EFFECT OF DIFFERENT AMINO ACIDS AT DIFFERENT CONCENTRATIONS ON MULTIPLICATION AND ROOTING STAGE OF *In vitro* PROPAGATION OF STRAWBERRIES (*Fragaria* X *Ananassa* Duch cv. Chandler)

S. EL-SHARABASY[1], FATMA ISSA[2], GEHAN HAMMAD[2] AND MAIADA EL-DAWAYATY[1]

1. *Central Laboratory for Date Palm Research and Dev1elopment, Agricultural Research Center, Giza, Egypt*
2. *Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA University), Giza, Egypt*

Strawberries are members of the genus *Fragaria* in the Rosaceae family, is a perennial, stoloniferous herb and a very popular and nutritious fruit. Strawberries are a chief berry crop that is cultivated globally. Strawberries have always been considered a popular delicious fruit that has been universally used for nutritious properties whether on its own or in combination with other foods for desert purposes. It possesses a unique flavour that can be savored for both fresh and frozen use. They comprise multiple components that are considered vital dietary staples. They are also an incredibly rich source of vitamin C (Hasan *et al*., 2010). The strawberry fruit retains high levels of ellagic acid, a known compound with anti-carcinogenic properties (ICAR news, 2005). Strawberries are usually propagated vegetatively using runners to obtain true to type plants; their popularity is reflected in their production of over 71 countries worldwide on approximately 50,000 acres (Biswas *et al*., 2007). One of the important goals of the agricultural policy is to increase the acreage of strawberry to meet the demand of local fresh market, processing and export. The strawberry fruits are rich of vitamin C, B1, B2, protein, calcium, potassium, copper and iron, most of the nutritious elements essential for human being (Nehra *et al*., 1994; Hasan *et al.*, 2010). They possess important transformation genes where the genetic engineering of strawberries has already been reported in multiple studies (Moradi *et al*., 2011).

The propagation of strawberries is achievedeither by runners or by *in vitro* micropropagation. Conventional propagation methods are slow, laborious, and expensive with many limitations and may not be recommended for effective and commercial multiplication (Ashrafuzzaman *et al*., 2013). Micropropagation of strawberries from runners was initially reported for achieving efficient generationandlarge numbers of disease free plants (Moradi *et al*., 2011). The micropropagation of strawberries through axillary shoot proliferation and regeneration *via* somatic embryogene-

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sis has also been previously reported for the demonstration of efficient plantlets (Donnoli *et al*., 2001; Kauahal *et al*., 2004; Rekha *et al*., 2012). The micropropagation of strawberry plants was introduced in order to prevent most of the transmissible diseases.

The growth and morphogenesis of plant tissue cultures can be improved by the addition of small amounts of some organic nutrients. These include some vitamins (not strictly animal vitamins), amino acids and certain indeterminate supplements. The amount of substances required for a successful culture varies according to the species and genotype, and is a reflection of the synthetic capacity of the explant. (Edwin *et al*., 2008).

Amino acids and vitamins have demonstrated a profound effect on tissue culturing systems of several species. The optimization of these compounds can stimulate the regeneration inrecalcitrant cultivars (Benson, 2000). Amino acids provide plant cells with a source of nitrogen that is assimilated by tissues and cells more efficiently than inorganic nitrogen sources.

Amino acid mixtures such as casein hydrolysate, L-glutamine, Lasparagine and adenine are frequently used as sourcesof organic nitrogen in culture media. Gerdakaneh *et al*. (2012) demonstrated that the type and concentration ofamino acid have imperative effects on the somatic embryogenesis process and embryo development of the strawberry cultivars. The most suitable and efficient amino acid source forembryo culturing of strawberries was proline. Gerdakaneh *et al.* (2009) also observed different responses in the induction of somatic embryos in various genotypes of strawberries. The literature suggests that optimal concentrations of diverse amino acids are speciesor genotype-dependent, which requires predetermination upon its respective use (Chukwuemeka *et al.*, 2005; Homhuana *et al.*, 2008; Han *et al.*, 2009).

Kadhimi *et al*. (2014) studied the classic and *de novo* plant tissue culturing techniques as well as the effect of some features, such as the media and development managers (plant hormones), vitamins, amino acids and the physical case of the media. These features mostly enhance the circulation of plants in tissue culturing and development of roots, rather than the induction of a callus, embryos, orany form of vegetative emergence, they are also involved in the fractional development of branches and roots; this development is particularly highlighted in the influence of these compounds on the micropropagation of plants.

