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Indirect Organogenesis is Useful for Propagation of Iranian Edible Wild Asparagus (*Asparagus officinalis* L.)

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Abstract: In order to establish an efficient *in vitro* propagation protocol for Iranian edible wild Asparagus, this experiment was carried out. For this purpose, explants were excised from spear lateral buds and cultured on MS medium containing 6% sucrose and different NAA and BAP concentrations for both callus induction and shoot development. The treatment inducing the formation of the highest number of shoots was achieved on a medium containing 0.015 mg/l NAA and 0.5 mg/l BAP. For root formation, media were supplemented with two IBA concentrations (1.25 and 1.5 mg/l), that 1.5 mg/l giving a higher rooting percentage (43%). These preliminary results showed that the treatment containing 0.015 mg/lNAA, 0.5 mg/l BAP and 1.5 mg/l IBA gave the highest number of plantlets. Also, The data present a relatively efficient method for *in vitro* culture of this valuable vegetable in Persia.

Key words: Asparagus, callus, *in vitro*, spear

INTRODUCTION

Asparagus is a large genus with over 150 different species of herbaceous perennials crop of high economic value with a chromosome number of 2n = 20. they are grown throughout the world but they originated mainly from Asia, Africa and Europe (Prohens *et al*., 2008). The most economically important Asparagus species is garden Asparagus (*Asparagus officinalis* L.), which is a highly prized vegetable (Stajner *et al*., 2002). Tender and unexpanded shoots, commonly called spears, are the edible organs of garden Asparagus (Rubatzky and Yamaguchi, 1997). Propagation of *Asparagus officinalis* L. by seed results in a low percentage of germination and clonal propagation by division of individual crowns is very slow as one plant gives only 2-4 new plants per year under optimum conditions in absence of any pest invasion of injured surface (Ornstrup, 1997), so micropropagation could be an alternative solution.

Within the Asparagus genus, micropropagation protocols have been extensively studied in *A. officinalis* (Murashige *et al*., 1972) and other species used mainly as ornamental or medicinal plants using media supplemented with MS (Murashige and Skoog, 1962) medium and various concentrations of auxins and cytokinins. Several methods of *in vitro* regeneration of Asparagus have been established namely: direct organogenesis (Murashige *et al*., 1972), indirect organogenesis (Reuther, 1984) and somatic embryogenesis (Reuther, 1977). Among the existing pathways of Asparagus *in vitro* regeneration, none of them are used on a large commercial scale for propagation, as regenerated plantlets have poor survival rate either at hardening or at field level (Desjardins, 1992).

Benmoussa *et al.* (1996) reported efficient establishment of callus and shoot culture in *A. densiflorus* cv. Sprengeri on MS medium supplemented with several auxins and cytokinins. Callus formation with subsequent shoot regeneration has also been reported for three other species: *A. plumosus* (Ghosh and Sen, 1994a), *A. verticillatus* (Ghosh *et al*., 1996) and *A. robustus* (Nayak and Sen, 1998); micropropagation without callus formation of *A. cooperi* was reported by Ghosh and Sen (1994b).

Several authors have pointed out that the major obstacle of Asparagus micropropagation protocols is not the establishment of shoot culture and shoot multiplication, but difficult root initiation. Chin (1982) reported considerable improvement of shoot and root development in *A. officinalis* with ancymidol.

The germplasm of *A. officinalis* in Iran is limited, and only some individual plants were found in Taleghan Mountains. To our knowledge, there are no reports of *in vitro* culture of *A. officinalis* L. in Persia. The aim of this study was to establish an efficient *in vitro* propagation protocol in order to preserve and multiply this valuable species, and to develop a method for efficient shoot culture that could be used for other biotechnological treatments. The main pathway of regeneration that was considered was indirect organogenesis as it has very high rate of regeneration.

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MATERIALS AND METHODS

Plant materials: Fifteen to twenty-cm long young spears emerging in the early spring were harvested from edible wild Asparagus genotypes of Taleghan Mountains in Iran at 2008. The basal (10 cm) region of these spears was discarded and the remaining portions were used as explants.

These segments were washed in running water and surface sterilized with a 1% solution of mercury chloride (Hg_2Cl_2) for 15 min. they were then washed thoroughly in sterile distilled water 3-4 times. Lateral buds (5-7 mm in length), including some primordial leaves, were excised aseptically from the spear segments and used as explants. The explants were cultured for callus and shoot initiation on MS basal medium containing 2 mg/l glycin, 100 mg/l myoinositol, 0.5 mg/l nicotinic acid, 2 mg/l thiamine-HCl, 0.5 mg/l pyridoxine-HCl, 7 g/l agar (Cina gen, Co. Iran), 6% sucrose, different combinations of NAA (0, 0.005, 0.01, 0.015 mg/l) and BAP (0, 0.25, 0.5, 0.75, 1 mg/l) for 4 weeks in light condition (1000 Lux) and at a temperature of 25±1ºC (Table 1).

In each subculture, vitrified and non-vigorous shoots were eliminated and only healthy shoots were cultured on rooting medium. Further subcultures were started with 2 shoots on a crown.

Culture condition: The pH of the medium was adjusted to 5.7±0.1 and autoclaved at 121ºC for 15 min. Fifty ml of medium was dispersed in baby food jars (250 ml capacity containing 50 ml of medium). Cultures were maintained in a growth chamber at 25±1ºC and the light intensity was 1000 Lux at daily periods of 16 hours.

Callus induction: Callus was induced in the food jars as from the first week of inoculation. After 28 days, the explants together with their primary calli were subcultured in media of same composition in food jars.

