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Research Paper

In Vitro Shoot Multiplication of *Asparagus Densiflorus* as Affected by Media, Sucrose and PH

Khetam A. Rasheed[1, *] and Suaad A. Yaseen[1]

[1] University of Duhok, Faculty of Agriculture and Forestry, School of Plant Production, Department of Horticulture, Duhok, Iraq

* Corresponding author, e-mail: (Khetam2001@yahoo.com)

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Abstract: *A successful and reproducible clonal propagation protocol from nodes culture derived from Asparagus densiflorus was achieved in this investigation. The results showed that nodes of Asparagus plant was cultured on the shoot regenerating medium, used modified MS medium full strength supplemented with 2mgl^{-1} BA with different concentrations of sucrose and pH levels for adventitious shoot regeneration. The results showed that MS medium full strength gave the highest percentage of explant responded to shoot proliferation (100%), number of shoots per explant, number of leaves per explant , mean length of shoots and the values were (24.625 shoot/explant, 39.125 leaves/ explant and 8.5cm) respectively. Also the results revealed that the media supplement with 30 gm l^{-1} sucrose showed the highest percentage of explant responded to shoot proliferation (100%). This sucrose concentration also showed the optimum result for number of shoots per explant, number of leaves per explant and average length of shoots (6 shoot/explant, 22.375 leave/ explant and 5.7125cm) respectively . Finally, the highest percentage of explant showing multiplication was observed on the media adjusted to pH 5.5 and 6.0. The results presented here proved to be suitable for the in vitro shoot multiplication of Asparagus densiflorus.*

Keywords: *In vitro*, *Asparagus densiflorus*, pH, sucrose.

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1. Introduction:

Asparagus plant *Asparagus densiflorus* belongs to Asparagus family *Asparagaceae*, is a native of South Africa (Lemake, 2011). *Asparagus densiflorus* is a branching perennial herb with tough green aerial stems which are sparsely covered with spines. The leaves are actually leaf-like cladodes, which are 0.8-2 cm long and 0.1-0.2 cm wide, and arise in groups of four or more from the stem. Occurring

in spring, the small white or pinkish-white flowers are 0.3-0.5 cm long and arise in clusters off the stem. The root system is a mat of fibrous roots with bulbous tubers, from which plants may re-sprout (Wolf, 1999).

Asparagus densiflorus can be readily propagated by separating the tubers in fairly large clumps, or by sowing the seed in spring or early summer (Jamieson, 2002). Although Asparagus plant is regarded as an easy to propagate plant, but its propagation through the *in vitro* micropropagation technology is advantageous due to tissue culture techniques have become an attractive field of biotechnological research. The benefits of these studies are particularly valuable in the areas of large-scale clonal propagation, crop improvement, the production of important plant compounds and the conservation of genetic resources. The development of shoot regeneration efficiency requires a better understanding of the influence of culture conditions on shoot regeneration (Debergh and Zimmerman, 1991).

In vitro growth and shoot multiplication may be affected by media, sucrose and pH of the shoot induction media. Sucrose is used as source of C and energy for optimum proliferation and growth of the *in vitro* grown cultures (Karim *et al*., 2003).

Sucrose is the main source of carbon energy for *in vitro* cultures. Plant cells and tissues in a culture medium lack autotrophic ability and therefore, need external carbon for energy (Razdan, 1993). The addition of an external carbon source to the medium enhances the proliferation of cells and regeneration of green shoots (Nowak *et al*., 2004). The optimal sucrose concentration in a medium should be sufficient to satisfy the basic energy requirements for cell division/ differentiation and not impose any negative osmotic effects on shoot formation (Stavarek *et al*., 1980). This indicates that sucrose acts not only as a carbon energy source in a medium but also as an osmotic (Nowak *et al*., 2004) and that different concentrations of sucrose are one of the factors controlling the induction and growth of shoots (Gibson, 2000).

Plant cells and tissues require an optimum pH for growth and development in cultures. The pH affects nutrient uptake as well as enzymatic and hormonal activities in plants (Bhatia and Ashwath, 2005). The optimal pH level regulates the cytoplasmic activity that affects cell division and the growth of shoots and it does not interrupt the function of the cell membrane and the buffered pH of the cytoplasm (Brown *et al*., 1979). The pH also influences the status of the solidifying agent in a medium: a pH higher than 6 produces a very hard medium and a pH lower than 5 does not sufficiently solidify the medium (Bhatia and Ashwath, 2005). The change of pH in cells or organs is due to the ions absorbed from the medium (Sakano, 1990). Therefore, it is necessary to optimize the sucrose concentration and pH level for maximum shoot regeneration because the sucrose concentration and pH level directly influence shoot regeneration.

