Role of endomycorrhizae and *Pseudomonas fluorescens* on the acclimatization of micropropagated *Stevia rebaudiana* Bert. plantlets

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*Stevia rebaudiana* Bert. is a non-caloric sweetener plant of medicinal and commercial values. This study was performed to micropropagate *Stevia* and improve its ability to withstand the sudden shocks of the environmental changes during acclimatization by using endomycorrhizae and/or *Pseudomonas fluorescens*. *In vitro* establishment of *Stevia* was superior by culturing shoot tips and nodal segments of mature plants on Murashige and Skoog medium supplemented with 0.5 mg/l 6-benzyl adenine and 1 mg/l β-naphthalene acetic acid, and nodal segments gave better response when compared to shoot tips. While 6-benzyl adenine alone at 1 mg/l was found to be the most promising concentration for shoots multiplication for four successive subculturing. The best rooting response was obtained on half strength Murashige and Skoog medium supplemented with 1 mg/l β-naphthalene acetic acid. Adding 8 ml/l mycorrhizal root extract gave 100% rooting with the highest mean number and length of roots and shoot height. Inoculation of plantlets with endomycorrhizal spores alone in non-sterilized soil increased the survival percentage to 90% and gave the highest mean number and length of both shoots and roots, as well as leaves number. Moreover, they recorded the highest values of total chlorophyll, NPK and relative water contents.

**Key words:** Stevia, micropropagation, mycorrhizae, mycorrhization helper bacteria.

**INTRODUCTION**

*Stevia rebaudiana* Bert. (Stevia) belongs to the family Asteraceae. It contains natural sweetening compounds that are non-calorific and 230 times sweeter than sucrose (Kinghorn, 1987). It is useful for hypoglycemia and diabetes, as well as nourishes and healing pancreas. Also, It is suitable as a row material for the production of food components, since it is a good source of carbohydrates, protein, fiber materials as well as...
dispensable and indispensable amino acids (Laribi et al., 2012).

Large-scale cultivation of Stevia is restricted because of the low germination percentage of seeds. On the other hand, propagation through seeds produces heterogeneous plants, resulting in variations in sweetening levels. Propagation by cutting is also limited, because of a lower number of individuals that can be obtained simultaneously from a single plant (Dey et al., 2013). Tissue culture techniques hold great promise for micropropagation, and enhancement the natural levels of in vitro Stevia plant (Jitendra et al., 2012). Considerable efforts have been directed to optimize the conditions for micropropagation of Stevia, but the acclimatization process of micropropagated plants remains the major hurdle, as it gave low success reports. Desiccation and wilting are the main causes of low survival. Plantlets develop within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in high level of humidity. These contribute a phenotype that cannot survive the environmental conditions when directly transferred in a greenhouse or field (Manjusha and Sathyaranayana, 2010).

Inoculation of endomycorrhizae to micropropagated plants has proved their effectiveness in the resistance of these plants to transplanting stress and improving the growth and mineral nutrient uptake especially phosphorus (Jaizme-Vega et al., 2002), as well as improving physiological parameters, such as increment in photosynthetic rates and stomatal conductance and protecting plant against biotic and abiotic stresses and disease (Arriagada et al., 2012; Bárzana et al., 2014).

Pseudomonas fluorescens inhabiting the rhizosphere of various plants and release auxins as secondary metabolites (Lee et al., 2004). These auxins induces the formation of additional root hair and/or lateral root (Tien et al., 1979), enhancing the ability of plants to take up nutrients from soil and increasing their yield. Ps. fluorescens is one of the most important mycorrhization helper bacteria as they affect the symbiotic establishment of mycorrhizal fungi on plant roots in various ways (Garbaye, 1994). The soil-borne Ps. fluorescens has received particular attention, because of their capacity to produce a wide range of enzymes and metabolites (Kapoor et al., 2012).

The present study was conducted to investigate the effect of endomycorrhizae and Ps. fluorescens on the survival and growth of micropropagated Stevia, either in vitro and ex vitro.

MATERIALS AND METHODS

Plant material and surface sterilization

Stevia shoots with terminal buds (10 cm in length) were obtained from a nursery in El-Mansurya, Egypt. After defoliating the shoots, they were cut into 2 cm nodal segments and 0.5-1 cm shoot tips. Surface sterilization of explants were done in 95% ethyl alcohol for 10 seconds, then, explants were immersed in sterile distilled water with two drops of Dettol for 10 min, then immersed into 1.05% sodium hypochlorite solution (NaOCl) for 10 min for shoot tips and 1.58% NaOCl solution for 15 min for nodal segments. After each treatment the explants were rinsed 5-6 times with sterile distilled water.

Culture media and growth conditions

Explants were cultured on Murashige and Skoog (MS) basal medium (Duchefa, Haarlem, Netherlands) (Murashige and Skog, 1962) with 30 g/l sucrose, 100 mg/l myo-inositol and solidified with 2 g/l phytage (Duchefa, Haarlem, Netherlands). Various concentrations and combinations of plant growth regulators (PGRs, Sigma Cell Culture, min. 90%, St. Louis, USA) were added to the culture medium according to the growth stage. The pH of the media was adjusted to 5.7±0.1 and autoclaved at 121°C at a pressure of 1.1 kg/cm² for 15 min (Harvey Sterilemax autoclave, Thermo Scientific, USA). Cultures were incubated in a temperature of 26±2°C under a photoperiod of 16 hours with a light intensity of 20 µmol/m²/s (F1409d/38, Toshiba), and under relative humidity of 60-65%.

