IN VITRO PROPAGATION OF F1 MALE HYBRID LINES IN *ASPARAGUS OFFICINALIS*

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For mass production of super male hybrid lines of *Asparagus officinalis* (Mary Washington 500 W' CV.), *in vitro* propagation system was initiated to obtain a sterile, healthy culture using shoot tip and single-node spear. Explants established on Murashige and Skoog (MS) medium supplemented with kinetin (KIN) at 1 mg l^{-1}, naphthalene acetic acid (NAA) at 0.2 mg l^{-1} and gibberellic acid (GA_3) at 0.5 mg l^{-1} showed 100% of survival, the highest number of thick, dark green shoots (8.5) and nodes (8.7). Multiplication of shoots was carried out using benzyl amino purine (BAP) or KIN. BAP containing media gave higher shoot multiplication rate with high vitrification ratio in a positive correlation with concentration. The vitrification ratio was declined by replacing BAP with the same concentration of KIN (2 mg l^{-1}). Adding the growth retardant; paclopatrazol (PP333) at 1.0 mg l^{-1} completely eliminated the vitrification. Direct somatic embryos from stem segment (internode) induced on MS medium supplemented with BAP at 1 mg l^{-1} and NAA at 0.1 mg l^{-1} and seedling regeneration occurred on MS medium with KIN at 1 mg l^{-1} and GA_3 at 0.5 mg l^{-1}. Medium supplemented with NAA, indole-3-butyric acid (IBA), PP333 and *Aspergillus niger* extract were tested for rooting. MS medium supplemented with IBA at 1.0 mg l^{-1} and PP333 at 0.5 mg l^{-1} was optimum for producing white-rooted plants (79.5%) within 54 days from old clusters of shoots, which were better, compared to young single shoots. Healthy rooted clusters from each treatment were successfully transferred to growth champers then greenhouse for acclimatization.

Keywords: micropropagation, direct somatic embryo, vitrification, rooting, paclopatrazol, *Aspergillus niger* extract

Asparagus officinalis L. is belonging to Liliaceae family (Prohens et al., 2008). The most economically important Asparagus species is garden Asparagus (*A. officinalis*), which is a highly prized vegetable (Stajner et al., 2002 and Afroz and Abul Hassan, 2008), grown in temperate climate regions

worldwide and widely cultivated, because of its edible spears of high economic, medicinal and food value. *A. officinalis* is native originated mainly from Asia, Africa and Europe (Prohens et al., 2008). It has been introduced and become naturalized in some parts of Europe, across Australasia and parts of North and South America (USDA, ARS, and National Genetic Resources Program, 2011). Asparagus is dioecious and highly heterozygous, traditionally propagated by seeds and the resulting seedlings contain equal proportions of male and female plants. Generally, male plants have more commercial advantages over the female plants. Male plants give much higher yields, are less susceptible to diseases than female plants and are longer lived. Female plants put much of their energy into producing copious quantities of seed (Lo´pez-Anido and Cointry, 2008 and Sarabi and Almasi, 2010). The female and super male parents cannot be propagated by seed, they can only be preserved by vegetative or asexual propagation (Chen, 2015). Male plants will never produce seeds, in order to obtain all-male hybrids; the production of super male is the key. Nevertheless, super male rarely exists in nature. Clonal propagation by division of the Asparagus crowns is possible, but the multiplication rate is low under optimal condition. In one year, only 2-4 new plants produce from one plant. When a superior male hybrid is identified, tissue culture is necessary, quick and a safe solution for large-scale production of the parents (Reuther, 1984).

In vitro propagation systems may be useful to develop virus eliminated and genetically stable plants. Expansively studies were carried out on several species of Asparagus that are considered as ornamental or medicinal plants such as *A.s officinalis*, through micropropagation, organogenesis and somatic embryogenesis (Stajner, 2013 and Carmona-Martin et al., 2014). *A. officinalis* is the first monocotyledonous species showed regeneration via somatic embryogenesis (Hisato and Masahiro, 1998). Direct somatic embryogenesis induced by tissue culture has a great potential for rapid and efficient clonal propagation. Somatic embryo involves the development of embryos from somatic cells and production of complete somatic plantlet. Somatic embryo maintains genetic stability in Asparagus and many studies focused on the production of direct somatic embryos (Levi and Sink, 1991; Conner and Abernethy, 1996 and Mustafa and Muhammad, 2017). Vitrification phenomenon (glassiness) or the malformation problem appears through multiplication stage, resulting in the occurrence of short and less chlorophyll stems in many kinds of plants (Phan and Hegedus, 1986). In vitrified plantlets, leaves have large vacuolated mesophyll cells, fewer stomata and less photosynthetic capacity. Their stem and leaves are often rigid, thick and breakable (Kevers et al., 2004 and Sharma and Mohan, 2006). Vitrified plants show difficulties in rooting and acclimatization and normal growing (Yadav et al., 2003 and Kevers et al., 2004). Growth retardants are able to modify the growth of plants when used

exogenously (Grossmann et al., 1985). Khunachak et al. (1987) used paclobutrazol (PP_{333}) as a growth retardant for improving Asparagus shoot-tips culture.