This study aimed to conduct the optimum type and concentrations of the three selected amino acids (tyrosine, arginine and glutamine) for an effort of enhancing *in vitro* regeneration of (*Fragaria X Ananassa* Duch cv. Chandler) during shooting and rooting stages and the further growth in greenhouse since there were no studies investigated for strawberry micropropagation in this approach.

MATERIALS AND METHODS

All experiments were conducted Biotechnology Laboratory, at The Central Laboratory for Date Palm Research and Development (CLDPRD). Agricultural Research Center, Giza. Fresh proliferation shoots were cultured for two relevant experiments**.**

1. Explant material

In vitro shoot clusters of (*Fragaria* X *Ananassa* Duch cv. Chandler) were used as experimental materials. Explants were collected from 4-6 weeks old virus free aseptic proliferated meristems, the *in vitro* propagation was carried out according to the protocol of Ashrafuzzaman *et al*. (2013) to investigate the effect of three amino acid compounds (Tyrosine, Arginine, or Glutamine) at different concentrations on the shooting and rooting stagesas well as the residual effects of the examined amino acid compounds on the harvested rooting plantlets for the acclimatization stage.

2. Shooting stage

Shoot clusters of *cv. Chandler* explants consist of (2-3) shoots at (1-1.5 cm) high without roots were cultured on MS media (Murashige and Skoog, 1962) which were supplemented with (0.5 mgl^{-1}) kinetin and various amino acids (Tyrosine, Arginine, or Glutamine) which were add separately at multiple concentrations (0, 25, 50 and 100 mg/L). Datarecorded in this stage included the number and lengths of all shoots every four weeks for two subcultures.

3. Rooting stage

Shoot clusters obtained from the shooting stage were transferred for culturing on MS nutrient medium supplemented with (1 mgl^{-1}) NAA (Naphthalene acetic acid) and (2.5 gl^{-1}) AC (Activated charcoal powder) along with the amino acids (Tyrosine, Arginine, or Glutamine) at the previously stated concentrations (0, 25, 50 and 100 mg/L). In this stage data recorded was regarding the root numbers and lengths (cm) after six weeks of culturing.In this experiment all MS nutrient culture media treatments in shooting and rooting stages were supplemented with (30 gl^{-1}) Sucrose, (0.1 mgl^{-1}), Thiamine HCL, (2 mgl^{-1}) Glycine, (0.5 mgl^{-1}) nicotinic acid, (0.1 mgl^{-1}) Myo-inositol. Media was solidified with (5 gl^{-1}) agar.

Previously prepared media was dispensed into large jars 250 ml at the rate of 60 ml per jar. The culture jars were immediately capped with polypropylene closure and then autoclaved at 121°C and 15 Ibs/in 2 for 20 min. The medium pH was adjusted to 5.7-5.8 prior to the addition of agar. Each jar contained one shoot cluster. After inoculation, the culture jars were maintained at a temperature of 25±2°C with a 16 hours/day photoperiod. Lighting was supplied using fluorescent lamps with 1000 lux for the multiplication stages and 3000 lux forthe rooting stages.

4. Acclimatization stage

Well rooted plantlets obtained from each treatment of the previous rooting stage were treated with the investigated amino acid compounds at the specified concentrations and transferred to plastic pots containing garden soil and compost at a ratio of 2:1 that was adequately moistened for respective hardening. They were left for four weeks in the greenhouse. Data recorded was relevant to the root numbers and lengths to estimate the residual effect of the different amino acid compounds at numerous concentrations during the acclimatization stage.

5. Statistical analysis

The experiments were carried out using completely randomized design. Each treatment consisted of three replicates, each of the three jars consisted of one explant. The results were analyzed using the analysis of variance and the means were compared using the Least Significant Difference (LSD) at the 5% level, all obtained data was subjected to analysis of variance by completely randomized design according to Snedecor and Cochran (1980).

RESULTS

In this investigation the effect of the three selected amino acid compounds (Tyrosine, Arginine, or Glutamine) at different tested concentrations (0, 25, 50 and 100 mg/L) showed significant results according to their influences on the multiplication, rooting and acclimatization stages of *Fragaria* X *Ananassa* Duch cv. Chandler *in vitro* propagation.