Micropropagation

Establishment stage

Shoot tips and nodal segments of Stevia were cultured on MS medium supplemented with 1 mg/l β-naphthalene acetic acid (NAA), individually, or in combination with 6-benzyl adenine (BA) at 0.5, 1, 1.5 and 2 mg/l, and MS medium without plant growth regulators (PGRs) was used as a control. Percentage of growth induction (%), mean number of axillary shoots/explant and mean length of axillary shoots (cm) were recorded after four weeks from culturing.

Multiplication stage

The in vitro established shoots were cultured on MS medium supplemented with BA at 0.5, 1, 1.5 and 2 mg/l, individually, or in combination with 0.5 mg/l kinetin (Kn), and MS medium free from cytokinins was used as control. Shoots were subcultured six times on the best medium every four weeks. Mean number and length (cm) of axillary shoots/explant were recorded for each subculture.

Rooting stage

Elongated shoots were excised and cultured on half strength MS medium supplemented with indole-3-butyric acid (IBA) or NAA at 0.1, 0.5, 1 and 1.5 mg/l, in addition to the control treatment of 1/2 MS medium without auxins. Data were recorded in terms of rooting percentage (%), mean number of roots/explant, mean length of roots (cm) and mean shoot height (cm) after four weeks from culturing.

Preparation of endomycorrhizal inoculant

Endomycorrhizal spores were originally extracted from soil around roots of maize grown in the Experimental Field of Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, Cairo, Egypt, using the wet sieving and decanting technique as described by Gerdemann and Nicolson (1963). The concentration of endomycorrhizal spore suspension was about 20 spores/ml, and

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250 spores/plantlet was used as a standard inoculum in the experiment.

Estimation of root infection with endomycorrhizae

Inoculated Stevia roots were collected after two months from infection. The percentage of root infection with endomycorrhizae was estimated by the method described by Phillips and Hayman (1970).

Preparation of mycorrhizal infected root extract (MIRE)

Ten gram from fresh roots of Stevia plants grown in the greenhouse and colonized with endomycorrhizae that exhibited 90% mycorrhizal infection, were excised. Mycorrhizal infected root extract (MIRE) was prepared using the method described by Sharma et al. (2005). The MIRE was sterilized by filtration using 0.2 µm filter.

Preparation of Pseudomonas free supernatant (PsFS)

Ps. fluorescens was obtained from Unit of Biofertilizers, Microbiology Department, Faculty of Agriculture, Ain Shams University. Microbial inoculant was maintained on King’s medium (King et al., 1954), supplemented with 0.01 g/l tryptophan at 25°C for 5-7 days. Culture containing 10^8 CFU/ml was centrifuged at 5500 rpm for 20 min. The Ps. fluorescens free supernatant (PsFS) was sterilized by filtration using 0.2 µm filter.

Estimation of PGRs in MIRE and PsFS

The MIRE and PsFS were examined for the presence of gibberellins (GA_3), zeatin and indole-3-acetic acid (IAA) using high-performance liquid chromatography (HPLC) system (Agilent 1100 series, Agilent Technologies, Germany) according to the method described by Tien et al. (1979).

In vitro application of MIRE and PsFS

Two concentrations of MIRE (4 and 8 ml/l) and PsFS (10 and 15 ml/l) were added to Stevia rooting media of 1/2 MS medium with and without 1 mg/l NAA. Media without MIRE and PsFS were served as control. Rooting percentage (%), number of roots/plantlet, length (cm) of roots and shoot height (cm) were recorded after four weeks from culturing.

Ex vitro inoculation of mycorrhizae and Ps. fluorescens

Endomycorrhizal spore suspension and Ps. fluorescens maintained in King’s medium supplemented with 0.01 g/l tryptophan containing 10^8 CFU/ml were used for the ex vitro inoculation of Stevia plantlets. Rooted plantlets were washed from the medium residues and transplanted ex vitro into pots containing sterilized and non-sterilized mixture of sand, peat moss and vermiculite (1:1:1 v/v/v). Plantlets were inoculated with 12.5 ml mycorrhizal spore suspension at a concentration of 20 spores/ml and 5 ml Ps. fluorescens at a concentration of 10^9 CFU/ml, either individually or in combination. Pots were covered with transparent polyethylene bags to maintain a high relative humidity around shoots, and placed in a greenhouse. Irrigation took place once a week with ½ MS medium. Relative humidity was reduced by gradual removal of the covers within two months. Cultures without mycorrhizal spore suspension and Ps. fluorescens were served as control. The survival percentage of plantlets (%), mean number of shoots/plantlet, length of shoots (cm), number of leaves/plantlet, number of roots/plantlet and length of roots (cm) were recorded after six months from transplanting in the greenhouse.

Biochemical analysis of the in vitro produced plantlets

Some biochemical analyses were carried out on the greenhouse acclimatized plantlets after six months.

Total chlorophyll

Chlorophyll content was measured in the leaves of acclimatized plantlets using Minolta chlorophyll meter SPAD-502 that expressed as SPAD.

Nitrogen, phosphorus and potassium (NPK) content

Six months old acclimatized plantlets were oven dried at 70°C until constant weight was obtained. Dried materials were ground to a fine powder. A constant weight (0.2 g) of oven dried plant samples were digested with concentrated sulfuric acid (98%); as well few drops of H_2O_2 were added. The mixture was placed on a hot plate until being colourless. The aliquot was completed to 50 ml volume with double distilled water (Murphy and Riley, 1962). Nitrogen content (%) was determined according to Bradford (1976) and expressed as mg/g dry weight. Phosphorus and potassium content was determined by the method described by Murphy and Riley (1962) and expressed as mg/g dry weight.

