organo-minerals compounds (Luz et al., 2010) on lettuce growth characteristics. However, there is almost no information concerning effects of organic materials, liming and soil conditioners on lettuce growth characteristics and nutrition.

A source of organic materials that may improve soil quality and productivity are soil conditioners. Several kinds of soil conditioners, produced from organic sources, such as organic composts, humus, and coal, are being sold in the market. These soil conditioners, which are sold in solid or liquid form, are mainly constituted of humic and fulvic acids, and may contain plant nutrients. These humic compounds may be absorbed by roots and transported to shoots, thus enhancing the growth of the whole plant (Lulakis and Petsas, 1995; García et al., 2014). Effects of soil conditioners on plants were reported to improve the soil chemically, releasing cations, complexing nutrients and toxic aluminum (Stevenson, 1994).

One soil conditioner of interest is leonardite, which is a secondary mineral originated from “soft coals”, commonly associated to lignite which is obtained normally at near-surface mining. Leonardite contains high amounts (> 80%) of humic acids, thus it is used, commercially, as a main source of these acids. Humic acid is extracted from leonardite is by using alkaline solutions, such as potassium hydroxide. Very few works showed the effects of soil conditioners in plant growth characteristics (Valšíková and Viteková, 2006). In addition, the great majority of the studies on the effects of humic substances on plants were held in nutrient solutions, using inert substrates and generally under laboratory conditions, and not on soils or field conditions (Zachariakis et al., 2001). The aim of this work was to evaluate effects of organic and mineral fertilizers, liming, and use of a soil conditioner produced from leonardite on the growth characteristics and nutrition of “iceberg” lettuce.

## MATERIALS AND METHODS

### Soil

Samples from 0 to 20 cm of a soil classified as Typic Dystrudepts (Soil Survey Staff, 1999), corresponding to the Cambissolo in the Brazilian classification system (EMBRAPA, 2009), were collected (Table 1). Lime was mixed to increase base saturation to 60%, aiming to increase soil pH to around 6, supply Ca and Mg for lettuce plants according to CESEMG (1999) recommendations. In 70 kg of soil, it was applied 56.4 g of dolomitic lime, equivalent to 1.612 t $ha^{-1}$ of lime.

### Fertilization

Chicken manure (CKM; Table 2) or organic compost (OCP), 24.19 and 54.83 g $kg^{-1}$, respectively, were mixed to the soil to provide the recommended nitrogen dose for lettuce (300 mg N $kg^{-1}$; Novais et al., 1991). The amount of organic fertilizers applied also considered N conversion from N total to mineral of 50% during the crop season (CFSEMG, 1999). Mineral fertilization (control) was performed as follows (Novais et al., 1991): 300 mg single superphosphate was mixed $kg^{-1}$ of soil. Nitrogen (N-urea, 300 mg N $kg^{-1}$), and potassium (300 mg K $kg^{-1}$) fertilization was split in four applications: 7, 15, 21, and 28 days after planting. Micronutrients (0.5 mg B $kg^{-1}$, 5.0 mg Zn $kg^{-1}$, 1.5 mg Cu $kg^{-1}$, 0.15 mg Mo $kg^{-1}$) were applied at once ten days after planting using soluble sources readly assimilable by lettuce plants. The soil conditioner (SCN; Table 2) extracted from leonardite, was composed of 229 g $L^{-1}$ of humic extract, in which 113 g $L^{-1}$ were humic acids, and 116 g $L^{-1}$ were fulvic acids (Marchi et al., 2008). The SCN was applied in its liquid form to the soil 7 days after planting. Application rates were: 0, 20 (recommended by SCN producer), 40, 100, and 200 L $ha^{-1}$. Based on plant population, application rates were: 0, 0.3986, 0.7972, 1.993, and 3.986 g $pot^{-1}$.

### Lettuce growth

Fertilized soil (2.75 kg $pot^{-1}$) was incubated for ten days before lettuce planting. Lettuce (iceberg, cv. Raider) was planted 35 days after germination, when plants presented four leaves. Plants were harvested 57 days after planting. After harvesting, lettuce height, circumference, total number of leaves, fresh and dry shoot weight, and fresh and dry shoot commercial (lettuce excluding external leaves) weight were measured. Lettuce leaves were dried in oven at 60°C for 48 h, digested in nitro-percloric acid, and analyzed for N, P, K, Ca, Mg, S, B, Cu, Fe, Mn, and Zn content by atomic absorption spectrometry or flame photometry (EMBRAPA, 1999).

### Experimental design and statistics

The experiment was completely randomized in a factorial scheme 5x3x2. In the first level, 3 types of fertilizers (CKM, OCP and mineral), in limed and unlimed soil were studied and, in the second level, five doses of soil conditioner (0, 20, 40, 100 and 200 L $ha^{-1}$), with five replicates. The analysis of variance was performed to test lettuce production as function of tested factors (soil conditioner, fertilizers and liming). Scott Knott test, at 5% of probability was performed.

## RESULTS AND DISCUSSION

The analysis of variance showed that lettuce height data were affected significantly with SCN, fertilizers and liming. Other lettuce characteristics, such as number of leaves, circumference, shoot fresh weight, and shoot commercial dry weight data were affected significantly only with fertilizers and liming. Data of shoot dry weight and shoot commercial fresh weight were affected significantly only with fertilizers.

When lime was not used, lettuce produced with mineral and CKM fertilizers was higher than lettuce produced with OCP. When lime was applied, lettuce height followed the order: mineral fertilization > CKM > OCP (Table 3). Mantovani et al. (2005) showed that high amounts of

**Table 1.** Soil physical and chemical characteristics[a].

| Characteristic[§] | Value |
|---|---|
| pH in water | 5.2 |
| P (mg $dm^{-3}$) | 0.6 |
| remaining phosphorus (mg $L^{-1}$) | 4.5 |
| K (mg $dm^{-3}$) | 12.0 |
| Ca ($cmol_c$ $dm^{-3}$) | 0.8 |
| Mg ($cmol_c$ $dm^{-3}$) | 0.2 |
| Al ($cmol_c$ $dm^{-3}$) | 0.5 |
| H+Al ($cmol_c$ $dm^{-3}$) | 3.2 |
| SB[(b)] ($cmol_c$ $dm^{-3}$) | 1.0 |
| t[(c)] ($cmol_c$ $dm^{-3}$) | 1.5 |
| T [(d)] ($cmol_c$ $dm^{-3}$) | 4.2 |
| V[(e)] (%) | 24.0 |
| m[(f)] (%) | 33.0 |
| Corg[(g)] (mg $g^{-1}$) | 14.1 |
| Zn (mg $dm^{-3}$) | 0.3 |
| Fe (mg $dm^{-3}$) | 25.0 |
| Mn (mg $dm^{-3}$) | 8.1 |
| Cu (mg $dm^{-3}$) | 0.5 |
| B (mg $dm^{-3}$) | 0.5 |
| S-sulphate (mg $dm^{-3}$) | 4.9 |
| Sand (g $kg^{-1}$) | 130.0 |
| Silt (g $kg^{-1}$) | 280.0 |
| Clay (g $kg^{-1}$) | 590.0 |

[a]Marchi et al. (2008); [§] EMBRAPA (1999); [b]SB = sum of basis; [c]t = cation exchange capacity at pH 5.0; [d] T = cation exchange capacity at pH 7.0; [e] V = basis saturation; [f] m = aluminum saturation; [g] Corg = soil organic carbon.

**Table 2.** Organic fertilizers and soil conditioner characteristics[a].

| Characteristic[b] | CKM[b] | OCP[c] | SCN[d] |
|---|---|---|---|
| pH in water | 8.90 | 7.60 | 14.30 |
| N-total (g $kg^{-1}$) | 25.80 | 12.00 | 4.00 |
| P (g $kg^{-1}$) | 25.75 | 4.24 | 34.44 |
| K (g $kg^{-1}$) | 22.28 | 6.81 | 37.12 |
| Na (g $kg^{-1}$) | - | - | 115.00 |
| Ca (g $kg^{-1}$) | 102.50 | 25.48 | 1.66 |
| Mg (g $kg^{-1}$) | 6.12 | 3.02 | 0.26 |
| S-sulphate (g $kg^{-1}$) | 5.23 | 5.23 | 7.50 |
| B (mg $kg^{-1}$) | 35.00 | 106.00 | - |
| Cu (mg $kg^{-1}$) | 68.00 | 43.00 | 0.00 |
| Fe (g $kg^{-1}$) | 2.18 | 48.38 | 102.10 |
| Mn (mg $kg^{-1}$) | 552.00 | 468.00 | 7.10 |
| Zn (mg $kg^{-1}$) | 503.00 | 473.00 | 16.20 |
| Humidity (dag $kg^{-1}$) | 16.58 | 6.16 | - |
| OC[(e)] (mg $g^{-1}$) | 94.00 | 128.00 | 59.00 |
| Density (g $cm^{-3}$) | - | - | 1.23 |

[a]Marchi et al. (2008); [b]Embrapa (1999); [b]CKM = dry chicken manure; [c]OCP = dry organic compost; [d]SCN = soil conditioner; [e]OC = organic carbon (dry basis).