Several authors pointed out that major obstacle of Asparagus micropropagation protocols is not the establishment and multiplication of shoots, but root initiation. None of the existing pathways for large commercial scale propagation of Asparagus is useful, as regenerated plantlets have poor survival hardening rate (Desjardins, 1992). Asparagus plants require vigorously growing roots for successful transplantation, the obstacles of Asparagus rooting *in vitro* can be categorized into three aspects: 1. low rooting rate, 2. big mount of abnormal roots, 3. low survival rate after transplanting to the greenhouse (Ren et al., 2012). From which shoots and roots are formed *in vitro*, two types of roots are formed: one is a white and vigorous root; the other is a transparent and less active root. The latter is easily formed but has no function as a root (Gonzalez, 2002; Lin et al., 2008 and Saharan, 2010). Also, auxin plays a key role in rooting, and exogenous auxins are required in many species. In many commercial propagation systems of other species, indole-3-butyric acid (IBA) is commercially used to induce roots on stem cuttings. IBA is most common used in Asparagus rooting *in vitro* (Chen, 2007; Behrooz and Kaveh, 2010 and Wang et al., 2010). Plant growth retardants are regarded as the most important group of plant growth regulators, their use in culture media significantly enhanced Asparagus *in vitro* culture and the field transplants (Rademacher, 2000). Incorporation of growth retardants can promote the growth of strong shoots and roots of Asparagus. Khunachak et al. (1987) investigated the effectiveness of some growth retardants including ancymidol, B-995, phosfon, Amo 1618, Cycocel (CCC) and PP_{333}.

Using fungus extracts as a potential for rooting of *in vitro* propagated culture is well known (other classes of root symbionts have the same potential, as reviewed by Nowak (1998). Paulo et al. (2003) reached rooting induction with a combination of auxin and hypertonic shock *in vitro*. *Aspergillus niger* is one of the most common species of the genus *Aspergillus*. *Aspergillus* species are known to produce a variety of secondary metabolites *in vivo* or *in vitro*, that act as co-markers in the taxonomy of Aspergilla, biocontrol effectiveness, root induction (Siddiqui et al., 2004). Moreover, *Aspergillus niger* is one of the fungi labelled with the GRAS (generally recognized as safe) status from the US Food and Drug Administration (Powell et al., 1994). It was previously used as an elicitation to enhance the accumulation of secondary metabolites in *in vitro* culture of several plant species (Mendhulkar and Vakil, 2013). Thus, it would appear likely that certain combinations of *Aspergillus niger* and procedures of micropropagation could be exploited for the improvement of root development of *A. officinalis in vitro*.

Not all types of explants are favorable for root stimulation. Fortes et al. (1997) studied the influence of young shoot number of Asparagus on rooting. It was reported that four-shoot explants rooted better than those with one, two or three shoots. Longer roots were also observed from the explant with four shoots. Wu et al. (2006) investigated the effect of plantlet age on the rooting rate. They found that plantlets older than four weeks (8, 12, 16 and 20 weeks) could have more rooting percentage (>75%) after sub culturing for two weeks or more.

The objective of this investigation was to develop an efficient, and economic *in vitro* propagation protocol, for rapid and large-scale propagation of *A. officinalis* high productivity male hybrid, as recommended for commercial production, as well as overcoming the problems of *in vitro* propagation of Asparagus, especially vitrification and rooting.

MATERIALS AND METHODS

The experiments were carried out in the Plant Tissue Culture Unit, Department of Genetic Resources, Desert Research Center, Cairo, Egypt.

1. Preparation of Plant Material

A. officinalis (Mary Washington 500 W' CV.) seeds were sown in an artificial medium (Peatmoss, vermiculite and sand) in 10 cm diameter pots (two seeds in each pot). After four weeks, plantlets were transferred to 20 cm diameter pots and one plantlet per pot.

Nodal segments of young spears, 8-10 cm long, from male hybrid line 10- to 12-week-old transplants, were taken as explants and washed carefully by soup solution and rinsed under tap water for about 40 min. Surface sterilization was carried out by immersing them in 0.05 mg l^{-1} mercuric chloride ($HgCl_2$) solution for 10 min. Then, explants were rinsed three times in double sterile distilled water. Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with vitamins supplemented with 100 mg l^{-1} myo-inositol, 30 g l^{-1} sources, 200 mg l^{-1} glutamine and 2.5 g ml^{-1} phytagel was used in all experiments with different plant growth regulators (PGRs) according to each stge. The pH of medium was adjusted to 5.7-5.8 prior to autoclaving (15 min at 121℃ and 1.5 kg cm^{-2} pressure). The cultures were incubated in a growth chamber at 25±2℃ at a 16-h photoperiod (2500-3000 Lux) provided by cool-white fluorescent lamps.

2. Establishment Stage

Sterilized shoot tips and node segments were cut into 0.3-0.5 cm segments and cultured on MS medium supplemented with different concentrations of kinetin (KIN; 0.5, 1.0 and 1.5 mg l^{-1}) in combination with 0.2 mg l^{-1} naphthalene acetic acid (NAA) and 0.5 mg l^{-1} gibberellic acid (GA_3), in addition to the MS medium without PGR as a control. Five

explants in each jar and three jars were considered as one replicate, and four replicates were used for each treatment. Soot formation percentage, number of shoots per propagule, shoot length and number of nodes were measured after 30 days of culturing.