Moisture content (%)

The moisture content (%) was determined as the difference between dry weight (DW) and fresh weight (FW) and calculated as [[FW - DW]/FW] x 100.

Statistical analysis

Experiments were subjected to completely randomized design. Variance analysis ANOVA was done using Costat software program. Treatments contained at least 10 replicates. The differences among means of treatments were tested for their significance at 5% level by using Duncan (1955) and multiple range tests as modified by Snedecor and Cochran (1990).

RESULTS AND DISCUSSION

Micropropagation

Establishment stage

Data recorded in Table 1 represent the effect of different concentrations of BA in addition to NAA at 1 mg/l on the in vitro establishment of Stevia from shoot tips and nodal segments. The percentage of growth induction reached 100% on all tested treatments for both explants. Shoot tips on MS medium with BA at 0.5 mg/l in combination with 1 mg/l NAA gave the highest significant mean
Table 1. In vitro establishment of shoot tip and nodal segment of Stevia on MS medium supplemented 1.0 mg/l NAA and different concentrations of BA. Growth induction reached 100% on all tested treatments.

| Treatments BA mg/l | Shoot tip | Nodal segment |
|------------------|-----------|---------------|
|                  | Mean number of axillary shoots/ explant | Mean length of axillary shoot (cm) | Mean number of axillary shoots/ explant | Mean length of axillary shoot (cm) |
| Control 0.0      | 4.7bc     | 1.30a         | 4.4c     | 0.66c         |
| 0.5              | 7.2b      | 1.03b         | 14.1a    | 1.54a         |
| 1.0              | 5.1b      | 0.70c         | 7.8b     | 0.89b         |
| 1.5              | 5.2bc     | 0.69c         | 6.5bc    | 0.87b         |
| 2.0              | 3.7c      | 0.54d         | 5.8bc    | 1.50a         |

Concerning the in vitro establishment of axillary shoots from nodal segments, the results in Table 1 show that MS medium supplemented with 0.5 mg/l BA and 1 mg/l NAA gave the highest significant mean number and length of axillary shoots, being 14.1 and 1.54 cm, respectively (Figure 1B). While MS control medium (free from PGRs) gave the lowest significant mean number and length of axillary shoots, being 4.4 and 0.66 cm, respectively.

These results are supported by Pratibha et al. (2010), who found that the nodal explants of Stevia cultured on 0.5 mg/l BA and 1 mg/l NAA recorded the best shoot establishment and proliferation. Singh and Singh (2005) recorded that cytokinins and auxins stimulate cell division even in non-meristematic tissues, as well as, parenchyma cell. Furthermore, the ratio of cytokinins to auxins controls cell differentiation. When the ratio is in the favor of cytokinins, shoot formation takes place, while for root formation will be in favor of auxins.

The length of axillary shoots has the priority in the establishment stage as the aim of this stage is to obtain a clean growing culture for further stages. Therefore, by comparing the two different explant types of Stevia used in the present study, it could be concluded that the nodal segments gave better response, since it gave higher mean number and length of axillary shoots, when compared to shoot tips.

**Multiplication stage**

Data recorded in Table 2 show the multiplication of the in...
**Table 2.** *In vitro* multiplication of Stevia shoots on MS medium supplemented with BA and Kn.

| Cytokinins conc. (mg/l) | Mean number of axillary shoots/ explant | Mean length of axillary shoots (cm) |
|-------------------------|----------------------------------------|-----------------------------------|
| Kn                      | BA                                     |                                   |
| 0.0                     | 15.2<sup>d</sup>                       | 2.16<sup>a</sup>                  |
| 0.5                     | 17.6<sup>abc</sup>                     | 1.76<sup>b</sup>                  |
| 1.0                     | 19.2<sup>a</sup>                       | 1.54<sup>ae</sup>                 |
| 1.5                     | 16.4<sup>bcd</sup>                     | 1.73<sup>bc</sup>                 |
| 2.0                     | 12.0<sup>g</sup>                       | 1.75<sup>bc</sup>                 |
| 0.5                     | 15.4<sup>cde</sup>                     | 1.40<sup>e</sup>                  |
| 1.0                     | 17.8<sup>ab</sup>                      | 1.86<sup>a</sup>                  |
| 1.5                     | 18.1<sup>ab</sup>                      | 1.44<sup>g</sup>                  |
| 2.0                     | 15.3<sup>cde</sup>                     | 1.65<sup>cde</sup>                |

**Table 3.** *In vitro* multiplication of Stevia during six successive subcultures on MS medium containing the best cytokinins for multiple shoots production.