**Table 3.** Lettuce height as a function of fertilizers, liming and doses of soil conditioner (SCN).

| Liming | Dose of SCN (L ha$^{-1}$) | Fertilization | | |
|---|---|---|---|---|
| | | CKM [(b)] | OCP[(c)] | Mineral |
| | | Height (cm) [(a)] | | |
| Without | 0 | 20.00[a] | 15.60[b] | 20.00[a] |
| | 20 | 20.50[a] | 15.20[b] | 21.10[a] |
| | 40 | 20.10[a] | 15.20[b] | 21.30[a] |
| | 100 | 20.60[a] | 15.30[b] | 21.60[a] |
| | 200 | 20.50[b] | 14.00[c] | 22.70[a] |
| With | 0 | 20.20[b] | 16.40[c] | 22.30[a] |
| | 20 | 19.50[b] | 16.60[c] | 22.90[a] |
| | 40 | 20.80[b] | 16.70[c] | 23.20[a] |
| | 100 | 20.00[b] | 15.70[c] | 23.20[a] |
| | 200 | 19.80[b] | 16.40[c] | 21.40[a] |

[a]Lettuce characteristics in the same rows followed by same letter were not significantly different (Scott Knott, p≤5%). [b]CKM = chicken manure; [c]OCP = organic compost

compost may decrease lettuce production due to nutrients in excess. A possible cause for the small lettuce height when produced with OCP may be excess of boron in this fertilizer. Average concentration of B in lettuce leaves produced with OCP was 212 and 160 mg kg$^{-1}$ in limed and unlimed soil, respectively (Table 4). An increase of B adsorption is observed after raising the soil pH (Soares et al., 2008). Therefore, with the addition of lime, B was less available to plant uptake. Symptoms of B toxicity in lettuce produced with OCP, such as those described by Choi et al. (2006) were noticed. Adequate values for B in lettuce leaves were reported to be 30 to 60 mg kg$^{-1}$ (van Raij et al., 1997). Except for B, the content of other nutrients in leaves of plants produced with all studied fertilizers were in adequate proportions (van Raij et al., 1997).

In unlimed soil, increases in pH were noticed after CKM and OCP were applied (Table 5). Increases in pH were probably the main responsible for, except by lettuce height, overall better lettuce characteristics with CKM than with mineral fertilizers. Organic fertilizers naturally increased soil pH, and worked as a pH buffer, preventing pH changes in soil. However, when liming was applied, lettuce produced with mineral fertilizers was taller than that produced with organic fertilizers. Even though mineral fertilization caused soil pH to be lower than organic fertilization (Table 5), Knight and Mitchell (1983) found that optimum lettuce growth occurred at pH 5.9. The pH was, therefore, better adjusted for the recommended lettuce production with use of mineral fertilizers than with organic fertilizers.

Liming did not influence significantly lettuce height with organic fertilizers, except with OCP at 200 L SCN ha$^{-1}$ (Table 6). Soil conditioner, at 200 L ha$^{-1}$ may have enhanced boron toxicity, decreasing lettuce height when produced with OCP. In the unlimed soil, lettuce produced with mineral fertilizers was higher with increases in SCN doses according to the equation:

$y = 20.53 + 0.0112x$ ($R^2 = 0.86$; $P<0.05$).

Where y= lettuce height, and X = amount of SCN applied

Although the amount of organic carbon added to the soil by SCN was small, it may show its role as soil conditioner, improving conditions for plant growing due to its chemical characteristics (Table 2). Lettuce quality and yield was reported to increase with use of SCN even in small quantities (Valšíková and Viteková, 2006).

Circumference and shoot fresh commercial weight are the most important lettuce characteristics affecting consumer purchase decisions (Mota et al., 2001). Lettuce number of leaves, circumference, shoot fresh weight, and shoot commercial dry weight were higher when produced with CKM than with OCP or mineral fertilizers (Table 7). Content of P, Ca, and Mg in leaves were higher in lettuce produced with CKM than with other used fertilizers, and it may have accounted to increase lettuce growth (Table 4). These characteristics did not change with liming, when lettuce was produced with CKM, except the number of leaves, which were increased in unlimed soil. When lettuce was produced with OCP, liming increased all of these characteristics, except circumference, which was not affected statistically by the treatments. When lettuce was produced with mineral fertilizers, only shoot fresh weight increased with liming, the other characteristics remained the same statistically. Differences between

**Table 4.** Nutrient content in plants.

| Liming | N[(a)] | P | K | Ca | Mg | S |
|---|---|---|---|---|---|---|
| | | | g kg$^{-1}$ | | | |
| | | | **Chicken manure** | | | |
| Without | 19.77±5.83[(b)] | 4.99±0.54 | 11.99±4.02 | 10.03±2.27 | 2.46±0.40 | 2.19±0.30 |
| With | 20.00±3.87 | 4.20±0.34 | 11.32±1.97 | 8.36±1.40 | 2.29±0.34 | 2.16±0.18 |
| | | | **Organic compost** | | | |
| Without | 19.75±2.84 | 3.42±0,.29 | 11.12±3.07 | 7.27±1.33 | 1.73±0.30 | 1.27±0.42 |
| With | 21.36±3.90 | 3.64±0.24 | 10.77±1.36 | 7.69±0.86 | 1.88±0.22 | 1.62±0.19 |
| | | | **Mineral fertilization** | | | |
| Without | 31.80±5.87 | 3.24±0.26 | 10.81±2.07 | 7.00±1.14 | 0.83±0.10 | 2.80±0.21 |
| With | 24.56±3.19 | 3.21±0.28 | 11.05±2.77 | 7.08±1.12 | 1.69±0.22 | 2.71±0.18 |
| | B | Cu | Fe | Mn | Zn | |
| | | | mg kg$^{-1}$ | | | |
| | | | **Chicken manure** | | | |
| Without | 59.79±12.50 | 10.33±1.02 | 90.95±13.96 | 111.85±24.49 | 25.17±2.28 | |
| With | 58.13±16.37 | 9.86±0.63 | 91.20±12.48 | 74.70±24.77 | 24.15±4.29 | |
| | | | **Organic compost** | | | |
| Without | 212.68±53.47 | 8.09±0.54 | 85.57±18.18 | 66.66±11.19 | 61.81±11.39 | |
| With | 160.37±34.73 | 8.47±0.89 | 97.00±34.93 | 61.21±12.04 | 38.63±4.95 | |
| | | | **Mineral fertilization** | | | |
| Without | 53.47±6.92 | 9.34±0.78 | 85.45±11.20 | 72.75±22.27 | 68.41±14.13 | |
| With | 53.02±10.35 | 8.58±0.64 | 89.38±16.40 | 59.33±18.44 | 50.42±9.13 | |

[a]EMBRAPA (1999); N-total; [b]mean ± standard error.

**Table 5.** Soil pH as a function of fertilizers within liming versus doses of soil conditioner (SCN).

| Liming | Dose of SCN (L ha$^{-1}$) | Fertilization | | |
|---|---|---|---|---|
| | | CKM[a] | OCP[b] | Mineral |
| | | pH | | |
| Without | 0 | 6.7 | 6.1 | 5.2 |
| | 20 | 6.3 | 6.0 | 5.2 |
| | 40 | 6.7 | 6.1 | 5.2 |
| | 100 | 6.6 | 6.2 | 5.2 |
| | 200 | 6.4 | 6.0 | 5.2 |
| With | 0 | 6.5 | 6.0 | 5.5 |
| | 20 | 6.7 | 6.1 | 5.5 |
| | 40 | 6.8 | 6.3 | 5.6 |
| | 100 | 6.8 | 6.3 | 5.4 |
| | 200 | 6.8 | 6.2 | 5.9 |

[a]CKM = chicken manure; [b]OCP = organic compost.

lettuce characteristics, in general, despite the use of lime, were very small within fertilizers (Table 7).