3. Multiplication Stage

Thick healthy initiated shoots (1-2 cm long with 2-3 nodes) were subcultured on MS medium supplemented with benzyl amino purine (BAP) or KIN (1, 2 and 3 mg l^{-1}) in combination with 0.2 mg l^{-1} NAA and GA_3 at 0.5 mg l^{-1}, in addition to MS medium without PGRs as a control. Explants were subcultured at least six times on the best multiplication medium every 30 days. Four explants in each jar and three jars were considered as one replicate, and four replicates were used for each treatment. Shoot formation percentage, number of shoots per propagule, and shoot length were measured.

4. Overcoming Vitrification

In order to overcome vitrification through multiplication stage BAP was exchanged by KIN (1 mg l^{-1}). The effect of adding PP_{333} at 0.5, 1.0, 1.5 and 2.0 mg l^{-1} was examined. Four culture tubes with one explant were considered as one replicate, and four replicates were used for each treatment. Survival percentage, normal explant percentage, shoot length and number of proliferated shoots were measured after 30 days of culturing.

5. Induction of Direct Somatic Embryos

Direct embryogenesis induction was examined from stem segment within 6-8 weeks on MS medium supplemented with BAP (0.5, 1.0, 1.5 and 2.0 mg l^{-1}) and NAA at 0.1 mg l^{-1}. Seedling regeneration was examined on MS media supplemented with 1 mg l^{-1} KIN and 0.1 mg l^{-1} NAA. Six explants in each jar and three jars were considered as one replicate, three replicates were used in four treatments. Number of induced somatic embryos, germination percentage and shoots length were measured after 75 days of culturing.

6. Rooting Stage

Three experiments were conducted for inducing healthy white-rooted *A. officinalis* plantlets, using clusters of 5-8 shoots. MS free medium or supplemented with NAA (0.5, 1.0) mg l^{-1} and IBA (0.5, 1.0 and 1.5 mg l^{-1}) or IBA at 1.0 mg l^{-1} with PP_{333} (0.5 and 1.0 mg l^{-1}). As well using *Aspergillus niger* fungus extract at 0, 50, 100, 150, 200, 250 and 500 mg l^{-1}. *Aspergillus niger* fungus extract was prepared according to Taha et al. (2009). Type of explants (young single shoot or old cluster of shoots) effect was also evaluated for rooting. Four culture tubes and four baby jars with one cluster of shoots were considered as one replicate, and three replicates

were used for each treatment. Time to induce roots, percentage of rooted explants, number of roots per plantlet and root length for white and transparent rooted explants were recorded after 20 weeks.

7. Acclimatization Stage

Healthy rooted clusters were transferred into 1:1 (v/v) sand: peat sterilized mixture in jars *in vitro* for two weeks and then transferred into small pots (10 cm) in diameter, under *in vivo* conditions. The pots were covered with polyethylene bags for 2-3 weeks to maintain humidity. Then plantlets were transferred into large pots for 3-4 months.

8. Experimental Design and Statistical Analysis

This study was performed in completely randomized design. Mean comparisons were conducted with Duncan's multiple range test and the statistical analysis was analyzed with SPSS software. All the primary observation data were registered in Microsoft EXCEL software.

RESULTS AND DISCUSSION

Seedlings of F1 male hybrid lines 10- to 12-week-old of *A. officinalis* cv. Mary Washington 500 W', were efficiently regenerated *in vitro*, using MS medium with or without different PGRs through five experiments.

1. Establishment Stage

Shoot tip and node segment explants were established on MS control medium or supplemented with different concentrations (0, 0.5, 1.0 and 1.5 mg l^{-1}) of KIN combined with 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} GA_3. Results in table (1) indicate that no significant differences between treatments for the percentage of shoot formation were recorded and it reached 100% on MS medium supplemented with 0.5 and 1.0 mg l^{-1} KIN. The highest mean number of proliferated shoots/propagule (8.5) and number of nodes (8.7) were recorded at 1.0 mg l^{-1} KIN. However, the highest average shoot length (11.15 cm) was obtained from control treatment (MS medium without PGRs), followed by 0.5 and 1.0 mg l^{-1} of KIN, respectively with significant differences between them. In the present study, KIN showed a significant effect on almost all parameters, especially at 1.0 mg l^{-1}. Ammirato (2004) reported that cytokinin at moderate concentrations enhances shoot development, as role for *in vitro* shoot propagation. Also, at low concentration of auxin (NAA) plus moderate concentration of cytokinin (KIN), good development of complete plantlets was shown from meristem tips of potato (Badoni and Chauhan, 2009). Similar results were also found by Guo et al. (1993), who reported that MS medium supplemented with KIN

and NAA was more suitable for axillary and lateral buds from selected *A. officinalis* male plants in order to optimize proliferation ability.

Table (1). Effects of different levels of KIN on establishment stage of *A. officinalis* after 30 days of culturing on MS medium supplemented with 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} GA_3.