| Subculture no. | Mean no. of axillary shoots/explant | Mean length of axillary shoots (cm) | Mean no. of axillary shoots/explant | Mean length of axillary shoots (cm) |
|----------------|-------------------------------------|------------------------------------|-------------------------------------|------------------------------------|
|                | 1 mg/l BA                           | 1.5 mg/l BA + 0.5 mg/l Kn          |                                     |                                     |
| 1<sup>st</sup> | 16.0<sup>c</sup>                    | 1.41<sup>ab</sup>                  | 14.0<sup>c</sup>                    | 1.14<sup>c</sup>                  |
| 2<sup>nd</sup> | 18.4<sup>abc</sup>                  | 1.45<sup>ab</sup>                  | 17.1<sup>abc</sup>                 | 1.25<sup>abc</sup>                |
| 3<sup>rd</sup> | 19.5<sup>a</sup>                    | 1.44<sup>ab</sup>                  | 18.3<sup>a</sup>                    | 1.15<sup>b</sup>                 |
| 4<sup>th</sup> | 20.7<sup>a</sup>                    | 0.41<sup>b</sup>                  | 18.0<sup>ab</sup>                  | 1.14<sup>b</sup>                |
| 5<sup>th</sup> | 10.9<sup>c</sup>                    | 1.45<sup>ab</sup>                  | 8.2<sup>c</sup>                    | 1.25<sup>abc</sup>                |
| 6<sup>th</sup> | 8.1<sup>d</sup>                     | 1.54<sup>a</sup>                  | 7.4<sup>d</sup>                    | 1.45<sup>a</sup>                |

*In vitro* established Stevia shoots using BA, individually, or in combination with Kn. The highest significant mean number of axillary shoots/explant was recorded with 1 mg/l BA alone, being 19.2 (Figure 1C). This result is supported by Ali et al. (2010) who mentioned that MS medium with 1 mg/l BA give maximum shoot multiplication response in Stevia. While, MS medium supplemented with 1.5 mg/l BA and 0.5 mg/l Kn gave 18.1 as mean number of shoots per explant and 1.44 cm as shoot length, which could be considered significantly for production of both. While MS medium without cytokinins gave the significantly highest mean length of axillary shoots, being 2.16 cm, but with lower mean number of axillary shoots per explant. Shoot multiplication increased with increase in concentration of BA and Kn, except for the highest concentration, which could be supra-optimum for the shoots to multiply. Cytokinins have been considered to be one of the most crucial components of plant tissue culture protocol, as optimum culture proliferation and shoot growth is impossible in their absence (Hamide and Mustafa, 2004) and treatments, which stimulated multiple shoot formation hindered shoot elongation. Shoot length is found to be the highest in medium supplemented with no or low dose of cytokinin (Waman and Bohra, 2016).

The six successive subculturing of Stevia shoots were attained on MS medium either supplemented with 1 mg/l BA only or with 1.5 mg/l BA, in combination with 0.5 mg/l Kn (Table 3). In both media the mean number of axillary shoots increased till the 4<sup>th</sup> subculture, then decreased in the 5<sup>th</sup> and 6<sup>th</sup> subcultures. MS medium supplemented with 1 mg/l BA alone found to be the most promising concentration for shoot multiplication of Stevia for four successive subcultures, as it gave 20.7 as mean number of axillary shoots per explant. In this respect, Prakash et al. (2006) successfully used this technique to increase the number of shoot buds in *Ptterocarpus santalinus*. They observed increase in shoot bud multiplication rate up to the 6<sup>th</sup> subculture stage.

**Rooting stage**

Rooting of Stevia was studied on ½ MS medium supplemented with different concentrations of IBA and NAA, as represented in Table 4. Data revealed that all the used concentrations gave 100% rooting. However, the concentration of 1 mg/l NAA gave the highest significant mean number (21.5) and length (3.55 cm) of roots with a mean shoot height of 4.8 cm (Figure 1D). It
was also noticed that by increasing the concentration of auxins (either IBA or NAA) the mean number and length of roots increased until the concentration of 1 mg/l. While the mean shoot height significantly increased by the increase of auxin concentration until 1.5 mg/l. These results are in harmony with that obtained by Ali et al. (2010) who found that the best in vitro rooting response of Stevia could be obtained on MS medium containing 1 mg/l NAA either from apical or nodal meristem, being 96%.

Roots are mostly induced in the presence of an auxin. IAA, IBA and NAA promoted rhizogenesis in general. However, their response differed according to the plant species and the physiological state of the explant. NAA showed the most positive effect on roots number and length among the different auxins used in this study. Such an effect of NAA was also observed in numerous studies, which have indicated that, among the common auxins, NAA is the most effective auxin for induction of root regeneration (Chae et al., 2016).

### PGRs content in the mycorrhizal infected root extract (MIRE) and *Pseudomonas* free supernatant (PsFS)

The PGRs content detected in the form of GA₃, zeatin and IAA recorded 12.391, 64.711 and 2.435 μg/ml in MIRE and 56.5, 205.8 and 14.02 μg/ml in PsFS, respectively. Results show that the concentrations of GA₃, zeatin and IAA were higher in PsFS than MRE.

### Effect of mycorrhizal infected root extract (MIRE) and *Pseudomonas* free supernatant (PsFS) on *in vitro* produced plantlets

This experiment was conducted to evaluate the efficiency of MIRE and PsFS on rhizogenesis of Stevia in vitro during rooting stage. All the treatments gave 100% rooting, as well as control. However, as recorded in Table 5, MS medium containing MIRE at 8 ml/l with 1 mg/l NAA gave the significantly highest mean number and length of roots, being 32.3 and 5.13 cm, respectively, as well as the significantly highest mean shoot height of 5.85 cm (Figure 2). Followed by MS medium supplemented with 15 g/l PsFS and 1 mg/l NAA, which gave 26.5 and 4.02 cm as mean number and length of roots, respectively, and 5.3 cm as mean shoot height. This result is similar to that obtained by Sharma et al. (2005), who found that endomycorrhizal root extract of *Morus alba* was equivalent to IBA in promoting plant rhizogenesis, root growth and proliferation. These results could be explained as endomycorrhizal fungi are able to produce several growth promoting substances (Hasan, 2002), which are capable to alter plant internal hormone balance (Scagel and Linderman, 1998).