Ali et al. (2007) reported that lettuce weight may be lower when there is low N availability, however, in the present experiment, N amount was, theorically, leveled to all treatments. Then, organic matter mineralization is the only way N availability could influence lettuce growth. Shoot dry weight of lettuce was the same when produced

**Table 6.** Lettuce heights as a function of liming within fertilizers versus doses of soil conditioner (SCN).

| Fertilization | Doses of SCN (L ha$^{-1}$) | Height (g) [a] | |
|---|---|---|---|
| | | Unlimed | Limed |
| CKM[b] | 0 | 20.00 [a] | 20.20 [a] |
| | 20 | 20.50 [a] | 19.50 [a] |
| | 40 | 20.10 [a] | 20.80 [a] |
| | 100 | 20.60 [a] | 20.00 [a] |
| | 200 | 20.50 [a] | 19.80 [a] |
| OCP[c] | 0 | 15.60 [a] | 16.40 [a] |
| | 20 | 15.20 [a] | 16.60 [a] |
| | 40 | 15.20 [a] | 16.70 [a] |
| | 100 | 15.30 [a] | 15.70 [a] |
| | 200 | 14.00 [b] | 16.40 [a] |
| Mineral | 0 | 20.00 [b] | 22.30 [a] |
| | 20 | 21.10 [b] | 22.90 [a] |
| | 40 | 21.30 [b] | 23.20 [a] |
| | 100 | 21.60 [b] | 23.20 [a] |
| | 200 | 22.70 [a] | 21.40 [a] |

[a]Lettuce characteristics in the same rows followed by same letter were not significantly different (Scott Knott, p≤5%). [b]CKM = chicken manure; [c]OCP = organic compost.

**Table 7.** Number of leaves, circumference, shoot fresh weight, and shoot commercial dry weight as a function of fertilizers and liming.

| Liming | CKM[b] | OCP[c] | Mineral |
|---|---|---|---|
| | | **Number of leaves[a]** | |
| Without | 31.52 [Aa] | 24.88 C[b] | 27.04 [Ba] |
| With | 30.04 [Ab] | 26.40 [Ba] | 27.04 [Ba] |
| | | **Circumference (cm)** | |
| Without | 40.77 [Aa] | 28.56 [Ca] | 32.48 [Ba] |
| With | 39.08 [Aa] | 30.59 [Ca] | 33.92 [Ba] |
| | | **Shoot fresh weight (g)** | |
| Without | 480.65 [Aa] | 165.58 [Cb] | 325.36 [Bb] |
| With | 475.44 [Aa] | 194.68 [Ca] | 359.23 [Ba] |
| | | **Shoot commercial dry weight (g)** | |
| Without | 15.22 [Aa] | 6.00 [Cb] | 12.34 [Ba] |
| With | 16.29 [Aa] | 7.31 [Ca] | 11.95 [Ba] |

[a]Lettuce characteristics in the same rows followed by same upper case letter, and the value in the same columns followed by same lower case letter were not significantly different (Scott Knott, p≤5%). [b]CKM = chicken manure; [c]OCP = organic compost.

with CKM and mineral fertilizers (Table 8). However, shoot commercial fresh weight, important lettuce characteristic which attract consumers, was higher when produced with CKM than with mineral fertilizers. This difference, between dry and fresh weight for lettuce is the water content. Malathi et al. (2005) showed that in cowpea

**Table 8.** Shoot dry weight and commercial fresh weight shoot as a function of fertilizers.

| Fertilization[(a)] | Shoot dry weight (g) | Shoot commercial fresh weight (g) |
|---|---|---|
| CKM | 23.86[a] | 288.96[a] |
| OCP | 14.59[b] | 079.52[c] |
| Mineral | 24.72[a] | 157.61[b] |

[a]Lettuce characteristics in the same columns followed by same letter were not significantly different (Scott Knott, p≤5%). [§]CKM = chicken manure; OCP = organic compost.

cowpea plants with increased levels of calcium in tissue, the water status was higher than in plants with low levels of Ca in tissue, while the rate of water loss after harvesting was lower in plants with higher levels of Ca in tissue. Calcium content in lettuce produced with CKM was higher than with mineral fertilizers (Table 7). Adequate values for Ca in lettuce leaves are from 15 to 25 g $kg^{-1}$ (van Raij et al., 1997). These levels are above those found in the present work for lettuce grown with all kinds of fertilizers. For lettuce produced with OCP, the weight for both characteristics was smaller than that produced with CKM and mineral fertilizers.

## Conclusions

(1) Lettuce growth characteristics were modified according to the soil management. When lime was applied, treatments with mineral fertilization produced higher lettuce plants than those produced with organic fertilizers. Lettuce height was also affected by the highest dose of SCN in unlimed soil.
(2) Lettuce number of leaves, circumference, shoot fresh weight, and shoot commercial fresh and dry weight, which are important characteristics for consumers, were higher when produced with CKM than with OCP or mineral fertilizers. Probably the increase in these characteristics were reflex of a rise in soil pH and in the higher amount of P added initially with the CKM than with the other treatments. Higher soil pH and P content probably led to an increase in the P, Ca, and Mg content in leaves of lettuce produced with CKM.

## Conflict of Interest

The authors have not declared any conflict of interest.

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# Tolerance of bio-fertilized *Delonix regia* seedlings to irrigation intervals

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**This work aims to investigate the effect of different bio-fertilizers (*Arbascular mycorrhizae* fungi, *Azotobacter chroorcoccum*, yeast strains and mixture of all inoculum) and irrigation intervals (3, 6 or 12 days) on the growth and chemical composition of *Delonix regia* seedlings grown in sandy soil. Pot experiments were conducted using a randomized complete blocks design with three replicates during two successive seasons of 2013 and 2014. The results indicated that dual bio-fertilizers led to significant increase in growth characters (plant height, root length, number of branches/plant, total fresh and dry weights/plant), microbial populations and AM fungi colonization (%), enzymatic activities, chemical composition (plant pigments, total carbohydrates, proline content, N, P, K) besides antioxidant enzymes such as catalase (CAT),and peroxidase (POD) compared to the un-inoculated seedlings (as control) at the recommended dose of NPK chemical fertilizers under the same conditions. Generally, these results undoubtedly confirm that dual bio-fertilizers could replace the use of chemical fertilizers and consequently improve the quality and quantity of *D. regia*.**

**Key words:** *Delonix regia*, *Arbascular mycorrhizae* fungi, *Azotobacter chroorcoccum*, yeast strains - growth characters, chemical composition.

## INTRODUCTION

Lack of fresh water resources for agriculture is the most important problem facing many countries in arid and semi-arid regions of Africa, such as Egypt. Thus there is a need to look for alternative methods to balance sustenance with demand (Wolters et al., 2013).

Using drought-tolerant trees in dry regions is one of many ways used to solve this problem. *Delonix regia* is one of the most important and common drought tree species in Egypt which also tolerates a wide variety of soils and conditions but needs to be well-watered until it gets established. The genus name is derived from the Greek words delos (meaning conspicuous) and onyx (meaning claw) referring to the appearance of the spectacular flowers. The tree is commonly cultivated in the tropics and subtropics, including Madagascar (Menninger, 1962). It plays a key role in regulating

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climate, resisting wind, sand, conserving water and soil (Du Puy et al., 2002). It is an ornamental tree found in streets and parks. It is fast-growing and develops an umbrella-shaped crown, making it a valuable shade tree. The wood is widely used as firewood and for making fence posts. It has an antioxidant potential (Auudy et al., 2003) and its seeds contain gum that may be used in food and textile industries. Its dried seeds can also be used as a binder in the manufacturing of tablets (e.g. Paracetamol). Its bark has medicinal properties (Little and Wadsworth, 1964; Webb et al., 1984).

Numerous studies have found that plants have mechanisms to cope with drought stress; they will become more tolerant to drought when associated with different soil microorganisms (Soliman, 2008; Aroca and Ruiz-Lozano, 2009). Beneficial microorganisms are a tool that enhances yield, plant growth and nutrient uptake under various environmental conditions such as salinity, drought and low fertility supply. Some endomycorrhizal fungi (*Arbuscular mycorrhizal* fungi) have been proven to improve drought stress; they colonize bio-trophically the root cortex and develop an extra-metrical mycelium that helps the plants to acquire mineral nutrients from the soil particularly those, which are immobile. They can under drought conditions stimulate growth-regulating substances, increase photosynthesis, improve osmotic adjustment, optimize hormonal balance and enhance water uptake (Colla et al., 2007).