Therefore the attempts of this present study were to determine the effects of MS media strength sucrose and pH on *in vitro* shoot formation and multiplication of *Asparagus densiflorus* over the cultural period. The objective of this present study has been to determine the optimum cultural conditions for production of genetically stable multiple shoots from the explants.

2. Materials and Methods

The current experiment was conducted in Plant Tissue Culture laboratory of the Horticulture Department, School of Plant Production, Faculty of Agriculture and Forestry, University of Duhok, Iraq. The plant material *Asparagus* (nodes) were taken from *in vitro* grown plantlets under controlled conditions. The nodes were cultured on MS medium (Murashige and Skoog, 1962) supplemented with full strength, half strength and quarter strength and supplemented with 0.4 mgl^{-1} Thiamine HCl, 2 mgl^{-1} BA, 30 gl^{-1} sucrose, 0.7% w/v agar and 100 mgl^{-1} Inositol. The pH of the medium was adjusted to 5.7± 0.1 using 0.1 N HCl and/or 0.1 N NaOH prior to autoclaving at 121°C temperature and 15 lb pressure for 20 min.

For testing the effects of sucrose, MS full strength with 0.7% agar at pH 5.5 was supplemented with 1.5, 2.5, 3 and 4.5 % sucrose.

Finally, different levels of pH (4.0, 5.0, 5.5 and 6.0) at 3% sucrose and 0.7% agar were used for testing the effects of pH on shoot proliferation. Explants were incubated in a controlled environment (25±1^{o}C, 16 h photoperiod, 1000 lux light intensity, 75±5% humidity). The materials were subcultured at 3-4 weeks intervals. After 6 weeks of culture, percentage of explants showing proliferation, the numbers of shoots and leaves per explants and the mean length of shoots were recorded. Ten replicates were assigned for each level of treatment and the experiment was designed according Completely Randomized Design (CRD). The comparison between means was carried out according to Duncan's multiple range test ($P < 0.05$) using a computerized program of SAS (SAS, 2001).

3. Results and Discussion:

All the studied factors were found to be highly effective on shoot growth and development in *Asparagus densiflorus*. Yang (1977) established a very good tissue culture technique for micropropagation of *Asparagus* using spears as explants. However, this protocol was lengthy and took 20 weeks. Using different explants as a source, several workers have developed protocols for *in vitro* culture of different *Asparagus* species (Štajner et al. 2002; Nayak and Sen 1998). To our best knowledge this is the first report for multiplication of *Asparagus adscendens* from nodal explants.

The use of aerial plant parts as a source of explants reduces the chances of contamination. So, the nodal explants were inoculated on to MS contain cytokinin for multiple shoot induction. The maximum number of multiple shoots was obtained in MS media full strength supplemented with 2mgl^{-1} BA after four to six weeks of incubation.

Table (1) shows that the highest percentage of explant responded to shoot proliferation and that was 100%. The medium having full strength media also produced the optimum result for number of shoots per explant, number of leaves per explant and mean length of shoots and the values were (24.625 shoot/explant, 39.125 leaves/ explant and 8.5cm) (Fig. 1 A).

The medium containing half and quarter strength MS salt showed the lowest percentage of explants number showing proliferation, number of shoots per explant, number of leaves per explant and mean length of shoots and the values were (88, 65%, 20.75, 13.375 shoot/explant and 27,18.5 leaves/ explant and 6.238, 4.688 cm) . These results confirmed that some plant species have enough levels of endogenous hormones and do not require a high level of exogenous growth regulators for plant regeneration (Hussey, 1982).

Table (1): Effects of MS medium strength on proliferation and growth of axillary shoot from nodal segments of *in vitro* proliferated shoots, Data were recorded after 6-7 weeks of culture at the best PH 5.7±1 and Agar 7gml^{-1} containing 2mgl^{-1} of BAP

MS medium strength	% of explants responded	Number of shoots/ explant	No. of leaves/explant	Mean length of shoots (cm)
Full (1/1)	100 a	24.625 a	39.125 a	8.5 a
Half (1/2)	88 b	20.750 a	27.0 b	6.238 b
Quarters (1/4)	65 c	13.375 b	18.5 c	4.688 c

***Different letters within columns represent significant differences according to Duncan's multiple range test at 5% level.**

Table (2) reveals to sucrose is an important factor for ***in vitro*** shoot proliferation. In this experiment different concentration of sucrose in MS medium were used for multiple shoot regeneration and development. Among the different sucrose concentrations in MS medium, the media having 30gml^{-1} sucrose showed the highest percentage of explant responded to shoot proliferation and that was 100% (Table. 2). The medium having 30 gml^{-1} sucrose also produced the optimum(high) result for number of shoots per explant, number of leaves per explant and mean length of shoots and the values were (6 shoot/explant, 5.7125 leave/ explant and 22.375 cm) respectively (Fig. 1 B).