Also, shoots treated with MIRE gave the best results in all growth parameters when compared to PsFS, which may be due to the presence of GA₃, zeatin and IAA in the PsFS in supra-optimal level for plants, that cause some inhibition in plant growth parameters (Kunkel and Chen, 2006; Zulfiqar et al., 2009). On the other hand, Peyvandi et al. (2010) found that length and numbers of adventitious and lateral roots of olive micro-shoots increased in vitro by using the supernatant of *Ps. fluorescens* maintained on King’s medium that supplemented with L-tryptophan.

### Effect of the endomycorrhizae and *Ps. fluorescens* on the acclimatized Stevia plantlets

Data recorded in Table 6, after six months from culturing Stevia plantlets in the greenhouse, show that inoculation of Stevia plantlets with endomycorrhizal spores and/or *Ps. fluorescens* in non-sterilized soil gave higher significant values on all parameters when compared within same treatments in sterilized soil. In this respect,

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**Table 4.** Effect of ½ MS medium containing different concentrations of IBA or NAA on the rooting of Stevia axillary shoots. Rooting percentage reached 100% on all tested treatments.

| Auxins conc. (mg/l) | Mean number of roots/ explant | Mean length of roots (cm) | Mean shoot height (cm) |
|---------------------|-------------------------------|--------------------------|------------------------|
| IBA                 | NAA                          |                          |                        |
| 0.0                 | 14.6bc                        | 1.5cd                    | 2.4h                   |
| 0.1                 | 8.1d                         | 0.816ef                  | 2.4h                   |
| 0.5                 | 13.1bc                       | 1.634g                   | 3.45i                  |
| 1.0                 | 14.3bc                       | 1.69g                    | 4.1d                   |
| 1.5                 | 12.7bc                       | 1.143h                   | 5.1b                   |
| 0.1                 | 10.1cd                       | 0.685i                   | 2.95j                  |
| 0.5                 | 15.4b                        | 1.29cd                   | 3.85e                  |
| 1.0                 | 21.5a                        | 3.55a                    | 4.8f                   |
| 1.5                 | 16.8b                        | 2.25b                    | 5.4a                   |
Table 5. Effect of MIRE and PsFS on the rooting of Stevia axillary shoots.

| Treatments | No. of roots/ explant | Roots length (cm) | Shoot height (cm) |
|------------|-----------------------|-------------------|------------------|
| Control    |                       |                   |                  |
| 1.0 mg/l NAA |                      |                   |                  |
| MIRE (ml/l) | 4.0                   | 20.6<sup>d</sup> | 3.55<sup>d</sup> |
|            | 8.0                   | 25.6<sup>c</sup> | 3.99<sup>bc</sup>|
|            | 10.0                  | 32.3<sup>c</sup> | 5.13<sup>a</sup> |
|            | 15.0                  | 22.9<sup>de</sup>| 3.81<sup>c</sup> |
|            |                       | 26.5<sup>b</sup> | 4.02<sup>b</sup> |
| PsFS (ml/l)| 0.0 mg/l NAA          |                   |                  |
| MIRE (ml/l) | 4.0                   | 21.8<sup>ef</sup> | 3.560<sup>d</sup>|
|            | 8.0                   | 22.6<sup>c</sup> | 3.90<sup>bc</sup>|
|            | 10.0                  | 23.7<sup>d</sup> | 3.90<sup>bc</sup>|
|            | 15.0                  | 21.8<sup>ef</sup>| 3.56<sup>d</sup> |
| PsFS (ml/l)| 15.0                  | 21.9<sup>ef</sup> | 3.63<sup>cd</sup>|

Table 6. Effect of MIRE and PsFS on the growth and development of non-sterile soil inoculated with endomycorrhizal spores and Ps. fluorescens in combination.

| Treatments | Survival percentage (%) | Mean number of leaves | Mean number and length of roots (cm) | Total chlorophyll (SPAD) | NPK (mg/g dry weight) | Relative water content (%) |
|------------|-------------------------|-----------------------|---------------------------------------|--------------------------|-----------------------|---------------------------|
| Control    |                         |                       |                                       |                          |                       |                           |
| 1.0 mg/l NAA |                      |                       |                                       |                          |                       |                           |
| MIRE (ml/l) |                         |                       |                                       |                          |                       |                           |
|            | 4.0                     | 90                    | 34                                   | 34.83                    | 1.16                  | 84.43                     |
|            | 8.0                     | 90                    | 34                                   | 34.83                    | 1.16                  | 84.43                     |
|            | 10.0                    | 90                    | 34                                   | 34.83                    | 1.16                  | 84.43                     |
|            | 15.0                    | 90                    | 34                                   | 34.83                    | 1.16                  | 84.43                     |
| PsFS (ml/l)| 0.0 mg/l NAA            |                       |                                       |                          |                       |                           |
| MIRE (ml/l) |                         |                       |                                       |                          |                       |                           |
|            | 4.0                     | 90                    | 34                                   | 34.83                    | 1.16                  | 84.43                     |
|            | 8.0                     | 90                    | 34                                   | 34.83                    | 1.16                  | 84.43                     |
|            | 10.0                    | 90                    | 34                                   | 34.83                    | 1.16                  | 84.43                     |
|            | 15.0                    | 90                    | 34                                   | 34.83                    | 1.16                  | 84.43                     |

Smith and Read (2008) showed that plantlets cultivated in non-sterile soils could develop endomycorrhizae and grew better than plantlets cultivated in sterilized soils, as they mentioned that sterilization by heat may be responsible for the production of toxic compounds, which are harmful for plant development. Also, Martins (2008) mentioned that endomycorrhizae formed in non-sterile soil are responsible for the increased performances of the plants, which may be explained by the presence of some soil microbiota that enhance the germination of endomycorrhizal spores.