KIN level (mg l^{-1})	Shoot formation (%)	No. of shoots/ propagule	Shoot length (cm)	No. of nodes/shoot	Notes
0.00	98.50[a]	6.88[b]	11.15[a]	8.56[a]	Thin, light green explants
0.50	100.00[a]	7.62[ab]	9.58[b]	7.96[a]	Slightly thick, green explants
1.00	100.00[a]	8.50[a]	8.85[c]	8.70[a]	Thick, dark green explants
1.50	99.25[a]	8.25[a]	6.58[b]	6.05[b]	Thick, dark green explants

Mean values followed by the same letter within a column are not significantly different. *P*= 0.05 = Least significant difference test at 0.05 level of probability.

2. Multiplication Stage

Mass production of *A. officinalis* was induced in the multiplication stage by subculturing *in vitro* propagated healthy stem segment for six times at least. MS medium without PGR (control) in addition to that supplemented with BAP or KIN at 1, 2 and 3 mg l^{-1} in combination with 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} GA_3 were used. Data presented in table (2) illustrate that, shoot formation percentage reached 100% with each of 1.0, 2.0 and 3.0 mg l^{-1} BAP and 1.0 and 2.0 mg l^{-1} KIN. It was observed that, BAP containing medium gave the maximum values of shoot number (9.5), and shoot length (12.13 cm) at 2.0 mg l^{-1}, but with high vitrification. On the other hand, KIN containing medium in the same concentration of 2.0 mg l^{-1} gave lower values (8.06 shoots and 8.62 cm length) but induced explants, which were normal, hard and green (Fig. 1). These results agree with Nemati et al. (2011), who focusing on the problem of vitrification in carnation (*Dianthus caryophyllus* L.), and reported that, there is a positive correlation between the concentration of cytokinin and vitrification, and adding BAP into the media resulted in more shoot multiplication and vitrification than KIN.

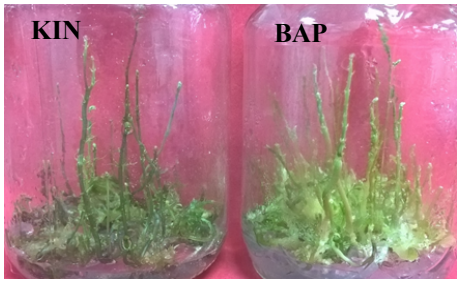

Fig (1). A composition between the effect of KIN and BAP at 2.0 mg l^{-1} on *A. officinalis* in the multiplication stage.

Table (2). Effect of different levels of BAP and KIN on the multiplication stage of *A. officinalis,* on MS medium supplemented with 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} GA_3.

Treatments	Shoot formation (%)	No. of shoots/ propagule	Shoots length (cm)	Notes
Control	99.72[a]	5.01[d]	10.62[b]	Normal soft explants
1.0 mg l^{-1} BAP	100.00[a]	7.07[c]	11.40[ab]	Soft explants with slight vitrification
2.0 mg l^{-1} BAP	100.00[a]	9.50[a]	12.13[a]	Soft, light green explants with vitrification
3.0 mg l^{-1} BAP	100.00[a]	8.15[b]	10.58[b]	Very soft, light green explants with high vitrification
1.0 mg l^{-1} KIN	100.00[a]	7.58[bc]	8.45[c]	Normal, green explants
2.0 mg l^{-1} KIN	100.00[a]	8.06[b]	8.62[c]	Normal hard, green explants
3.0 mg l^{-1} KIN	96.75[b]	7.50[bc]	6.99[d]	Hard, dark green explants with slightly vitrification

Mean values followed by the same letter within a column are not significantly different. *P*= 0.05 = Least significant difference test at 0.05 level of probability.

3. Vitrification

Regarding vitrification or malformation problem, which appears during the multiplication stage, in the present study, BAP was replaced by KIN to overcoming vitrification. Results recorded in table (3) exhibit the effect of adding PP_{333} to the multiplication medium at 0.5, 1.0, 1.5 and 2.0 mg l^{-1}. Concerning the survival percentage, it recorded 100% on MS

(control) medium without PGRs and that supplemented with 0.5 mg l^{-1} PP_{333}, and then decreased in a negative correlation with PP_{333} levels, with significant differences. The normal explants percentage was increased significantly in a positive relationship with PP_{333} levels and gave the highest percentage of 100% at media containing 1.5 and 2.0 mg l^{-1} PP_{333}. Shoot length and number of proliferated shoots gave their highest mean values (10.75 cm and 7.81, respectively) at MS medium without PGRs (control) and the lowest mean values at 2.0 mg l^{-1} PP_{333} in a significantly negative correlation. Although, from the obtained results, it can be noticed that, PP_{333} at 1.0 mg l^{-1} was the optimum concentration with moderate mean values of survival (87.8%), normal explants percentage (88%), shoot length (7.62 cm) and number of proliferated shoots (3.94), with normal thick, dark green explants (Fig. 2). These results are explained by Chin (1982), who referred that addition of growth retardants and gibberellin inhibitors improve shoot and root thickness and plantlets can survive when transferred to soil. Also, Khunachak et al. (1987) used PP_{333} as a growth retardant to improve *A. officinalis* shoot-tip culture. In the same trend, Guo et al. (1993) reported that the combinations of PP_{333}, IBA, NAA and KIN on MS medium can be applied on axillary bud proliferation.