Some yeast species (*Saccharomyces cerevisiae* and *Rhodotorula mucilaginosa*) and *Azotobacter* spp. have evolved different strategies to adapt with the changes in their environment. They can combat high osmolarity by enhancing transcription (Treuner-lange et al., 1997) or by the presence of some stress enzymes like catalases and peroxidases or organic and inorganic compounds.

Therefore, this study aims to investigate the effect of specific bio-fertilizers on increasing the drought resistance of *D. regia* seedlings under irrigation intervals.

## MATERIALS AND METHODS

The present study was carried out at the Experimental Laboratories of the Natural Resources Department, Institute of African Research and Studies, Cairo University, and at the Microbiology Department, Soils, Water and Environment Institute (SWERI), Agricultural Research Center, Giza, Egypt, during the two successive seasons of 2013 and 2014.

### Plant material

Seeds of *D. regia* (Bojer ex Hook.) Raf. were obtained from the Faculty of Agriculture, Giza, Egypt. They were soaked in hot water (90°C) for 10 s followed by 24-h imbibitions, to accelerate germination (Millat and Mustafa, 1989). On the first of June, the seeds were then sown in 8-cm plastic pots filled with sandy soil. After two weeks, in both seasons, the seedlings (15 cm tall) were transplanted into plastic pots (30 cm diameter) filled with 6 kg of the same sandy soil. The physical and chemical characteristics of the soil are shown.

### Soil analysis

The soil texture was sandy having the following characteristics: Coarse sand, 30.82%; fine sand, 62.61; silt, 1.22%; clay, 5.35%; pH, 7.75; EC, 1.15 ds/m; organic matter, 0.08%; available N, 6.9 (ppm); available P, 6.2 (ppm); available K, 64 (ppm); $CaCO_3$, 0.26%; and water holding capacity, 14.5%.

### Treatments

At the beginning of the experimental, pots were supplied with recommended dose of NPK at a rate of 2.1 g/pot ammonium sulphate (20.5%) as nitrogen. Phosphorus was added as superphosphate (15.5%) at a rate of 1.2 g/pot and potassium was added as potassium sulphate at a rate of 0.3 g/pot. The seedlings were divided into seven treatments. The first one was un-inoculated control plants whereas the second treatment received only full dose of NPK after one month of each season. In the third treatment, the plants inoculated with mixed spores of AMF from genera (*Glomus*, *Gigaspora*and *Acaulospora*) (500 spores/g) at a rate of 3 g/hole, where spores dressed in a hole around the rhizosphere were attached to secondary roots (Massoud et al., 2009). Once the mycorrhizal symbiosis was established, *A. chroococcum*as a fourth treatment was prepared by growing on modified Ashby's medium $10^8$ CFU/ml for 5 days (Abdel Malak and Ishac, 1968); whereas yeast strains as fifth and sixth treatments (*S. cerevisiae* and *R. mucilaginosa*) were also incubated at 28°C on rotary shaker at 150 rpm for 48 h in conical flasks (250 ml) containing 100 ml of glucose peptone yeast (GPY) medium (Difco, 1985). Then both were individually applied monthly at a rate of 5 ml/ pot. In addition, the mixture of previous inoculums (AMF, *A. chroococcum*, yeast strains) as seventh treatment was inoculated; it was isolated from very dry soil located at Tushka Valley Region, where there are great variations between the temperatures at day and night in winter and summer.

### Irrigation intervals

The plants were irrigated every 3, 6, or 12 days. At each irrigation, the plants were watered till 100% of field capacity (F.C.). The soil moisture tension was measured before each irrigation using micro tension meters, and the quantity of water needed to reach 100% F.C. was calculated, as described by Richards (1949).

### Experimental design

This experiment was factorial, conducted using a randomized complete blocks design with three replicates. The study included 21 treatments [7 treatments×3irrigation intervals], with each block consisting of 105 plants (5 plants/treatment).The treatments were applied regularly until the termination of each season (1$^{st}$ September in both seasons).

### Growth parameters

Plant height, root length, number of branches/plant, as well as total fresh and dry weights /plant were recorded.

### Microbial populations and AM fungi colonization

The population dynamics of total bacterial and yeast count in the rhizospheric zone of *D. regia* roots was determined by the plate count technique according to Reinhold et al. (1985). While

*Azotobacter* spp. population counts in the rhizospheric zone of *D. regia* roots were determined using the most probable number (CFU/g rhizosphere) method described by Cochran (1950).

The percentage of AM fungi colonization in plant root tissues was also determined as described by Phillips and Hayman (1970).

#### Enzymatic activities determinations

Nitrogenase activity ($N_2$-ase) in rhizosphere (roots) was measured as described by Somasegaran and Hoben (1994). The dehydrogenase activity was also estimated according to Skujins and Burns (1976).

#### Photosynthetic pigments, total carbohydrates and proline determinations

In addition, chemical analysis of fresh leaves samples was conducted to determine total chlorophyll (a+b) and carotenoids contents, using the method described by Nornai (1982). The content of total carbohydrates in dried leaves samples was determined using the method described by Dubois et al. (1956). The proline content in fresh leaves was also determined according to Bates et.al. (1973).

#### Determination of elements

Dried leaves samples were digested to extract nutrients as described by Piper (1950), and the extract was analyzed to determine its contents of nitrogen (using the modified micro-Kjeldahl method as described by Pregl (1945), phosphorus according to Jackson (1967) and potassium estimated photometrically using a Jenway flame photometer, according to Chapman and Pratt (1961).

#### Activities of antioxidant enzymes

Preparation of the enzymes extraction of leaves tissues was carried out at 40°C at 3:1 buffer: fresh weight (v/v) in a pastel and mortar with 100 mM potassium phosphate buffer (at pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol and 5% (w/v) insoluble polyvinyl pyrolidone. The homogenates were centrifuged at 10000 rpm for 30 min and then the supernatants were stored in separate aliquots at 8°C (Vitoria et al., 2001). Antioxidant enzymes were assayed as follows; Catalase (CAT) by measuring the decrease in absorbance due to disappearance of $H_2O_2$at 240 nm according to Chance and Maely (1955), Peroxidase (POD) by spectrophotometry according to Amako et al. (1994). Enzymes activities were expressed as units/min/mg protein.

All the obtained data were subjected to statistical analysis of variance, and the means were compared using the "Least Significant Difference (L.S.D.)" test at the 5% level, as described by Little and Hills (1978).

## RESULTS AND DISCUSSION

### Growth parameters

The obtained results revealed that, the prolonged irrigation intervals had an adverse effect on the growth of *D. regia* plants, regardless of the effect of inoculation treatments (Table 1). In both seasons, prolonging irrigation intervals (12 days) steadily resulted in significant reduction in the values recorded for all of the growth parameters (plant height, root length, number of branches/plant, as well as total fresh and dry weights /plant). However, prolonged irrigation intervals from 3 to 6 days caused only a slight (insignificant) reduction in the mean values recorded for some studied growth parameters, whereas longer irrigation intervals (12 days) resulted in significant reduction in the values recorded for all of the vegetative characteristics.

It can be concluded that the reduction in the growth may be attributed to the participation of water in the cell division, cell expansion and cell enlargement. In addition, water stress reduction causes a decrease in transport of cytokinin from root to shoot and/or increase in Abscisic acid in leaf; these changes in the balance of hormones cause change in the extensibility in cell wall and these affect generally growth enlargement (Siddique et al., 2000; Ouma, 2005; Luvaha, 2005). This result has also been confirmed by Oyun et al. (2010) on *Acacia senegal*, and Liu et al. (2011) on apple.

The growth parameters of *D. regia* under different treatments as shown in Table 1 undoubtedly revealed that seedlings inoculated with dual bio-fertilizers significantly showed higher values in growth parameters compared to un-inoculated (control) plants at the recommended dose of NPK under the same conditions, in both seasons. Total fresh weight/plant was increased by 60.01 and 59.24% in the first and second seasons, respectively, over control plants.