Shoot culture: After 28 days, the explants from the callus induction media were subcultured in similar growth media in jam jars. After 28 days, five treatments giving the greatest numbers of shoots of size 3 mm or more were selected and their shoot numbers counted.

Rooting: Rooting of shoots from the selected treatments was tried on two different media, both containing MS basal medium with 6% sucrose. The two media differed only in their IBA content: containing 1.25 mg/l and 1.5 mg/l, respectively. The regenerated shoots were placed at a frequency of 2 per jar containing 50 ml of medium. Shoots from each of the five selected treatments were used to set up four replicates for each of the two rooting media. After inoculation of shoots on the rooting media (2 shoots per jar), the jars were placed in the culture room and the rooting percentage was recorded after 40 days.

Table 1: Different Combinations of auxin and cytokinin concentrations of the 20 treatments used during callus induction and shoot culture on basal media

	Concentration of BAP (mg/l)				
Concentration of NAA (mg/l)	0.00	0.25	0.50	0.75	1.00
0.000	A	B	C	D	E
0.005	F	G	H	I	J
0.010	K	L	M	N	O
0.015	P	Q	R	S	T

Table 2: Mean shoot production per explant for the five best treatments

Treatment	Mean shoot number per explant
R (0.015 NAA / 0.5 BAP)	15.50 [a]
S (0.015 NAA / 0.75 BAP)	6.25 [b]
B (0.25 BAP)	5.20 [b]
Q (0.015 NAA / 0.25 BAP)	4.75 [b]
N (0.01 NAA / 0.75 BAP)	2.60 [b]

(Mean separation by protected LSD at 0.5% level)

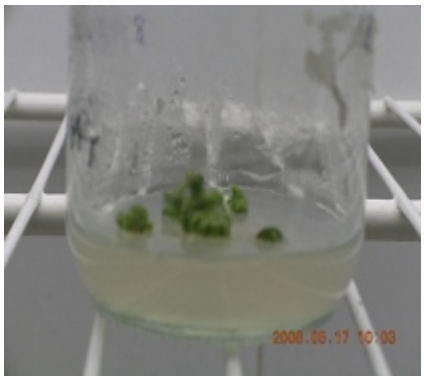

Fig. 1: Close view of pale green compact callus from which shoots were developed (magnification: X 0.8)

Growth of regenerated plantlets to transplantable size: Shoots that have rooted successfully in the rooting media were transferred to 50 ml of MS basal medium in jam jars, to allow the plants to grow to transplantable size. After a period of 30 days, the plantlets were hardened in the lab in trays containing sand, peat and vermiculite in the ratio of 1:1:1 by volume. The trays were all enclosed in plastic bags (with few holes for aeration) to maintain high humidity level.

RESULTS

Calli were induced in all of the 20 media as from the first week of inoculation. The calli proliferated very quickly to form compact masses that ranged from pale-yellow to light green in color. Thick green shoots developed from the callus masses as from the 4 weeks of culture (Fig 1).

After three months of culture, the five best treatments with the highest average number of shoot of size 3 mm or more per explant were found to be R, S, B, Q and N (Table 2). The results showed the importance of both auxin and cytokinin for callus and shoot induction in *A. officinalis*.

For rooting of regenerated shoots, 1.5 mg/l of IBA was most appropriate, as shoots from the five treatments

gave higher rooting percentage (43%) than with 1.25 mg/l of IBA (32.5%).

According to Yang and Clore (1974), rooting percentage increases when shoots are allowed more time on rooting media. In their studies they showed that shoots regenerated from stem segments cultured for 20 weeks had a higher rooting percentage (92.2%) when allowed to root for 8 weeks. The method used by Yang and Clore (1974), however, involved a longer culture period, which is a major drawback. Data obtained from Yang and Clore's (1974) study clearly indicate that the age of the shoots and the rooting time should be optimised to increase rooting frequency.

The small number of plantlets regenerated by this pathway, could be attributed to the too short exposure of the explants to the auxin-rich media. This implies an inadequate time period for the dedifferentiation process to occur. Other important factors that might have negatively affected the pathway are poor culture condition and inappropriate culture medium.

DISCUSSION

To our knowledge, this is the first report for *in vitro* culture of edible wild Asparagus in Iran. Indirect organogenesis was applied for the first time to obtain preliminary information on preserving and mass propagating of this plant. The data present a relatively efficient method for *in vitro* culture of this limited and valuable vegetable in Persia.

These introductory results showed that the treatment containing 0.015 mg/l NAA, 0.5 mg/l BAP and 1.5 mg/l IBA gave the highest number of plantlets. However, this number is still low for an efficient use in mass propagation. This could be attributed mainly to the low, erratic rooting behavior and the inability of most regenerated plantlets to develop a crown (Dore, 1988). It has also been noted that most of the *in vitro* regenerated plantlets that survive in the field have reduced yield (Desjardins, 1992). These problems are mainly due to excessive stress suffered by regenerated plantlets during their *in vitro* developmental stages (Desjardins, 1992). Some important factors responsible for the low survival of regenerated plantlets are: the plant growth regulators used; sugar content in culture medium and light intensity. Our method of regeneration is far from optimum. Nevertheless, it provides a starting point for future researches on indirect organogenesis for micropropagation.

This work is being oriented towards the type and concentration of plant growth regulator used both for induction of callus and shoot initiation. Furthermore, for the potential use of any *in vitro* regenerated plantlets for micropropagation, it is essential to confirm their genetic stability. RAPD analysis and chromosome counting are two important tools that are currently being used on regenerated plantlets to test if they are true to type.

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