Table (3). Effect of different levels of PP_{333} on vitrification of *A. officinalis* shoots after 30 days of culturing on MS medium supplemented with KIN.

PP_{333} level (mg l^{-1})	**Survival (%)**	**Normal explants (%)**	**Shoot length (cm)**	**No. of shoots/jar**	**Notes**
0.0	100.00[a]	46.75[d]	10.75[a]	7.81[a]	Thin, Light green and vitrified explant
0.5	100.00[a]	73.31[c]	8.31[b]	6.15[b]	Thin, hard, green and lower vitrified explants
1.0	87.80[b]	88.00[b]	7.62[b]	3.94[c]	Normal thick, dark green explants with root
1.5	80.89[c]	100.00[a]	6.44[c]	1.81[d]	Thick, short and dark green explants with root
2.0	62.75[d]	100.00[a]	3.02[d]	1.31[d]	Hard, very short and yellow green explants with root

Mean values followed by the same letter within a column are not significantly different. *P*= 0.05 = Least significant difference test at 0.05 level of probability.

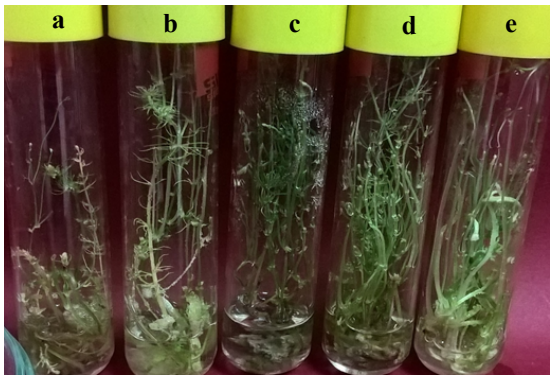

Fig. (2). Shoots of *A. officinalis* on MS medium supplemented with deferent levels of PP_{333}, **a.** 2.0 mg l^{-1} PP_{333}, **b.** 1.5 mg l^{-1} PP_{333}, **c.** 1.0 mg l^{-1} PP_{333}, **d.** 0.5 mg $l^{-1}PP_{333}$, and **e.** control treatment.

4. Direct Somatic Embryogenesis

Direct somatic embryogenesis involves the development of embryos from somatic cells and results in the production of complete plantlet with the potential to grow into a whole plant. Direct somatic embryos were induced from stem segment explants on MS medium without PGRs (control) or with BAP (0.5, 1.0, and 2.0 mg l^{-1}). Data presented in table (4) and fig. (3) illustrate that, MS medium supplemented with 1.0 mg l^{-1} of BAP and NAA at 0.1 mg l^{-1} was the most suitable medium for inducing direct somatic embryogenesis in *A. officinalis*, which gave the highest mean number of induced somatic embryos (87 per jar) and percentage of germinated somatic embryos (93%) with significant differences between other treatments (Fig. 4). Mean length of germinated shoots were increased significantly with BAP concentration in a positive correlation. The highest mean value was 5.56 cm, recorded at 2.0 mg l^{-1}. Similar results are investigated by Bojnauth et al. (2003), who induced somatic embryogenesis from nodal explants of *A. officinalis* on MS medium supplemented with 0.015 mg l^{-1} of NAA and BAP at 0.5 mg l^{-1}, which gave the highest plantlet regeneration rate (83.3%). Also, it was noticed that adding 0.5 mg l^{-1} of PP_{333} reduced vitrification during somatic induction and germination (Fig. 4).

Table (4). Effects of different levels of BAP on direct induction and regeneration of somatic embryos from internodal explant on MS medium.

BAP level (mg l^{-1})	No. of induced somatic embryos	Germination (%)	Shoot length (cm)	Notes
0.0	0.00	0.00	0.00	Shoots proliferation with callus
0.5	63.00^{b}	84.46^{b}	4.56^{b}	Direct somatic embryos inducing internodes
1.0	87.00^{a}	93.00^{a}	5.18^{ab}	Direct somatic embryos without vitrification
2.0	49.00^{c}	65.90^{c}	5.56^{a}	Direct somatic embryos with vitrification

Mean values followed by the same letter within a column are not significantly different. *P*= 0.05 = Least significant difference test at 0.05 level of probability.