These results provide a plausible mechanism of how the dual bio-fertilizers led to increase in growth parameters in the control. This rise of their beneficial effects on seedlings represented in tolerance to drought produces some growth promoting substances (Gibberellins, IAA and Abscisic acid, etc.), and vitamins which have favorable effects on root development (Alexander, 1977; Dobbelaere et al., 2003). Hyphae produced by AM fungi, which are microscopic tubes, colonize plant roots and grow out into the soil further than root hairs. The hyphae help in retaining moisture around the root zone of plants, and also increase nutrients uptake to the plant (especially diffusion of limited nutrient like P). Moreover, yeast contents of micro and macronutrients stimulate the plant to build up dry. In addition, they reduce diseases caused by root pathogens matter (Morte et al., 2001; Ortas et al., 2002; Hesham and Mohamed, 2011). Supportive evidence for this view was reported by Ibrahim (2009) on Flame seedless and Superior grapevines and Ibrahim et al. (2010) on Balady guava trees.

### Microbial populations, mycorrhizal colonization (%), and enzyme activities

Data presented in Figures 1 and 2 also revealed that prolonged irrigation intervals had an adverse effect on the mean number of microbial populations (total microbial,

**Table 1.** Influence of bio-inoculants and irrigation intervals on the growth parameters of *D. regia* during 2013 and 2014.

| Treatment (T) | Irrigation intervals (I) | Growth parameters | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Plant height (cm) | | Root length (cm) | | Stem diameters (cm) | | Number of branches/plant | | Total fresh weight (g/ plant) | | Total dry weight (g/plant) | |
| | | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd |
| **Control** | | 46.20 | 42.00 | 54.00 | 50.67 | 1.00 | 0.70 | 8.00 | 5.00 | 63.33 | 58.83 | 29.67 | 26.42 |
| Full NPK | | 86.33 | 84.23 | 88.00 | 83.00 | 1.40 | 1.10 | 9.33 | 7.00 | 95.26 | 89.98 | 45.30 | 43.99 |
| AMF | | 100.27 | 99.00 | 106.67 | 101.33 | 2.10 | 1.70 | 14.33 | 13.67 | 103.89 | 97.83 | 50.28 | 47.92 |
| *Azotobacter chroococcum* | 3 Days | 91.17 | 88.00 | 95.00 | 92.33 | 1.70 | 1.30 | 11.33 | 9.00 | 97.40 | 91.39 | 47.04 | 44.03 |
| *Rhodotorula mucilaginosa* | | 83.10 | 79.00 | 83.00 | 80.00 | 1.30 | 1.05 | 8.33 | 7.00 | 91.19 | 86.72 | 44.60 | 41.03 |
| *Saccharomyces cerevisiae* | | 95.00 | 91.73 | 102.33 | 100.00 | 1.90 | 1.40 | 12.00 | 10.67 | 99.61 | 94.35 | 48.81 | 45.84 |
| Mixture of all | | 108.50 | 42.00 | 109.33 | 105.67 | 2.40 | 2.00 | 16.33 | 14.33 | 105.53 | 99.30 | 51.43 | 49.65 |
| **Control** | | 87.22 | 84.15 | 48.33 | 42.33 | 0.80 | 0.60 | 7.00 | 3.67 | 59.04 | 51.63 | 26.19 | 23.48 |
| Full NPK | | 40.33 | 38.00 | 70.67 | 66.33 | 1.23 | 0.80 | 8.00 | 5.00 | 82.07 | 72.65 | 39.04 | 34.33 |
| AMF | | 78.00 | 73.33 | 91.33 | 90.00 | 1.90 | 1.40 | 12.67 | 10.33 | 97.65 | 88.02 | 46.16 | 41.36 |
| *A. chroococcum* | 6 Days | 97.80 | 95.00 | 83.67 | 81.67 | 1.60 | 1.10 | 9.67 | 6.67 | 90.55 | 80.59 | 43.61 | 37.63 |
| *R. mucilaginosa* | | 85.00 | 82.13 | 74.33 | 70.00 | 1.40 | 1.00 | 8.33 | 5.33 | 87.26 | 75.80 | 41.97 | 35.90 |
| *S. cerevisiae* | | 80.67 | 77.00 | 88.00 | 85.00 | 1.70 | 1.30 | 11.33 | 8.00 | 94.75 | 83.85 | 45.71 | 40.89 |
| Mixture of all | | 89.00 | 95.00 | 95.00 | 92.67 | 2.00 | 1.60 | 15.33 | 12.00 | 98.35 | 90.55 | 47.51 | 43.28 |
| **Control** | | 29.40 | 26.8 | 33.00 | 29.33 | 0.60 | 0.30 | 4.67 | 2.33 | 39.76 | 37.46 | 16.88 | 14.73 |
| Full NPK | | 59.67 | 54.13 | 67.33 | 64.00 | 0.80 | 0.60 | 5.33 | 3.67 | 74.35 | 67.64 | 35.18 | 29.82 |
| AMF | | 82.00 | 80.67 | 86.33 | 80.00 | 1.25 | 1.14 | 10.67 | 8.00 | 91.56 | 85.80 | 45.11 | 40.57 |
| *A. chroococcum* | 12 Days | 71.00 | 66.50 | 75.00 | 73.67 | 1.09 | 1.03 | 7.33 | 5.33 | 85.80 | 77.63 | 40.23 | 35.82 |
| *R. mucilaginosa* | | 66.17 | 61.80 | 70.00 | 68.33 | 1.00 | 0.90 | 6.00 | 4.00 | 80.25 | 72.12 | 36.79 | 34.06 |
| *S. cerevisiae* | | 74.90 | 68.73 | 82.33 | 76.67 | 1.19 | 1.10 | 9.00 | 6.67 | 88.90 | 81.47 | 42.12 | 39.74 |
| Mixture of all | | 89.10 | 86.17 | 89.33 | 84.33 | 1.50 | 1.20 | 11.67 | 9.33 | 93.92 | 86.87 | 45.63 | 42.10 |
| **LSD (0.05)** | | | | | | | | | | | | | |
| I | | 2.30 | 2.00 | 5.82 | 3.77 | 0.32 | 0.18 | 2.40 | 0.94 | 5.05 | 5.53 | 2.91 | 4.24 |
| T | | 2.49 | 2.60 | 6.63 | 7.48 | 0.24 | 0.24 | 0.87 | 0.74 | 5.67 | 9.36 | 2.99 | 4.80 |
| I X T | | 4.32 | 4.50 | 11.48 | 12.96 | 0.42 | 0.41 | 1.51 | 1.28 | 9.82 | 16.22 | 5.19 | 8.32 |

*Azotobacter* spp. and yeasts count), mycorrhizal colonization (%), as well as enzyme activities (nitogenase and dehydrogenase activities), regardless of the effect of inoculation treatments. In both seasons, prolonged irrigation intervals steadily reduced the values recorded for these parameters. This reduction can be attributed to the role of water in enhancing the microbial activities (Ouma, 2007). Preceding results are in harmony with those obtained by Soliman (2008) on *Acacia nilotica*, who reported that the nitrogenase and dehydrogenase activities were decreased with

prolonged irrigation intervals.

In general, microbial population, mycorrhizal colonization (%) and enzyme activities in the rhizosphere of *D. regia* were significantly affected by dual bio-fertilizers, as compared to the control which had the highest microbial populations, mycorrhizal colonization (%), as well as enzyme activities (Figures 1 and 2). Many studies have shown that the power of dual bio-fertilizers is due to their production of secondary metabolites that are essential for the growth of almost all the microorganisms, Nitrogenase and other proteins involved in nitrogen fixation (Brill, 1980; Muthuselvan and Balagurunathan, 2013). In addition, AMF development could be enhanced by supplying yeast vitamin B12, as it stimulates it (Boby et al., 2008). Also, AM fungi stimulate the activity of beneficial soil microorganisms (Boby and Bagyaraj, 2003) and root exudation is modified both qualitatively and quantitatively by *A. mycorrhizal* symbiosis. This leads to increase in mycorrhizal infection (Garg and Manchanda, 2009). Supportive evidence for this view was reported by Harisudan et al. (2010).

## Chemical composition

### *Total chlorophyll and carotenoids content*

Data recorded in the two seasons (Table 2) revealed that prolonged irrigation intervals had an adverse effect on the total chlorophyll and carotenoids contents in the leaves of *D. regia* plants, regardless of the effect of inoculation treatments. In both seasons, the contents of total chlorophyll and carotenoids were reduced steadily as the irrigation intervals were prolonged daily to 6 or 12 days. Drought stress causes reduction of the $CO_2$ concentration in leaf internally. This is a result of stomata closure, changes in chlorophyll content, chlorophyll components and damage of the photosynthetic apparatus. All these led to the reduction rates of leaf photosynthetically. Also, producing reactive oxygen species (ROS) such as $O_2$- and $H_2O_2$ can lead to lipid peroxidation and consequently, chlorophyll destruction (Foyer et al., 1994; Iturbe Ormaetxe et al., 1998; Lawlor and Cornic 2002; Flexas et al., 2004). Similar reductions in the chlorophylls content were reported by Mafakheri et al (2010), on *Cicer arietinum* and Arjenaki et al (2012) on *Triticum aestivum*.