As shown in Table 6, inoculation of non-sterilized soil with endomycorrhizal spores alone recorded the significantly highest values of survival percentage (90%), mean number and length of shoots (5.8 and 38.95 cm), mean number of leaves (34), mean number and length of roots (35 and 21.83 cm), total chlorophyll (33.83 SPAD), NPK (1.16%, 0.72 and 43.02 mg/g dry weight) and relative water content (84.43%), when compared to all tested treatments (Figure 3). Followed by those inoculated by endomycorrhizal spores in combination with *Ps. fluorescens* in non-sterilized soil that gave 90% survival percentage, 4.6 and 30.50 cm as mean number and length of shoots, respectively, 28.2 as mean number of leaves, 31.30 and 18.30 cm as mean number and length of roots, respectively, 29.813 SPAD as relative chlorophyll content, 0.98%, 0.61 and 41.40 mg/g dry weight as NPK content, respectively, and 78.76% as relative water content. These results are in agreement with those reported by Estrada-Luna et al. (2000), who showed that mycorrhizal Guava plantlets had greater shoot length, leaf area, leaf, stem, root dry mass, mineral levels of P, Mg, Cu, and Mo. While, Earanna (2007) mentioned that inoculation with arbuscular mycorrhizae and plant growth promoting rhizobacteria improve the growth and biomass of several plants by supplementing plant with nutrients and producing growth hormones.

Arbuscular mycorrhizal fungi can also benefit plants by stimulating the production of growth regulating substances, increasing photosynthesis, improving osmotic adjustment under drought and salinity stresses as well as enhance root enzymes activity, shoot nutrient and pigments content (chlorophyll and carotenoid), and water use efficiency (Wang et al., 2008; Manaf and Zayed, 2015). Also, Estrada-Luna and Davies (2003) mentioned that arbuscular mycorrhizal fungi help plantlets to recover rapidly during acclimatization and gave great growth during post-acclimatization. Also, in this respect, Gray and Smith (2005), Glick et al. (2007) and Manaf and Zayed (2015) mentioned that *Ps. fluorescens* have substantial effects on plant growth, particularly under stress conditions, and play an important role in plant physiology by secretion of PGRs (auxins, cytokinins, and gibberellins), which enhance various stages of plant growth or synthesize enzymes that modulate plant growth and development as well as improving nutrients uptake, and enhancement of stress resistance.

As mentioned in the present study, inoculation

Figure 2. *In vitro* rooting of Stevia on ½ MS medium supplemented with (A) 1 mg/l NAA only and (B) 1 mg/l NAA in addition to 8 mg/l MIRE.
Table 6. Effect of mycorrhizal spores and *Ps. fluorescens* on acclimatization and total chlorophyll, NPK and relative water contents in Stevia plantlets acclimatized in sterilized and non-sterilized soil, after 6 months of culture.

| Treatments                                      | Survival % | No. of shoots/ explant | Length of shoots (cm) | No. of leaves/ explant | No. of roots/ explant | Length of roots (cm) | Total chlorophyll content (SPAD) | N content (%) | P content (mg/g) | K content (mg/g) | Relative water content (%) |
|-------------------------------------------------|------------|------------------------|-----------------------|------------------------|-----------------------|-----------------------|-------------------------------|---------------|-----------------|-----------------|--------------------------|
| Non-sterilized soil                             |            |                        |                       |                        |                       |                       |                               |               |                 |                 |                          |
| Control                                         | 20c        | 0.3d                   | 3.00e                 | 3.8a                   | 13.67e                | 9.83d                 | 22.090                        | 0.78b         | 0.41c           | 24.18b          | 50.89g                   |
| Endomycorrhizal spores                          | 90a        | 5.8a                   | 38.95a                | 34.0b                  | 21.83a                | 33.830                | 0.86a                         | 0.72a         | 0.72a           | 43.02b          | 84.43g                   |
| *Ps. fluorescens*                                | 50abc      | 1.7cd                  | 13.85cde              | 14.22de                | 20.70d                | 13.17c                | 26.850                        | 0.86c         | 0.47bc          | 29.55b          | 61.83c                   |
| Endomycorrhizal spores + *Ps. fluorescens*       | 90a        | 4.6ab                  | 30.50ab               | 28.2eh                 | 31.30b                | 18.30b                | 29.813                        | 0.98b         | 0.64ab          | 41.40b          | 78.76b                   |
| Sterilized soil                                 |            |                        |                       |                        |                       |                       |                               |               |                 |                 |                          |
| Control                                         | 10c        | 0.2d                   | 1.50e                 | 2.2e                   | 11.70e                | 7.67e                 | 21.088                        | 0.72c         | 0.40c           | 22.20b          | 45.52g                   |
| Endomycorrhizal spores                          | 70a        | 4.4ab                  | 25.00bc               | 22.6abcd               | 27.67c                | 17.50b                | 28.413                        | 0.95c         | 0.60ab          | 39.12b          | 75.47c                   |
| *Ps. fluorescens*                                | 30bc       | 1.0d                   | 6.55de                | 6.8bc                  | 17.67d                | 10.83d                | 24.713                        | 0.84d         | 0.43bc          | 25.95f          | 58.93f                   |
| Endomycorrhizal spores + *Ps. fluorescens*       | 70ab       | 3.1bc                  | 21.95bcd              | 18.0bcd                | 20.67d                | 16.30b                | 27.813                        | 0.91d         | 0.53b           | 38.20f          | 70.86d                   |

Figure 3. Stevia plantlets, in the greenhouse, (A) inoculated with mycorrhizal spores in non-sterilized soil. (B) inoculated with mycorrhizal spores + *Ps. fluorescens* in non-sterilized, and (C) in soil without biofertilizers (control treatment).