Shoot number

From data in Table (1) and Fig. (1), the supplementation of the different types of amino acids separately at the studied concentrations to the MS nutrient medium during multiplication stage enhanced significantly the shoot number of the cultured explant of (*Fragaria* X *Ananassa* Duch cv. Chandler). When the three selected amino acids were added separately to the culture nutrient medium at the tested concentrations (25, 50 and 100 mg/L) the highest significant result ofshoot number/explant of cultured strawberry cv. Chandler was obtained at (25 mg/L) for each studied amino acid compound (6.66) followed significantly by the addition of concentration of (50 mg/L or 100 mg/L) for each studied amino acid compound (5.33 and 3.66, respectively with significant difference in between). Control culture mediums without the addition of amino acid compounds recorded the lowest significant result (3.00) of increasing in shoot number/ explant, and they seemed weaker than the other explants which were cultured on the nutrient medium supplemented with the selected amino acid compounds at the different concentrations.

Data indicated that when Tyrosine was added to culture nutrient medium, the highest significant result was obtained in increasing shoot number/explant (5.25) followed by the result of addition of Arginine or addition of Glutamine (4.50 and 4.25, respectively without significant re-

sults in between). The interaction between concentration of amino acids and the type of amino acids exhibited significant results where the addition of tyrosine at (25 mg/L) to the culture nutrient medium was the highest significant result in stimulating the number of shoots/explant (8.00).

Shoot length

Data in Table (2), clearly revealed that there was no significant difference among the tested concentrations (25, 50 and 100 mg/L) of each amino acid type added to culture nutrient medium an increase in shoot length/explant after two subcultures during multiplication stage. However, tyrosine was the best amino acid added to nutrient medium with regards to inducing the shoot length of (*Fragaria* X *Ananassa* Duch cv. Chandler) cultured explants during multiplication stage. Regarding the interaction of the different concentrations of amino acids and types of amino acids tyrosine gave the most significant difference at (50 and 100 mg/L) in increasing in shoot lengths of the cultured explants of (*Fragaria* X *Ananassa* Duch cv. Chandler).

Root number

Data in Table (3) and Fig. (2) revealed that the significant effect of the studied concentrations for the three selected amino acids (Tyrosine, Arginine and glutamine) when supplemented to the nutrient medium increased the root number of the cultured explants after six weeks during the rooting stages. It was clearly observed that when the lowest concentration (25 mg/L) was added to nutrient medium for each of the selected amino acid compounds, the highest significant results for increasing the root number/explant were obtained (4.55). There were no significant differences between the supplementation of the three selected types of amino acids to the nutrient medium at (50 and 100 mg/L) with regards to the development of new roots for the cultured explants of (*Fragaria* X *Ananassa* Duch cv. Chandler).

When assessing the effect of the amino acid types it can be noted from the data that tyrosine was the most preferred amino acid added to culture nutrient medium for increasing the root number/explant significantly especially when compared with glutamine supplementation. Control mediums showed no enhancement in the development of new roots for the cultured explants after six weeks during the rooting stages. Data interaction studies between the numerous concentrations and assessed types of amino acid compounds illustrated that tyrosine at (25 mg/L) gave the highest significant result for increasing root number/explant of (*Fragaria* X *Ananassa* Duch cv. Chandler).

Root lengths

Data in Table (4) demonstrated that after six weeks during rooting stages the addition of any studied amino acid compounds at the different studied concentrations to nutrient medium enhanced significantly the root length of the cultured explant when compared to the control and

other treatments. Also, tyrosine was the best amino acid added to nutrient culture medium in significantly enhancing the root length/explant at (1.97) when compared to the other investigated amino acid compounds Arginine and Glutamine (1.33 and 0.91, respectively).

Root number

Regarding to Table (5), data indicated that increases in the root number/explant after six weeks of culturing at greenhouse conditions for the (*Fragaria* X *Ananassa* Duch cv. Chandler) plantlets received from the nutrient culture medium supplemented with (25 mg/L) for each tested amino acid compound the most significant result in increasing in root number/ explant (5.33) followed by the results obtained when (50 or 100 mg/L) were added to culture nutrient medium for each tested amino compound (4.22 and 3.44, respectively). It was observed that plantlets received from the control treatment exhibited the worst result in increasing root number/explant after six weeks of culturing in the greenhouse.

For data in Table (5), tyrosine addition to the culture nutrient medium during the *in vitro* propagation stage illustrated the highest significant result in increasing the root number/explant for growing plantlets during the acclimatization stage. Interaction studies among the treatments proved that the residual effect of tyrosine at (25 mg/L) stimulated the increase in root number/ explant for the received plantlets of (*Fragaria* X *Ananassa* Duch cv. Chandler) growing in greenhouse to achieve the highest results.