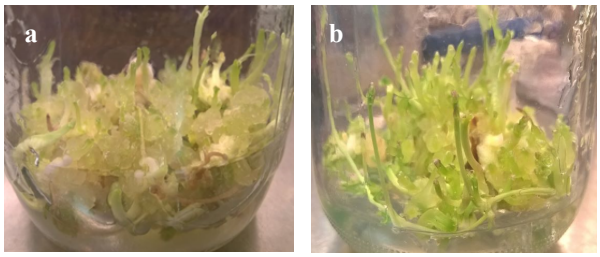

Fig. (3). a. direct somatic induction on MS medium supplemented with 1.0 mg l^{-1} BAP and NAA at 0.1 mg l^{-1}. **b.** Somatic embryos germination on MS medium supplemented with 1.0 mg l^{-1} KIN and NAA at 0.2 mg l^{-1}

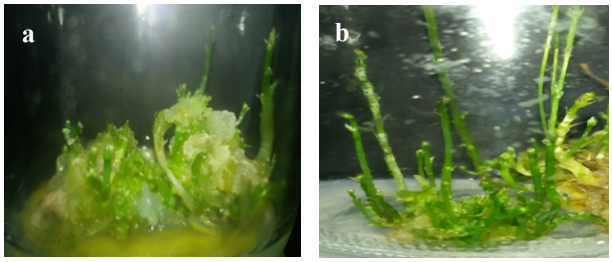

Fig. (4). a. Direct somatic induction on MS medium supplemented with 1.00 mg l^{-1} BAP, NAA at 0.1 mg l^{-1} and 0.5 mg l^{-1}.
b. Somatic embryos germination on MS medium supplemented with 1.00 mg l^{-1} KIN, NAA at 0.2 mg l^{-1} and 0.5 mg l^{-1}.

6. Rooting Stage

For successful transplantation of *A. officinalis in vitro* produced plantlets, it is required to improve vigorous storage roots (white-root). In vitro *A. officinalis* plantlets with few, weak, thin fibrous roots (transparent root) may be a big problem, they not only have poor survival rates, but the growth of the plants that do establish in soil is very slow (Gonzalez, 2002). There are many factors affecting *A. officinalis* rooting. Only with the healthy explant materials, proper culture medium and growth conditions can *A. officinalis* plantlets be produced and later successfully grown in the greenhouse (Ren et al., 2012). Transparent and white-rooted plantlets of *A. officinalis* were induced *in vitro*, using MS medium without PGRs (control) or that supplemented with NAA (0.5 and 1.0 mg l^{-1}), IBA (0.5, 1.0 and 1.5 mg l^{-1}) individually or also IBA at 1.0 mg l^{-1} with the growth retardant PP_{333} (0.5 and 1.0 mg l^{-1}). It is noticeable that the transparent roots were formed first and then followed by white roots. Data presented in table (5) show that, the transparent roots were formed at all treatments at different times. The rooted explants percentage reached 100% at 1.0 mg l^{-1} IBA and 0.5 mg l^{-1} and 1.0 mg l^{-1} PP_{333}, and the highest mean root per plantlet (9.69) also was recorded at 1.0 mg l^{-1} IBA and 0.5 mg l^{-1} PP_{333}, but root length was the highest (13.50 cm) at MS control medium.

Table (5). Effect of different PGRs levels (NAA, IBA and PP_{333}) on rooting stage of *A. officinalis* on MS medium.

Treatments	Time to induce roots (days)	White rooted plantlets			Transparent rooted plantlets		
		Rooted explants (%)	No. of roots/ plantlet	Root length (cm)	Rooted explants (%)	No. of roots/ plantlet	Root length (cm)
Control	143.75^{e}	0.00	0.00	0.00	70.16^{cd}	5.25^{c}	13.50^{f}
0.5 mg l^{-1} NAA	98.75^{d}	0.00	0.00	0.00	49.83^{f}	1.53^{d}	10.42^{e}
1.0 mg l^{-1} NAA	98.00^{d}	0.00	0.00	0.00	54.70^{ef}	1.90^{d}	10.53^{e}
0.5 mg l^{-1} IBA	72.50^{c}	20.3^{d}	1.46^{d}	0.83^{d}	65.10^{de}	5.75^{c}	8.98^{de}
1.0 mg l^{-1} IBA	65.00^{c}	40.50^{c}	2.52^{c}	2.13^{bc}	78.10^{bc}	4.81^{c}	8.24^{cd}
1.5 mg l^{-1} IBA	62.50^{bc}	51.25^{b}	4.25^{b}	2.81^{b}	86.93^{b}	4.85^{c}	7.15^{bc}
1.0 mg l^{-1} IBA+ 0.5 mg l^{-1} PP_{333}	53.75^{ab}	79.50^{a}	5.45^{a}	3.96^{a}	100^{a}	9.69^{a}	6.28^{ab}
1.0 mg l^{-1} IBA+ 1.0 mg l^{-1} PP_{333}	43.75^{a}	74.88^{a}	2.65^{c}	1.94^{c}	100^{a}	7.88^{b}	4.84^{a}

Mean values followed by the same letter within a column are not significantly different. *P*= 0.05 = Least significant difference test at 0.05 level of probability

The time for inducing roots (transparent or white) was decreased significantly by using various PGRs. The longest time period (143.75 days) for inducing roots was MS control medium, and the shortest time period (43.75 days) was 1.0 mg l^{-1} IBA and 1.0 mg l^{-1} PP_{333}. Concerning the effect of the different concentrations of PGRs induced white-rooted plantlets, it was noticed that, the percentage of white-rooted plantlets, number of white roots/plantlet, and white root length were increased significantly by increasing IBA concentration. While, the highest mean values were 79.50%, 5.45 and 3.96 cm, respectively were recorded with 1 mg l^{-1} IBA and 0.5 mg l^{-1} PP_{333} (Fig. 5). Control MS medium and NAA concentrations didn't record any significant values.