Data presented in Table 2 also revealed that in both seasons, the total chlorophyll and carotenoids contents were affected by dual bio-fertilizers, as compared to the control plants. Plants inoculated with the dual bio-fertilizers had the highest total chlorophyll and carotenoids contents, followed by plants inoculated with AMF, then un-inoculated plants, in both seasons. These augmentations in the total chlorophyll and carotenoids content were in the favor of control plants which recorded 69.78 and 66.88%, respectively.

Similar results have been reported by El-Khateeb et al. (2011) who stated that chlorophyll and carotenoids content in *Acacia saligna* were improved by inoculation with A. mycorrhizal fungi under water stress; also, Mazhar et al. (2010) on *Jatropha curcas*. The promotion of the synthesis and accumulation of chlorophyll may be attributed to the dual inoculation of AM fungi with other beneficial microorganisms that enhance mineral nutrition such as N, which is an essential component in the structure of porphyrines, which are found in many metabolic active compounds, including chlorophylls. And also, the role of cytokine yeast that delays the aging of leaves. It does this by reducing the degradation of chlorophyll, leading to increase in chlorophyll content. Thus, it helps in higher photosynthetic rate (Castelfranco and Beale, 1983; Feng et al., 2002; Boby et al., 2008).

### *Total carbohydrates percentage and proline content*

The data in Table 2 also showed that, in both seasons, prolonged irrigation intervals steadily increased the total carbohydrates percentage and proline content. These increments were 38.10 and 41.18%, respectively over control plants.

This behavior may be attributed to a reduction of carbohydrates translocation from leaves to other plant parts under drought conditions and/or the lesser consumption of carbohydrates in the leaves (El-Khateeb et al., 1991). Also, Hoekstra et al. (2001) mentioned that a high carbohydrate concentration decreases water potential, contributes in preventing oxidative damage, and maintains the structure of proteins and membranes under moderate dehydration during drought period.

The increase in the proline content of plants irrigated at long intervals is in agreement with the findings of El-Quesni et al. (2012) who reported that the proline content in leaves, stems and roots of *Matthiola incana* significantly increased as a result of decreased soil moisture level. This confirms that proline can biochemically adapt to stress condition.

The mean total carbohydrates percentage and proline content in leaves of *D. regia* were affected by dual bio-fertilizers (Table 2). In both seasons, plants inoculated with dual bio-fertilizers had the highest total carbohydrates percentage and proline content, followedby plants inoculated with AMF, *S. cerevisiae*, *A. chroococcum* and *R. mucilaginosa*, full NPK, and then un-inoculated plants. The favorable effect of biofertilization on the synthesis and accumulation of carbohydrates and proline may be attributed to the increase in the chlorophylls content of inoculated plants, and to the role played by nitrogen in the structure of porphyrine molecules (as previously mentioned), which are found in the cytochrome enzymes essential in photo-synthesis. This increase in the contents of chlorophylls and cytochrome enzymes results in an increase in the rate of photosynthesis, and a promotion in carbohydrate synthesis and accumulation

**Table 2.** Influence of bio-inoculants and irrigation intervals on the chemical analysis of *D. regia* during 2013 and 2014.

| Treatment (T) | Irrigation intervals (I) | Chemical analysis | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total chlorophylls (a+b) content (mg/g fresh matter) | | Carotenoids content (mg/g fresh matter) | | Total carbohydrates (% of dry matter) | | Proline content (µ moles/g fresh matter) | | N (% dry matter) | | P (% dry matter) | | K (% dry matter) | |
| | | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd | 1st | 2nd |
| **Control** | | 1.15 | 1.09 | 0.55 | 0.52 | 9.00 | 7.00 | 11.00 | 10.00 | 1.49 | 1.40 | 0.23 | 0.20 | 1.40 | 1.37 |
| Full NPK | | 1.20 | 1.18 | 0.58 | 0.59 | 11.00 | 8.00 | 13.00 | 11.00 | 1.60 | 1.58 | 0.27 | 0.25 | 1.41 | 1.39 |
| AMF | | 1.65 | 1.49 | 0.81 | 0.73 | 21.00 | 17.00 | 25.00 | 21.00 | 2.49 | 2.41 | 0.49 | 0.43 | 2.31 | 2.09 |
| *A. chroococcum* | **3 Days** | 1.58 | 1.45 | 0.78 | 0.70 | 18.00 | 12.00 | 20.00 | 17.00 | 2.33 | 2.31 | 0.35 | 0.33 | 2.00 | 1.88 |
| *R. mucilaginosa* | | 1.43 | 1.37 | 0.69 | 0.65 | 15.00 | 10.00 | 17.00 | 15.00 | 1.90 | 1.85 | 0.28 | 0.27 | 1.55 | 1.52 |
| *S. cerevisiae* | | 1.60 | 1.46 | 0.80 | 0.72 | 20.00 | 14.00 | 23.00 | 19.00 | 2.46 | 2.36 | 0.39 | 0.38 | 2.10 | 1.95 |
| Mixture of all | | 1.68 | 1.53 | 0.83 | 0.77 | 23.00 | 19.00 | 28.00 | 23.00 | 2.66 | 2.56 | 0.50 | 0.47 | 2.44 | 2.17 |
| **Control** | | 0.97 | 0.85 | 0.46 | 0.41 | 12.00 | 8.00 | 15.00 | 11.00 | 1.42 | 1.36 | 0.19 | 0.16 | 1.38 | 1.33 |
| Full NPK | | 1.05 | 0.99 | 0.51 | 0.49 | 13.00 | 10.00 | 18.00 | 15.00 | 1.58 | 1.55 | 0.25 | 0.23 | 1.40 | 1.36 |
| AMF | | 1.50 | 1.35 | 0.74 | 0.65 | 24.00 | 20.00 | 31.00 | 26.00 | 2.34 | 2.34 | 0.35 | 0.34 | 1.70 | 1.64 |
| *A. chroococcum* | **6 Days** | 1.46 | 1.30 | 0.73 | 0.63 | 19.00 | 17.00. | 26.00 | 23.00 | 2.27 | 2.18 | 0.29 | 0.28 | 1.63 | 1.60 |
| *R. mucilaginosa* | | 1.35 | 1.28 | 0.65 | 0.61 | 15.00 | 12.00 | 21.00 | 17.00 | 1.82 | 1.77 | 0.23 | 0.21 | 1.31 | 1.29 |
| *S. cerevisiae* | | 1.49 | 1.33 | 0.72 | 0.65 | 21.00 | 19.00 | 30.00 | 24.00 | 2.30 | 2.26 | 0.33 | 0.31 | 1.66 | 1.61 |
| Mixture of all | | 1.54 | 1.36 | 0.77 | 0.68 | 27.00 | 23.00 | 33.00 | 29.00 | 2.57 | 2.51 | 0.40 | 0.38 | 1.75 | 1.70 |
| **Control** | | 0.83 | 0.70 | 0.38 | 0.33 | 15.00 | 13.00 | 22.00 | 16.00 | 1.34 | 1.31 | 0.14 | 0.11 | 1.27 | 1.25 |
| Full NPK | | 0.89 | 0.73 | 0.43 | 0.35 | 18.00 | 15.00 | 29.00 | 25.00 | 1.44 | 1.37 | 0.20 | 0.16 | 1.35 | 1.27 |
| AMF | | 1.34 | 1.19 | 0.66 | 0.57 | 32.00 | 25.00 | 44.00 | 43.00 | 2.18 | 2.10 | 0.28 | 0.27 | 1.53 | 1.45 |
| *A. chroococcum* | **12 Days** | 1.28 | 1.15 | 0.64 | 0.56 | 27.00 | 21.00 | 40.00 | 38.00 | 2.00 | 1.94 | 0.25 | 0.23 | 1.49 | 1.33 |
| *R. mucilaginosa* | | 1.18 | 1.10 | 0.58 | 0.53 | 21.00 | 17.00 | 36.00 | 31.00 | 1.64 | 1.58 | 0.22 | 0.21 | 1.38 | 1.31 |
| *S. cerevisiae* | | 1.31 | 1.17 | 0.65 | 0.56 | 31.00 | 23.00 | 41.00 | 40.00 | 2.17 | 2.07 | 0.27 | 0.25 | 1.51 | 1.42 |
| Mixture of all | | 1.39 | 1.20 | 0.68 | 0.59 | 34.00 | 29.00 | 49.00 | 45.00 | 2.21 | 2.16 | 0.31 | 0.29 | 1.59 | 1.47 |
| **LSD (0.05)** | | | | | | | | | | | | | | | |
| **I** | | 0.18 | 0.03 | 0.09 | 0.01 | 6.99 | 5.94 | 5.84 | 6.07 | 0.54 | 0.94 | 0.05 | 0.06 | 0.03 | 0.05 |
| **T** | | 0.16 | 0.19 | 0.08 | 0.09 | 5.96 | 3.96 | 8.52 | 6.28 | 0.98 | 0.93 | 0.07 | 0.06 | 0.33 | 0.09 |
| **I X T** | | 0.28 | 0.33 | 0.14 | 0.16 | 10.32 | 6.85 | 14.75 | 10.87 | 1.69 | 1.62 | 0.12 | 0.11 | 0.57 | 0.15 |