Root length

Data in Table (6) showed that increase the root length of the received plantlets development for six weeks during acclimatization stage affected the previous treatments of different selected amino acid concentrations significantly at (25, 50 or 100 mg/L) in the nutrient culture medium during *in vitro* growth without significant differences between each amino acid concentrations. But they were still significantly higher in stimulating the increase in root length/explant for received plantlets when compared to the control treatment that showed the shortest root length/explant (1.33) for plantlets growing *in vivo*.

According to the residual effect of type of amino acid supplemented in nutrient culture medium during *in vivo* stage on the root length of received plantlets in greenhouse for six weeks. Tyrosine amino acid gave the highest significant result as shown in Table (6) (2.083) where glutamine addition gave the lowest result (1.25) in stimulating root length of received plantlets. It can be noted here that there was a significant effect among the studied interaction treatment; data revealed that plantlets received from tyrosine at (50 mg/L) showed the best results in increasing root length during the greenhouse growth stage after six weeks.

DISCUSSION

This investigation clearly established that amino acids play a vital role in the induction and development of the maximum number of multiple shoots. The results demonstrated that factors such as the type of amino acids and the amounts employed in the culturing process provided significant effects on the induction of multiple shoots. Tyrosine (25 mg/L) promoted the highest shoot and root stimulation amongst the examined amino acids. Previously conducted studies have shown a positive effect of amino acids on shooting processes (Karlidag *et al.*, 2009; Martin-Mex *et al.*, 2005).

In the present study, during the acclimatization stage the plantlets were treated with varying concentrations of several amino acids. Subsequently, the successfully grown plantlets were transferred to the greenhouse to examine its *in vivo* induction once the medium conditions were altered. The amino acid tyrosine provided the best results in terms of both leaf as well as root numbers. Cultured cells are normally capable of synthesizing all of the essential amino acids, the addition of certain amino acids or amino acid mixtures may be used to further stimulate cell growth. The use of amino acids is particularly important for establishing cell cultures and protoplast cultures (Amin *et al.*, 2007; Karlidag *et al.*, 2009).

Amino acids provide plant cells with an immediate source of nitrogen, which can generally be taken up by the cells more rapidly than inorganic nitrogen (El-Shiaty *et al.*, 2004). Nitrogen originating from amino acids is assimilated quicker into the carbonic skeletons during the metabolism and synthesis of the proteins, when compared to other inorganic N sources. Oliva *et al.* (2014) and Kadhimi *et al*. (2014) reported that for strawberry *in vitro* production, in order to get disease-free plants and harvest plants that are identical to the parent or the manufacture of new kinds with a potent potential the micropropagation strawberries is affected by the essential basics of the media, such as hormones, vitamins, amino acids, the physical state and the power source to the media.

In this investigation the effect of the three selected amino acids compounds (Tyrosine, Arginine, or Glutamine) at different tested concentrations showed significant results according to their influences on the multiplication stage,rooting stage and acclimatization stage of (*Fragaria* X *Ananassa* Duch cv. Chandler). It could be indicated that at 25 mg/L among other tested concentrations (0, 50 and 100 mg/L) for any selected amino acids (Tyrosine, Arginine, or Glutamine) supplemented in culture nutrient medium the highest significant results were achieved during multiplication stage, rooting stage and acclimatization stage as mentioned by Amin *et al.* (2007) and Karlidag *et al.* (2009) that cultured cells are normally capable of synthesizing all of the essential amino acids, the addition of certain amino acids or amino acid mix-

tures may be used to further stimulate cell growth. Also the optimal required concentration is essential for optimal growth. El-Shiaty *et al*. (2004) confirmed that when amino acids are added alone, care must be taken, as they can be inhibitory to cell growth. Examples of amino acids that are generally harnessed in the culture media to enhance cell growth are glycine at 2mg/liter, glutamine up to 8 mM, asparagine at 100 mg/liter, L-arginine and cysteine at 10 mg/liter and L-tyrosine at 100 mg/liter.