A. officinalis micropropagation often resulted in weak plants with spindly roots and shoots, which were difficult to establish in the soil. It observed that addition of PP_{333} as a growth retardant with IBA was effective in inducing white-rooted plantlets and improved shoot and root morphological characters. The similar observation was recorded by Chin (1982), who referred that addition of growth retardants, gibberellin inhibitors, improve shoot and root thickness, and therefore the plantlets can survive when transferred to soil. In the same way, also Ren et al. (2012), reported considerable improvement of shoot development in *A. officinalis* with ancymidol, but PP_{333} gave slightly higher rooting rate than ancymidol. Wu et al. (2006) showed that the growth retardant PP_{333} (1 mg l^{-1}) mixed with BAP as well as KIN were the appropriate medium for rooting culture pretreatment. Also, Alsalihy et al. (2004) found that 1.5 mg l^{-1} of IBA gives the highest number of roots and the longest roots and IBA (0.75 mg l^{-1}) with PP_{333} (0.06 and 0.12 mg l^{-1}) were the optimal for the rooting of peach rootstock. On the other hand, Górecka et al. (1998) found that the highest rooting rate (30%) was observed from *A. officinalis* plantlets cultured on the medium with ancymidol. Where, Behrooz and Kaveh (2010) reported that IBA at 1.5 mg l^{-1} gives higher rooting percentage (43%) of *A. officinalis*. Wang et al. (2010) showed that *A. officinalis* rooting ability had the greatest sensitivity to NAA at 0.2 mg l^{-1}, through which 35.5% rooting rate was obtained. On the contrary, 0.5 mg l^{-1} IBA was more beneficial to the growth of roots.

An experiment was conducted to study the effect of *Aspergillus niger* extract concentration (50, 100, 150, 200, and 250 mg l^{-1}) on white-root induction. Table (6) illustrates that, the highest values of the mean white-root percentage of 32.50% and highest mean white-root number of plantlets (3.00) were recorded at 200 mg l^{-1} of *A. niger* extract. It had no significant effect on inducing white-root explants after 80 days compared to PGRs (IBA and PP_{333}) (Fig. 6). Taha et al. (2009) studied the effect of *Aspergillus niger* as biotic stress on *Catharanthus roseus* callus growth parameters. The best results of callus growth parameters were observed with 0.15% and the highest value of total alkaloids resulted in 0.25% of *Aspergillus niger*.

Also, it was observed that, old cluster of shoots were better compared to young single shoots. Using old cluster of shoots reduced the induction of roots for all treatments. These results are confirmed by Shen et al. (1995), who found that when both a cluster of shoots (with 2–5 shoots) and a single shoot were used as explants, higher rooting percentage (78.9%) was observed from shoot clusters, compared to single shoot tips, which only gave 25.7% rooting. On the other hand, Gebler (2005) stated that none of the single shoots of *A. officinalis* could develop storage roots. Conner et al. (1990) described that the ideal type for micropropagated *A. officinalis* plantlets should consist of an *in vitro* minicrown with 4–6 shoots, 1–2 cm long and 3–4 storage roots, 4–5 cm long.

Fig. (5). Rooting of *A. officinalis* on MS medium supplemented with **a.** IBA at 0.5 mg l^{-1}, **b.** IBA at 1.0 mg l^{-1}, **c.** IBA at 1.5 mg l^{-1}, **d.** IBA at 1.0 mg l^{-1} + PP_{333} at 0.5 mg l^{-1} and **e.** IBA at 1.0 mg l^{-1} + PP_{333} at 1.0 mg l^{-1}.

Table (6). Effects of different concentration of *Aspergillus niger* extract on *in vitro* rooting of *A. officinalis* after 80 days.

***A. niger* extract level (mg l^{-1})**	**White rooted explants**		**Transparent rooted explants**	
	Rooted explants (%)	**No. of roots/ plantlet**	**Rooted explants (%)**	**No. of roots/ plantlet**
Control	0.00	0.00	30.00^{c}	9.67^{a}
50	12.50^{c}	1.00^{bc}	40.00^{bc}	8.50^{ab}
100	16.25^{c}	2.00^{ab}	45.00^{bc}	10.50^{a}
150	20.00^{bc}	1.50^{b}	56.25^{ab}	5.00^{b}
200	32.50^{a}	3.00^{a}	65.00^{ab}	4.50^{b}
250	27.50^{ab}	1.75^{b}	72.50^{a}	7.25^{ab}

Mean values followed by the same letter within a column are not significantly different. *P*= 0.05 = Least significant difference test at 0.05 level of probability.