This may be mainly due to the fact that high sugar levels available in the culture medium may speed up cell division thus leading to an increase in the volume and weight of tissues cultured, as suggested by other researchers (Chong and Taper, 1972).

Also noted in same table (2) the medium containing 15 gml^{-1}sucrose showed the lowest percentage of explant number showing proliferation, number of shoots per explant, number of leaves per explant and mean length of shoots and the values were (55%, 2 shoot/explant, 12 leave/explant and 2.375 cm) respectively.

Lower sucrose concentrations (3 and 4%) were significantly better during the multiplication stage in terms of shoot production rate and their subsequent growth, which was consistent with the findings of (Rugini & Verma 1983 and Rugini 1984) who suggested that 3% sucrose should be used in all culture stages for the in vitro multiplication of *Asparagus densiflorus*
From the present investigation it was observed that different concentrations of sucrose affected *in vitro* growth of *Asparagus densiflorus* shoots variously.

The optimum sucrose concentration as an efficient carbon source has been examined in tissue culture**s** of some plant species, such as *Paederia foetida* (Amin *et al*., 2003) and *Elaeocarpus robustus* (Rahman *et al*., 2004), in which 30 g L^{-1} sucrose enhanced shoot growth and development. Lower concentrations of sucrose have been shown to be less effective for adventitious shoot formation. On the other hand, the detrimental effect of a high sucrose concentration on shoot formation implies that the osmotic level in the medium may be inhibitory to further shoot development. Thus, high concentrations of sucrose seem to inhibit shoot growth and development. These observations are supported by another report (Nowak *et al*., 2004).

Table (2): Effects of sucrose on proliferation and growth of axillary shoot from nodal segments of *in vitro* proliferated shoots. Data were recorded after 6-7 weeks of culture on MS medium full strength containing 2mgl^{-1} of BAP

Sucrose concentrations gmL^{-1}	% of explants responded	Number of shoots/ explant	No. of leaves/plant	Mean length of shoots (cm)
15	55 d	2 d	12 c	2.375 d
25	75 c	3 c	15.625 bc	3.5625 c
30	100 a	6 a	22.375 a	5.7125 a
45	95 b	4.625 b	18.875 ab	4.5875 b

***Different letters within columns represent significant differences according to Duncan's multiple range test at 5% level.**

Table (3) showing that the pH of the culture medium is an important factor for proliferating shoots *in vitro*. In the absence of pH regulation, the ionization of acidic and basic groups causes considerable changes in structure that affect their function at the cellular level (Sakano, 1990). Every species requires an optimum pH which can promote maximum shoot formation. *In vitro* multiple shoot development depends upon some other factors rather than cytokinin, auxins and gibberellins. The pH of the culture medium is an important factor for the *in vitro* proliferation and healthy culture growth.

Among these pH levels in table (3), the highest percentage of explant showing proliferation was observed on the media adjusted to pH 5.5 and 6.0.and that was 100%.The second highest percentage of explant showing proliferation was observed on media having 5.0 pH and it was 78%. The lowest frequency of explant showing proliferation was observed on the media where pH was adjusted to 4.0 where the proliferation frequency ranged from 57%. Number of shoots per explant was highest in medium having pH 5.5 and the value was (6.5 shoot /explants) (Fig. 1C).

Our results indicated that pH 5.5 was the optimum during all stages in both cultivars and either low or high pH levels caused serious abnormalities, producing shorter shoots with narrow, curled and sharp-pointed leaves.

In same table (3) noted lowest in medium having 4 pH about 2.125 shoot /explants .From the present investigation it was revealed that lower (4.0) pH level hindered multiple shoot proliferation. *In vitro* proliferation of Azadirachta indica (Gautam *et al*., 1993), Plantago ovata (Barna and Walklu, 1988) and Smilax zeylanica (Jha *et al*., 1987) shoots was increased significantly when the pH the culture media was adjusted at 5.8 before autoclaving.

The lower and higher pH level hindered multiple shoot proliferation. The pH in medium is another important culture condition for *in vitro* shoot regeneration. The enzymatic and hormonal activities in plants and nutrient uptake are largely affected by pH level in the tissue cultures (Bhatia and Ashwath, 2005). The effects of medium pH are more significant for early differentiation of proembryogenic cell aggregates. Low pH (*ca.* 3-4) of media seems to prevent differentiation of pro-embryogenic cell aggregates, whereas higher pH levels (5-5.5) favor the formation of globular structures. (Moura-Costa *et al*., 1993). The solidification with gelling agent in the media is influenced by the pH level. The pH levels more than 6 produce hard-solidified media and pH levels lower than 5 give unsatisfactory solidification (Bhatia and Ashwath, 2005). *In vitro* propagation has possibility to offer highly efficient techniques for propagating elite fruit plants. It is well known that among many factors affecting in vitro plant regeneration, sucrose concentration and pH level are major important conditions.