Optimization of the amino acid content can bring about different morphogenetic responses however; higher concentrations of amino acids have been shown to be general growth inhibitors in *Cicerarietinum* (John and Mukherjee, 1997) However tyrosine amino acid exhibited high potential to induce the regeneration of strawberry explants during multiplication stage, rooting stage and acclimatization stage as shown in studied Data which indicated that when tyrosine was added to culture nutrient medium at 25 mg/L the highest significant results were obtained in increasing in shoots and roots number /explant. This was on line with Yamada *et al.* (1986) stated that tyrosine has been used to stimulate morphogenesis in cell cultures but should only be used in an agar medium. The role of amino acids in growth and differentiation is known to a considerable extent. The effect of the tyrosine amino acid and the results that demonstrated the highest efficacy are correlated towards the work of (Bayya, 2002) where they found that supplementing the culture medium with glutamine, tyrosine and asparagine encouraged for maximum explants development and direct regeneration of date palm as the most significant results was obtained with the amino acid tyrosine whereas in other opinion

Ageel and Elmeer (2011) showed that growth of date palm callus tissue was significantly stimulated by the addition of aminoacids specifically glutamine This stimulation suggested that organic nitrogen was a growth-limiting factor in date palm cultures The inclusion of glutamine decreased the culture lag phase, which indicated that glutamine was much more readily assailable than inorganic nitrogen.

Sarker *et al*. (2003) found that 20 µM of tyrosine combined with other growth regulators showed successful regeneration and *in vitro* rooting. This is also quite apparent in the present study where the most successful rooting properties were shown in the amino acid tyrosine, whether in terms of root numbers or root lengths. According to Oliveira *et al.* (2009), direct amino acid absorption by the roots offers an advantage to plants because they do not need to metabolize the mineral nitrogen (nitrate and ammonium), thus directing more energy for rooting. However, excess amino acids can cause substrate acidification, which could lead to plant toxicity.

CONCLUSION

This paper highlighted on the effect of different types of amino acids at differ-

ent studied concentrations added to MS nutrient medium during the regeneration of (*Fragaria* X *Ananassa* Duch cv. Chandler) which approved that tyrosine amino acid exhibited high potential to induce the regeneration of strawberry explants during multiplication stage, rooting stage and acclimatization stage when added to culture nutrient medium at 25 mg/L. The available information from different studies suggests that optimal concentrations of different amino acids may be species or genotype-dependent, which needs to be determined before recommending its use. There is insufficient studies on the effect of different amino acids on strawberry micropropagation much efforts is need in this approach in order to receive plantlets with high quality. This study has provisioned successful results however the assessment of a larger interval of concentrations could have provided more significant results and enabled various statistical analysis techniques for the assessment of efficiency that was not possible with these experiments.

SUMMARY

The Present study was carried out for the purpose of enhancing strawberries (*Fragaria* X *Ananassa* Duch cv. Chandler) *via In vitro* regeneration. Since amino acid compounds provide a vital role in plant tissue culturing, this study was conducted for the purpose of identifying the optimum conditions and concentrations from three selected amino acid compounds. Tyrosine, Arginine, and Glutamine were added at different concentrations (0, 25 50 and 100 mg/L), separately and supplemented with standard MS nutrient culture medium. The results demonstrated that tyrosine at (25 mg/L) significantly promoted the shooting and rooting growths as well as the acclimatization stage (*in vivo*) development. Supplementation of the MS culture medium with arginine and/or glutamine at the assessed concentrations yielded results that were much better than the control culture nutrient medium for the induction of the shooting and rooting stages providing healthy plantlets.

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Table (1): The Effect of different types of amino acids compounds at different concentration supplemented in MS nutrient medium on shoot number of cultured explant of (*Fragaria X Ananassa* Duch cv. Chandler) after two subculture during multiplication stage.

Amino acid concentrations mg/L (A)	Amino acid (B)			Mean
	Tyrosine	Arginine	Glutamine	
Control	3.00	3.00	3.00	3.00 d
25	8.00	7.00	5.00	6.66 a
50	6.00	5.00	5.00	5.33 b
100	4.00	3.00	4.00	3.66 c
Mean	5.25 a	4.50 b	4.25 b	

LSD at 0.05% A = 0.3605 B = .4163 AB = 1.210.
Means with the same letters are not significant at 0.05 level of significant.

Table (2): The Effect of different type of amino acids compounds at different concentration supplemented in MS nutrient medium on shoot length of cultured explant of (*Fragaria* X *Ananassa* Duch cv. Chandler) after two subcultures during multiplication stage.

Amino acid concentrations mg/L (A)	Amino acid (B)			Mean
	Tyrosine	Arginine	Glutamine	
Control	1.23	1.23	1.23	1.23 b
25	2.00	2.00	1.00	1.66 a
50	2.50	1.66	0.83	1.66 a
100	2.83	1.33	0.41	1.52 ab
Mean	2.14 a	1.55 b	0.87 c	

LSD at 0.05% A=0.3408 B=0.393 AB=0.6815
Means with the same letters are not significant at 0.05 level of significant.