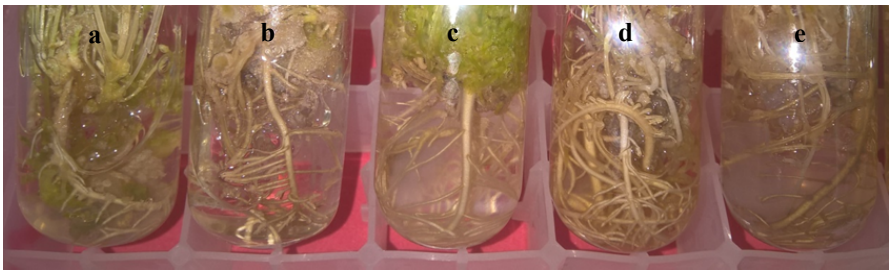

Fig. (6). Rooting of *A. officinalis* using different concentrations of *Aspergillus niger* extract, **a.** 50 mg l^{-1}, **b.** 100 mg l^{-1}, **c.** 150 mg l^{-1}, **d.** 200 mg l^{-1} and **e.** 250 mg l^{-1}.

6. Acclimatization stage

Healthy rooted clusters were successfully transferred to a sterilized mixture of 1:1 v/v sand: peat for three weeks in a growth chamber, then gradually acclimatized in the greenhouse with a percentage of survival of about 34% after four months.

Fig. (7). *A. officinalis* in the acclimatization stage under greenhouse conditions.

CONCLUSION

In order to develop an efficient and economic *in vitro* propagation protocol, for rapid and large-scale propagation of *A. officinalis* male hybrid, from nodal explants, MS medium supplemented with KIN at 1 mg l^{-1}, NAA at 0.2 mg l^{-1} and GA_3 at 0.5 mg l^{-1} for the establishment stage and MS medium supplemented with 2 mg l^{-1} KIN and 0.2 mg l^{-1} NAA for the multiplication were the best treatments. For overcoming the problems of vitrification, adding the growth retardant PP_{333} at 1.0 mg l^{-1} to the multiplication medium eliminated the vitrification definitely. Also, MS medium supplemented with BAP at 1 mg l^{-1} and NAA at 0.1 mg l^{-1} was the suitable treatment for direct somatic embryogenesis. For *in vitro* rooting,

IBA at 1.0 mg l^{-1} and PP_{333} at 0.5 mg l^{-1} was the optimum treatment for producing white-rooted plants (79.50%). Acclimatization was successful when transplants were transferred into greenhouse with a percentage of survival of about 34%.

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الإكثار المعملي للجيل الأول للهجين المذكر في الأسبرجس

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من أجل إنشاء نظام فعال لإكثار الجيل الأول للهجين المذكر في الأسبرجس معمليًا صنف .Mary Washington 500 W 'CV تم البدء باستخدام البراعم الجانبية للعقد الساقية في ست تجارب. أولًا. تمت تجربة البدء على بيئة موراشيجي وسكوج MS المزودة ب ١ مليجرام/لتر من الكينيتين بالإضافة إلى ٠.٢ مليجرام/لتر نفثالين حمض الخليك ومليجرام/لتر ٠.٥ من حمض الجبرليك والتي أعطت أعلى نسبة من البقاء (١٠٠٪)، وأعلى عدد من الأفرع الخضرية (٨.٥) وعدد العقد (٨.٧). ثانيًا. تم إجراء عملية التضاعف للأفرع السميكة الخضراء الداكنة باستخدام ٢ مليجرام/لتر من البنزيل أمينو بيورين و٠.٢ مليجرام/لتر من نفثالين حمض الخليك، حيث تم الحصول على أقصى عدد للأفرع (٩.٥) وطول الفرع (١٢.١٣ سم) مع نسبة عالية من التزجج. بإستبدال البنزيل أمينو بيورين بالكينيتين أنتجت نباتات سميكة نوعًا وخضراء داكنة مع انخفاض في قيم النمو، حيث كانت قيمة عدد الأفرع ٨.٠٦ وطولها ٨.٦٢ سم. وتم إجراء عملية التضاعف لست مرات على الأقل. ثالثًا. تم تخطي مشكلة التزجج نهائيًا بإضافة الباكلوباترازول بتركيز ٠.٥ مليجرام/ لتر لمكونات بيئة التضاعف. رابعًا. كان أفضل عدد من الأجنة الجسدية المباشرة من العقد الساقية (٨٧) بإضافة ١ مليجرام/لتر من البنزيل أمينو بيورين مع ٠.١ مليجرام من نفثالين حمض الخليك. خامسًا. لإنتاج الجذور التخزينية البيضاء الهامة واللازمة لنجاح عملية الأقلمة خارج المعمل. أجريت ثلاث تجارب منفصلة بإستخدام منظمات النمو (حمض الإندول بيوتريك، نفثالين حمض الخليك، الباكلوباترازول) وكذلك تم استخدام مستخلص فطر العفن الأسود (*Aspergillus niger*) وكانت بيئة موراشيجي وسكوج MS المزودة ب ١ مليجرام /لتر من حمض الإندول بيوتريك المضاف لها ٠.٥ مليجرام /لتر من الباكلوباترازول أعطت أفضل نسبة من الجذور التخزينية البيضاء (٧٩.٥) في أقصر مدة زمنية (٥٣.٧٥ يوم). تمت عملية الأقلمة بنجاح في الصوبة الزجاجية بنسبة ٣٤٪ تقريبًا.