The effect of mycorrhizal fungi alone gave the best results than inoculation with mycorrhizal fungi in combination with *Ps. fluorescens*, which was discussed by Bisht et al. (2009), who found that the mycorrhizal infection was lower when the plant growth promoting rhizobacteria was applied in combination with arbuscular mycorrhizal fungi as well as lower photosynthetic and transpiration rates. Also, Ravanskov et al. (1999) reported the
negative effect of mycorrhizae (Glomus intraradices) on the population size of *Ps. fluorescent* in rhizosphere.

Conclusion

The present study clearly demonstrated a successful and efficient micropropagation protocol for Stevia plant and solved the main difficulty that limits the commercial production of the plant by presenting the benefits of using arbuscular mycorrhizal fungi for enhancing the growth and survival of micropropagated Stevia plantlets. Mycorrhization can be combined by micropropagation to provide a sustainable tool to facilitate difficult propagating plants adaptation to *ex vitro* conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations

- **BA**, 6-benzyl adenine; **GA**<sub>3</sub>, gibberellic acid, **HPLC**, high-performance liquid chromatography; **IBA**, indole-3-butyric acid; **K**, kinetin; **MIRE**, mycorrhized infected root extract; **MS**, Murashige and Skoog; **NAA**, β-naphthylacetic acid; **NaOCl**, sodium hypochlorite solution; **PGRs**, plant growth regulators; **PsFS**, *Ps. fluorescent* free supernatant.

REFERENCES

Ali A, Gull I, Naz S, Afghan S (2010). Biochemical investigation during different stages of *in vitro* propagation of *Stevia rebaudiana*. Pak. J. Bot. 42(4):2827-2837.

Artilagada C, Manquel D, Cornejo P, Soto J, Sampedro I, Ocampo J (2012). Effects of the co-inoculation with saprobe and mycorrhizal fungi on *Vaccinium corymbosum* growth and some soil enzymatic activities. J. Soil Sci. Plant Nutr. 12(2):283-294.

Bárzana G, Aroca R, Biennert GP, Chaumont F, Ruiz-Lozano JM (2014). New insights into the regulation of aquaporins by the arbuscular mycorrhizal symbiosis in maize plants under drought stress and possible implications for plant performance. Mol. Plant Microbe Interact. 27(4):349-363.

Bisht R, Chaturvedi S, Srivastava R, Sharma AKa, Johari BN (2009). Effect of arbuscular mycorrhizal fungi, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* on the growth and nutrient status of *Dalbergia sissoo* Roxb. Trop. Ecol. 50(2):231-242.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(1-2):248-254.

Chae RSC (2016). Influence of auxin concentration on *in vitro* rooting of *Chrysanthemum morifolium*. Biosci. Biotechnol. Res. Asia 13(2):833-837.

Dey A, Kundu S, Bandyopadhyay A, Bhattacharjee A (2013). Efficient micropropagation and chloroholone chloride induced stevioside production of *Stevia rebaudiana* Bertoni. C. R. Biol. 336(1):17-28.

Duncan DS (1955). Multiple ranges and multiple “F” test. Biometrics 11:1-42.

Earanna N (2007). Response of *Stevia rebaudiana* to Biofertilizers. Karnataka J. Agric. Sci. 20(3):616-617.

Estrada-Luna, Davies FT (2003). Arbuscular mycorrhizal fungi influence water relations, gas exchange, abscisic acid and growth of micropropagated chile ancho pepper (*Capsicum annuum*) plantlets during acclimatization and post-acclimatization. J. Plant Physiol. 160(9):1073-1083.

Estrada-Luna, Davies Jr F, Egilla J (2000). Mycorrhizal fungi enhancement of growth and gas exchange of micropropagated guava plantlets (*Psidium guajava*) during *ex vitro* acclimatization and plant establishment. Mycorrhiza 10(1):1-8.

Garbaye J (1994). Transley review No. 76. Helper bacteria: A new dimension to the micorrhizal symbiosis. New Phytol. 128:187-210.

Gerdemann JW, Nicolson TH (1963). Spores of mycorrhizal endogone species extracted from soil by wet sieving and decanting. Trans. Br. Mycol. Soc. 46(2):235-244.

Glick BR, Todorovic B, Czarny J, Cheng Z, Duan J, McConkey B (2008). Promotion of plant growth by bacterial ACC deaminase. Crit. Rev. Plant Sci. 26(5-6):227-242.

Gray E, Smith D (2005). Intracellular and extracellular PGP: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biol. Biochem. 37(3):395-412.

Hamide G, Mustafa P (2004). *In vitro* propagation of some new banana types (*Musa spp.*). Turk. J. Agr. For. 28:355-361.

Hasan HAHH (2002). Gibberellin and auxin – indole production by plant root – fungi and their biosynthesis under salinity – calcium interaction. Acta Microbiol. Hung. 49:105-118.