Table (3): Effect of different types of amino acids compounds at different concentration supplemented in MS nutrient medium on root number of cultured explant of (*Fragaria* X *Ananassa* Duch cv. Chandler) after six weeks of the rooting stage.

Amino acid concentrations mg/L (A)	Amino acid (B)			Mean
	Tyrosine	Arginine	Glutamine	
Control	1.00	1.00	1.00	1.00 c
25	6.00	5.33	2.33	4.55 a
50	5.00	4.00	1.33	3.44 b
100	4.66	3.33	1.00	3.00 b
Mean	4.16 a	3.41 a	1.41 b	

LSD = 0.05% A=0.6547 B= 0.7560 AB=1.309
Means with the same letters are not significant at 0.05 level of significant.

Table (4): The effect of different types of amino acid compounds at different concentrations supplemented in MS nutrient medium on root length of cultured explant of (*Fragaria* X *Ananassa* Duch cv. Chandler) after six weeks during rooting stage.

Amino acid concentration mg/L (A)	Amino acid (B)			Mean
	Tyrosine	Arginine	Glutamine	
Control	1.00	1.00	1.00	1.00 b
25	2.25	1.58	1.00	1.61 a
50	2.33	1.41	0.91	1.55 a
100	2.33	1.33	0.75	1.47 a
Mean	1.97 a	1.33 b	0.91 c	

LSD = 0.05% A=0.2074 B= 0.2395 AB=0.4148

Means with the same letters are not significant at 0.05 level of significant.

Table (5): The residual effect of adding different concentrations of different studied type of amino acids compounds to MS cultural nutrient medium on increasing in root number of (*Fragaria* X *Ananassa* Duch cv. Chandler) plantlets after four weeks during acclimatization stage.

Amino acid concentration mg/L (A)	Amino acid (B)			Mean
	Tyrosine	Arginine	Glutamine	
Control	2.00	2.00	2.00	2.00 c
25	7.00	6.33	2.67	5.33 a
50	6.00	4.67	2.00	4.22 b
100	5.00	3.67	1.67	3.44 b
Mean	5.00 a	4.16 b	2.08 c	

LSD =0.05% A= 0.9771 B=0.5642 AB=0.9771

Means with the same letters are not significant at 0.05 level of significant.

Table (6): The residual effect of adding different concentrations of different studied type of amino acids compounds to MS cultural nutrient medium on increasing in root length of (*Fragaria* X *Ananassa* Duch cv. Chandler) plantlets after four weeks during acclimatization stage.

Amino acid concentration mg/L (A)	Amino acid (B)			Mean
	Tyrosine	Arginine	Glutamine	
Control	1.33	1.33	1.33	1.33 b
25	2.33	2.00	1.33	1.88 a
50	2.50	1.66	1.16	1.77 a
100	2.16	1.83	1.16	1.72 a
Mean	2.08 a	1.70 b	1.25 c	

L.S.D =0.05% A= 0.2981 B= 0.3443 AB=0.5963

Means with the same letters are not significant at 0.05 level of significant.

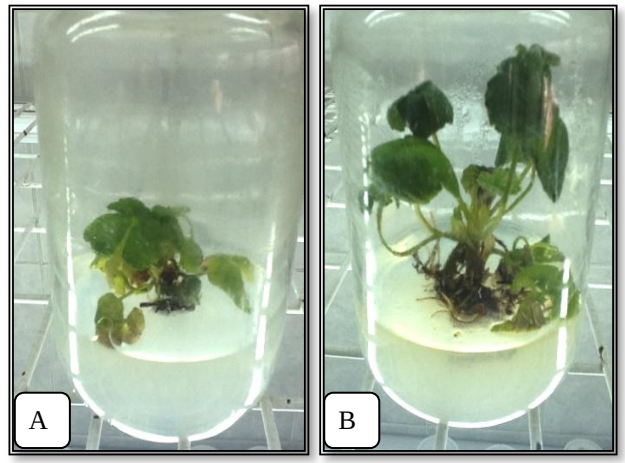

Fig. (1): (A) Shoot number on control MS medium (B) Shoot number on MS medium supplemented with 25 mg/L tyrosin.

Fig. (2): (A) Root number on control MS medium. (B) Root number on MS medium supplemented with 25 mg/L tyrosine.