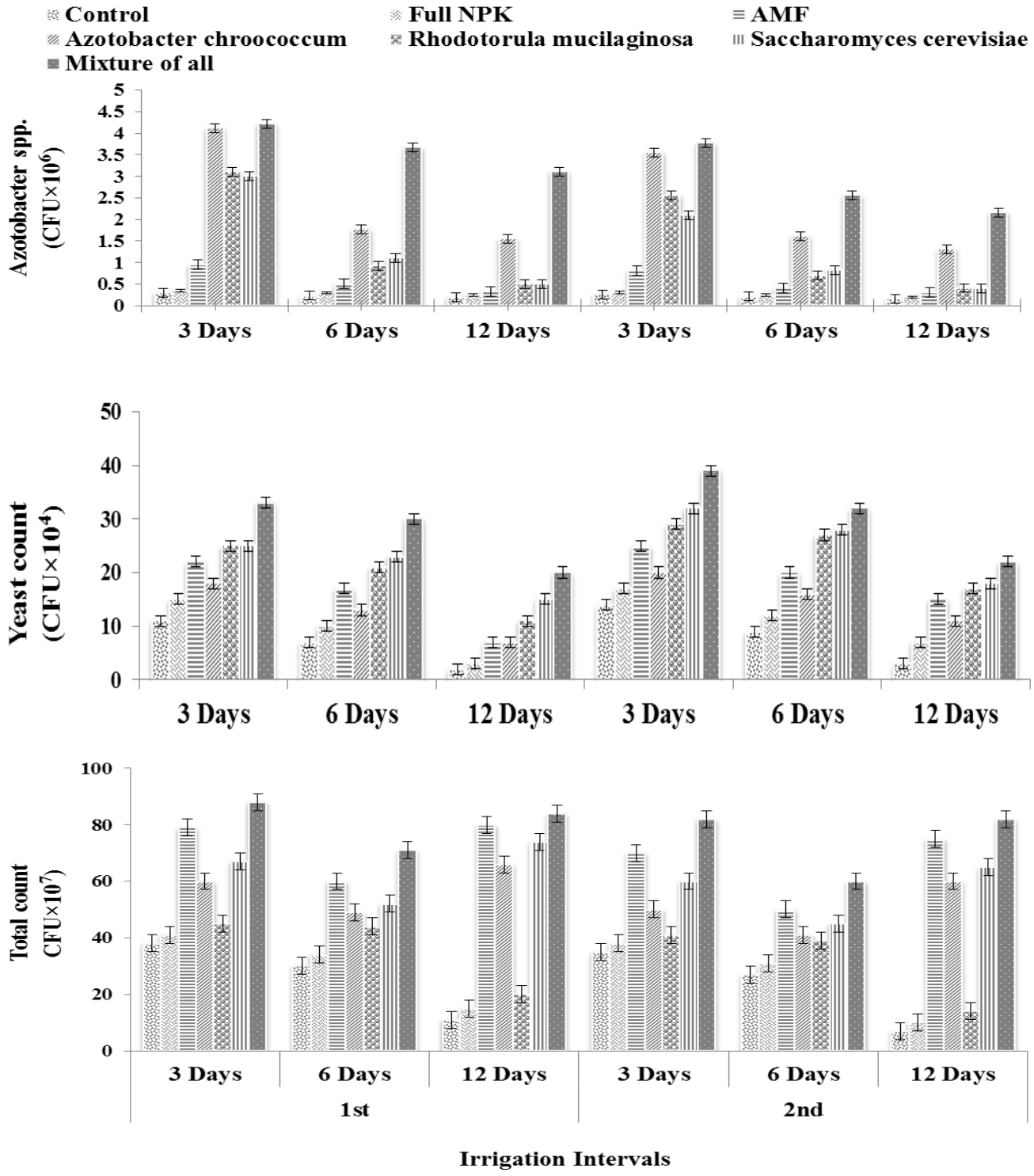


**Figure 1.** Effect of biofertilization and irrigation intervals on microbial population in the rhizosphere of *D. regia*during 2013 and 2014.

(Devlin, 1975). The obtained results are in agreement with the findings of Khalid (2006) on *Ocimum americanum* and *O. basilicum* who found that the total carbohydrates increased when the plants were inoculated with myvorrhizal fungi under water stress.

***N, P and K (%)***

The results in Table 2 also showed that the N, P and K percentages decreased steadily with prolonged irrigation intervals. Accordingly, the lowest percentages of the

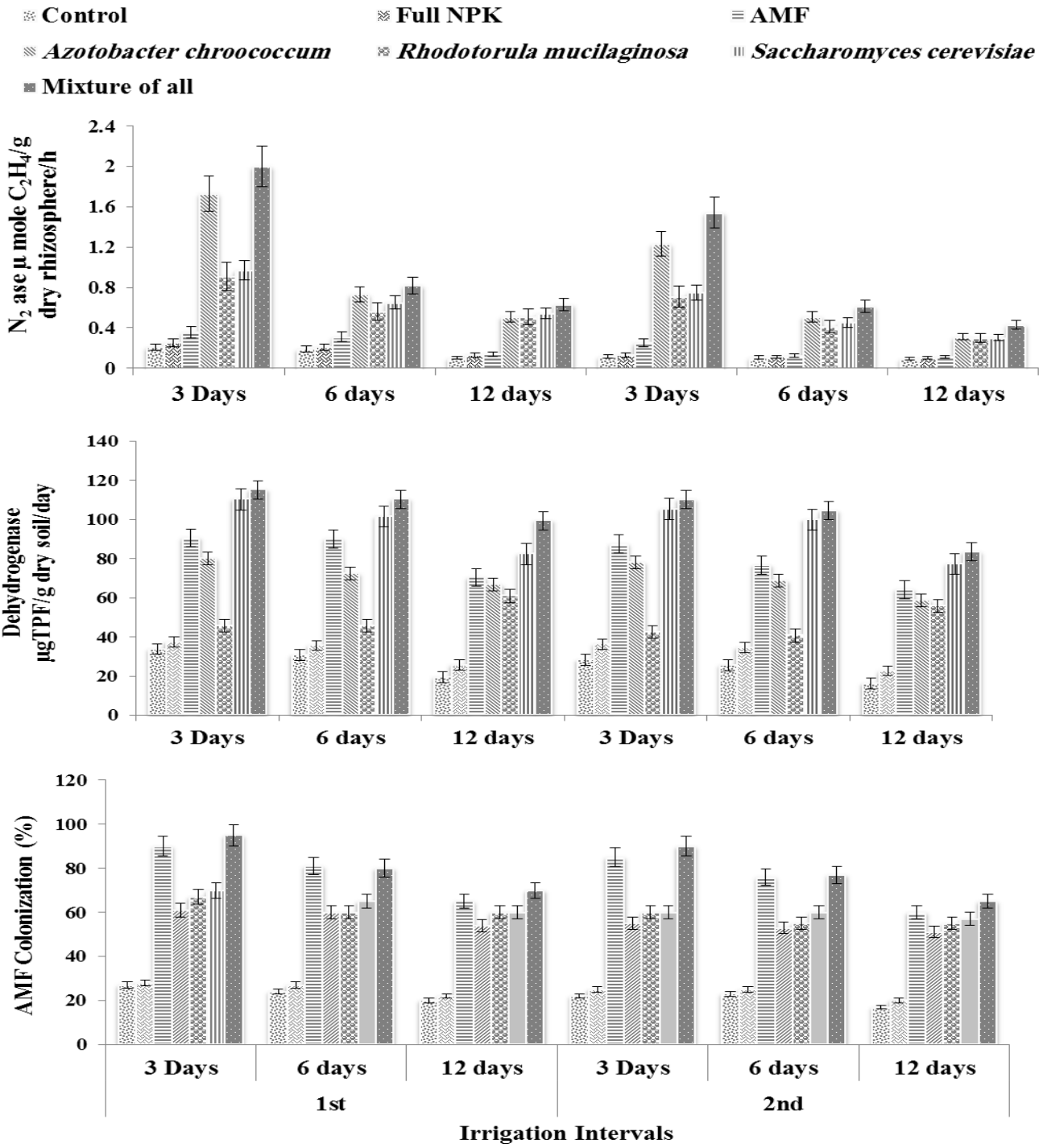


**Figure 2.** Effect of biofertilization and irrigation intervals on mycorrhizal colonization (%), dehydrogenase and nitrogenase enzyme activities during 2013 and 2014.