Plant regeneration through tissue culture technique would be a noble alternative for improving the quality and faster production of Asparagus. In vitro culture techniques permit the shoot induction and multiplication under aseptic condition with reduced space requirements because of the small size of explant. It has been demonstrated that sucrose and pH on the medium play an important role on in vitro growth and development of shoots. Therefore, the present report showed that in MS medium full strength containing BAP, sucrose 30 gm I-1, agar 6 gm I-1 and pH 5.5-6.0 proved more effective on shoot multiplication.

Table (3): effects of PH on proliferation and growth of axillary shoot from nodal segments of in vitro proliferated shoots. Data were recorded after 6-7 weeks of culture on MS medium full strength containing r 2mgl-1 of BAP

pH levels of the medium	% of explants responded	Number of shoots/ explant	No. of leaves/explant	Mean length of shoots (cm)
4.0	57 d	2.125 d	12.25 c	2.6625 d
4.5	78 c	3.25 c	17.25 b	3.4 c
5.5	100 a	6.5 a	22.5 a	5.8375 a
6.0	100 b	5.125 b	20 ab	4.85 b

***Different letters within columns represent significant differences according to Duncan's multiple range test at 5% level.**

Conclusion

This study found that an optimum concentration of sucrose (3%) and pH level (5.8) can gave the best performance on shoot development. The results obtained in this study provide some information on tissue culture, which helps the further advanced researches on this ornamental plant.

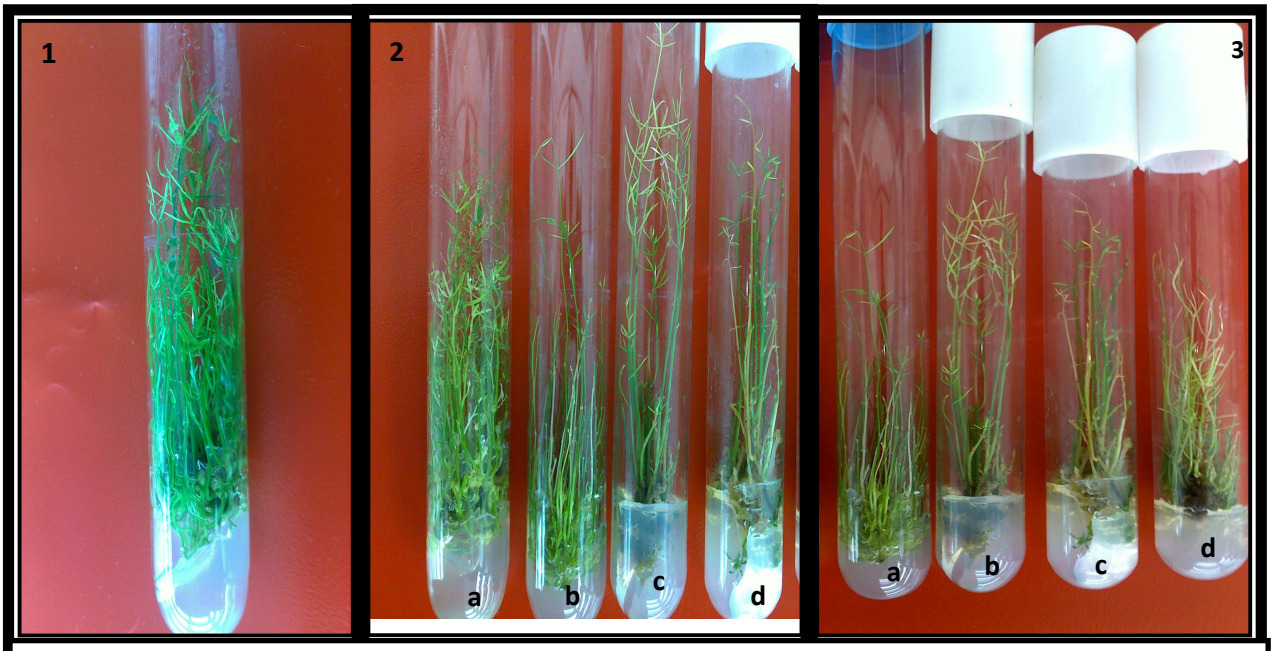

Fig 1: Shoot Multiplication from nodal explant as affected by media, Sucrose and pH of *Asparagus densiflorus*.
1- Growth and shoot multiplication from nodal explant on MS medium full strength containing BAP+ 7 gm l^{-1} agar
2- Development of multiple shoots on MS medium containing BAP and different concentrations of sucrose (a. 30 b. 45 c. 25 d. 15 gm l^{-1}) from the nodal explants.
3- Formation and multiplication of shoots on MS medium with BAP at different level of pH (a. 5.5 b. 6 c. 4.5 d. 4) from the nodal explant.

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