Jaimez-Vega M, Esquivel Delamo M, Tenoury Dominguez P, Rodriguez Romero A (2002). Effects of mycorrhization on the development of two cultivars of micropropagated banana. Informatia 11(1):25-28.

Jitendra M, Monika S, Ratan SD, Priyanka G, Priyanka S, Kaur DJ (2012). Micropropagation of an Anti-diabetic Plant *Stevia rebaudiana* Berti, (Natural Sweetener) in Hadoti Region of South-East Rajasthan, India ISCA. J. Biol. Sci. 1(3):37-42.

Kapoor R, Ruchi, Kumar A, Pratush A and Kaur M (2012). Indole acetic acid production by fluorescent *Pseudomonas* isolated from the rhizospheric soils of *Malus* and *Pyrus*. Rec. Res. Sci. Technol. 4:6-9.

King EO, Ward MK, Raney DE (1954). Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

Kinghorn AD (1987). Biologically active compounds from plants with reputed medicinal and sweetening properties. J. Nat. Prod. 50(6):1009-1024.

Kunkel BN, Chen Z (2006). Virulence Strategies of Plant Pathogenic Bacteria. In. The Prokaryotes. Springer, Pp. 421-440.

Laribi BR, Kouki K, Bettaieb T (2012). *In vitro* propagation of *Stevia rebaudiana* Berti, A non caloric sweetener and diabetic medicinal plants. J. Med. Arom. Plants 2(2):332-339.

Lee C, Encarnacion MF, Zentella MC, Flores LG, Fscamilla JEa, Kennedy C (2004). Indole-3-actic acid biosynthesis deficient in *Glucobacter bactobacter diazotrophicus* strains with mutation in cytochrome c biogenesis genes. J. Bacteriol. 186:5384-5391.

Manal HH, Zayed MS (2015). Productivity of cowpea as affected by salt stress in presence of endomycorrhizae and *Pseudomonas fluorescens*. Ann. Agric. Sci. 60(2):219-228.

Manjunatha AVMa, Sathyavanarayana BN (2010). Acclimatization studies in stevia (*Stevia rebaudiana* Bert.). Acta Hortic. 865:129-133.

Martins A (2008). *In vitro* Mycorrhization of Micropropagated Plants: Studies on *Castanea sativa* Mill. In. “Siddiquzi QA, Akhtar MS, Futai K (eds.), Mycorrhize Sustainable Agriculture and Forestry. Springer. Pp. 319-334.

Murashtik H, Skoog F (1962). Arevised medium for rapid growthand bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-493.

Murphy JA, Riley JH (1962). Ammodified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta 27:31-36.

Peyvandi M, Farahani F, Hosseini MM, Noormohamadi Z, Atail Sa, Asgharzade A (2010). *Pseudomonas fluorescens* and its ability to promote root formation of olive microshoots. Int. J. Plant Prod. 4(1):63-66.

Phillips JMa, Hayman DS (1970). Improved procedures for clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc.
Prakash E, Sha Valli Khan PS, Sreenivasa Rao TJVa, Meru ES (2006). Micropropagation of red sanders (*Pterocarpus santalinus* L.) using mature nodal explants. J. For. Soc. 11(5):329-335.

Pratibha G, Satyawati S, Sanjay S (2010). Micropropagation of *Stevia rebaudiana* (natural sweetener) using kinetin for steviol glycoside production. Res. J. Biotechnol. 5(1):63-67.

Ravanskov S, Nybroe Oa, Jakobsen I (1999). Influence of an arbuscular mycorrhizal fungus on *Pseudomonas fluorescens* DF57 in rhizosphere and hyphosphere soil. New Phytol. 142:113-122.

Scagel CFa, Linderman RG (1998). Relationships between differential *in vitro* indole-acetic acid or ethylene production capacity by ectomycorrhizal fungi and conifer seedling responses in symbiosis. Symbiosis 24:13-24.

Sharma S, Kashyap Sa, Vasudevan (2005). *In vitro* rhizogenesis of *Morus alba* by mycorrhizal extracts under saline stress. Eur. J. Hortic. Sci. 70(2):79-84.

Singh DKa, Singh SK (2005). Physiology and Post-Harvest Management of Horticultural Crops Geeta Somani Agrotech Publishing Academy, Udaipur, India.

Smith SE, Read DJ (2008). Mycorrhizal Symbiosis. 3rd ed. Elsevier Ltd., Academic Press.

Snedecor GWa, Cochran WG (1990). Statical Methods. 8th ed. Iowa State University Press, Ames, Iowa, USA.

Tien TM, Gaskin Sa, Hubbell DH (1979). Plant growth substances produced by *Azospirillum brasiliense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl. Environ. Microbiol. 37:1016-1024.

Waman AA, Bohra P (2016). Effect of various cytokinins and auxins on *in vitro* regeneration of plantlets from isolated bud clumps of Silk Banana var. Nanjanagud Rasabale (*Musa AAB*). Indian J. Plant Physiol. 21(1):64-69.

Wang C, Li X, Zhou J, Wang G, Dong Y (2008). Effects of arbuscular mycorrhizal fungi on growth and yield of cucumber plants. Commun. Soil Sci. Plan. 39(3-4):499-509.

Zulfiqar B, Abbasi NA, Ahmad T, Hafiz IA (2009). Effect of explant sources and different concentrations of plant growth regulators on *in vitro* shoot proliferation and rooting of avocado (*Persea americana* Mill.) cv."Fuerte". Pak. J. Bot. 41(5):2333-2346.