three nutrients were found in plants irrigated every 12 days; whereas the highest percentages were found in plants irrigated after 3 days, regardless of the effect of inoculation treatments. These results are in agreement with the findings of Jaleel et al. (2008) who indicated that water stress reduces growth by affecting various physiological and biochemical processes, such as ions uptake, translocation, and nutrient metabolism.

The results presented in Table 2 revealed that, in both seasons, *D. regia* plants inoculated with the dual bio-fertilizers had the highest N, P and K percentages in their leaf tissues, compared to un-inoculated (control) plants.

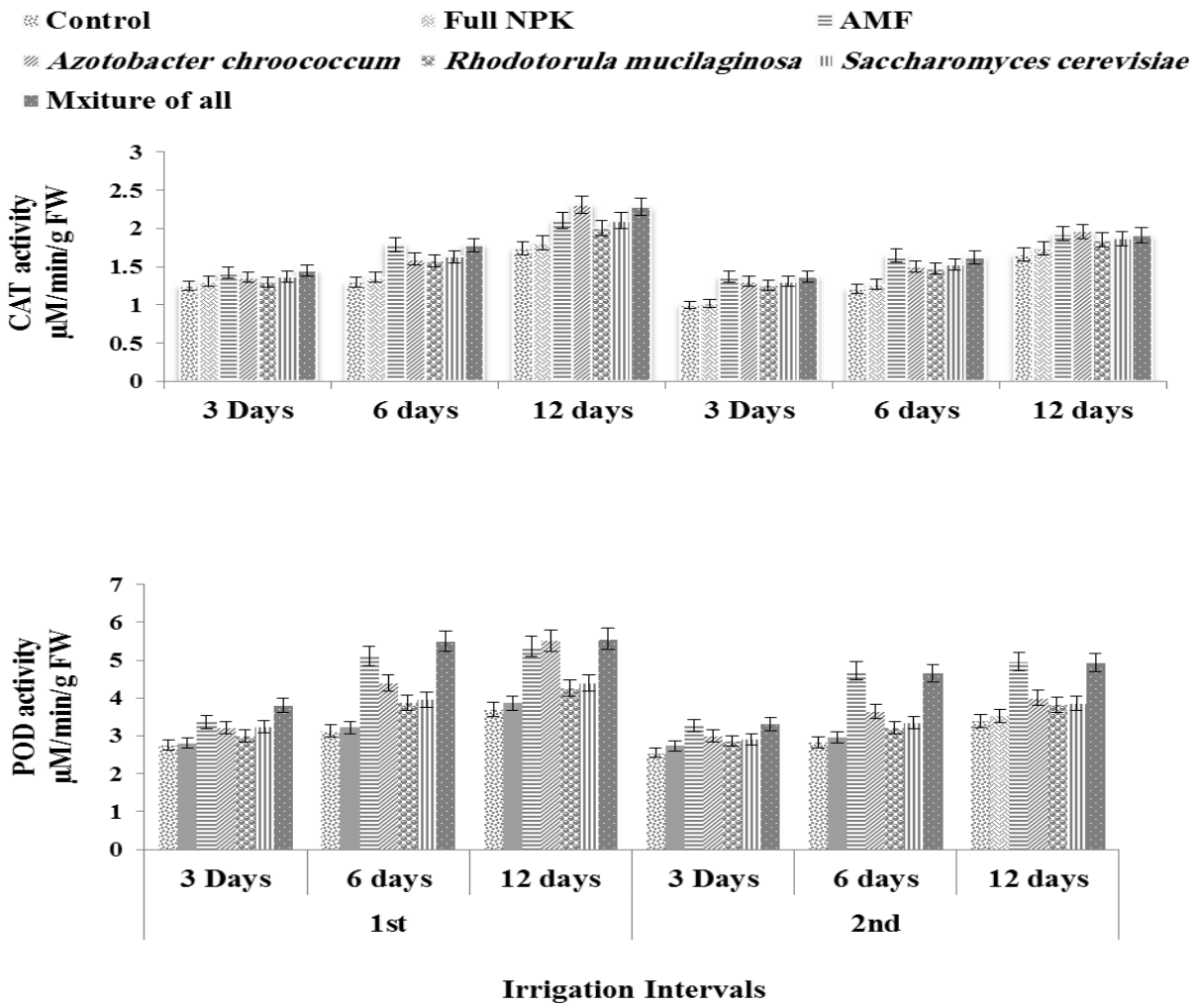


**Figure 3.** Effect of biofertilization and irrigation intervals on the activities of antioxidant enzymes (CAT and POD) of *D. regia* during 2013 and 2014.

These augmentations with reference to main macro-elements N, P and K were 55.36, 44.33 and 60.09%, respectively, in control plants. These results are in agreement with the findings of Jayakumar and Tan (2006) who indicated that seedlings of *Acacia mangium* inoculated with different strains of *Bradyrhizobium* had higher P contents compared to un-inoculated seedlings. AM fungi interact with other soil microbes like free-living nitrogen fixers and phosphate solubilizes to improve their efficiency for the biochemical cycling of elements to the host plants. Also, PGPR strains convert unavailable minerals and organic compounds into forms available to plants. In addition, PGPR strains usually have been found to increase the root length and root biomass and better developed root system. This may increase the mineral uptake in plants. This process increases nutrient uptake and availability of nutrients in the rhizosphere, resulting in an increase in plant growth and yield, as reported by Siddiqui and Mahmood (1999), Gupta et al. (2002) and Khalid et al. (2004).

**Activities of antioxidant enzymes**

Our findings showed that by increasing the irrigation intervals (12 days), the activities of catalase (CAT) and peroxidase (POD) enzymes are significantly increased (1.74 and 3.70 µM/min/g FW, respectively, in the first season;1.66 and 3.39 µM/min/g FW, respectively, in the second season) compared to the respective 3 days (1.25 and 2.75 µM/min/g FW, respectively, in the first season; 1.00 and 2.55 µM/min/g FW, respectively, in the second season) (Figure 3). Abiotic stress such as drought causes damage directly or indirectly to plants, through re-active oxygen species (ROS) formation, which increases by increase in the severity of drought conditions. This leads to the increase of tolerance to oxidative stress (Farooq et al., 2009). The increase in the activity of CAT

and POD enzymes is in agreement with those found in *Helianthus annuus* (Nazarlil et al., 2011) and *Boehmeria nitea* (Huang et al., 2013), subjected to different watering regimes.

In this study, dual bio-fertilizers led to a significant increase in the CAT and POD enzymes compared to un-inoculated (control) plants (Figure 3). Borde et al. (2012); Heidari and Golpayegani (2012) and Morteza et al. (2013) concluded that bio-fertilization can prevent oxidetive stress by increasing activities of antioxidant enzymes during periods with intense photosynthesis; elevated activity could be correlated with increased stress tolerance. Therefore, application of dual bio-fertilizers can be an important tool in *D. regia* cultivation to overcome drought stress conditions and can protect plants from drought conditions. The same previously mentioned trend was observed by other authors (Ruiz-Lozano et al, 2001; Alguacil, 2003; Saravanakumar et al., 2011).

It can be recommended to inoculate soil with *A. mycorrhizal* fungi combined with *Azotobacter* spp. and yeasts for increasing the tolerance of young seedlings of *D. regia* as well as enhancing growth, nutrition status and activities of antioxidant enzymes under drought condition, besides their safety for either environment or human health.

## Conflict of Interest

The authors have not declared any conflict of interest.

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