Micropropagation of *Asparagus officinalis L.* through callus development

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Abstract. In order to develop an in vitro propagation process for production and conservation, in this study, we investigated the influence of disinfectants and plants growth regulators (PGR) on the infection rate of explants, the ability of callus formation, and the regeneration of shoot and root from Asparagus officinalis L. trunk segments. We obtained the best results when disinfecting trunk segments explants combined with alcohol 70° in 5 minutes and NaClO in 5 minutes with the lowest contamination rate of 3.7%. Callus was formed well in MS medium supplemented with 3% sucrose; 0.7% agar, pH= 5.8; 1.0 mg/l BAP and combined with 0.5 mg/l NAA, 1.5 mg/l BAP and 0.75 mg/l NAA. The highest rate of shoot formation was 87.2% when the callus continued to grow in the environment supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA. Roots were formed after 4 weeks of shoot cultivation in MS medium supplemented with 0.5 mg/l NAA with the highest rooting rate of 74.59%.

1 Introduction

Asparagus officinalis L. is a plant with high nutritional and economic value. *A. officinalis* L. is known as a diuretic and is used to treat urinary problems such as cystitis. The active ingredients from the asparagus plant are also useful in the treatment of arthritis, being a mild laxative and sedative. Asparagus is also a rich source of glutathione, a potent antioxidant known to boost the immune system, reduce inflammation, and maintain liver health [1,2].

Propagation of asparagus can be carried out through budding from trunk segments or seeds, but this is a relatively slow process resulting in very few germinated seedlings [3]. In order to meet the increasing demand for varieties for this plant, in vitro propagation is a method with many advantages to producing a large, uniform, and disease-free plants quantity in a short time, serving the purpose of agricultural production and scientific research [4,5]. In this paper, we present the results of research on the propagation of asparagus through callus generation from young shoots.

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2 Materials and methods

Plant material: In the study, young asparagus trunk segments were used from different asparagus varieties.

Sterilization: After collecting young asparagus explants with size 20.0 - 30.0 mm, wash them with soap for 10 minutes and then rinse with distilled water 3 times. Explants were disinfected with 70° alcohol for 30 seconds, 1 minute, 3 minutes, and 5 minutes, then washed with sterile distilled water 3 times. Explants were further sterilized for 5 min in NaClO solution (5%). Continue rinsing 3 times with sterile distilled water. The explants were cut into small pieces with a thickness of 1.0 - 2.0 mm and inoculated on MS medium [6] at 25±2oC, under 16 hours of illumination.

Callus introduction: Investigation of the ability to induce callus production of asparagus explants was carried out on MS medium supplemented with 2,4-D (concentration 0-2.0 mg/l) or supplemented with a Simultaneous combination of BAP (6-Benzylaminopurine) with a concentration of 1.0-4.0 mg/l and NAA (1-Naphthaleneacetic acid) with a concentration of 0.5-2.0 mg/l.

Shoot induction: The shoot regeneration efficiency from asparagus callus was evaluated on MS basal medium supplemented with BAP (concentration 1.0-4.0 mg/l), NAA (concentration). 0.5-2.0 mg/l). After forming, shoots were rapidly multiplied on MS medium supplemented with 4.0 mg/l Kinetine.

Rooting production: The ability to induce roots in vitro from asparagus shoots was evaluated on ¼ MS, ½ MS, MS, and MS media supplemented with NAA with concentrations in the range of 0.5-2.0 mg/ l.

Data recording and analysis: Sixty explants were established in each treatment. Each treatment was replicated 3 times. The treatments were arranged in a completely randomized design. Experimental data were statistically processed using computer software

3 Results and discussion

3.1 Effects of sterilizers on A. officinalis explants

The results from Table 3 show that the percentage of contaminated explants decreased with increasing sterilization time from 30 seconds to 5 minutes. Specifically, when using 70% ethyl alcohol (EtOH) sterilization formula combined with 5% NaClO solution for 5 minutes, the sterilization efficiency was highest with the infection rate of 3.7±1.62%.

Table 1. Effects of different concentrations and combinations of surface sterilizers on trunk segments of *A. officinalis.*

Sterilization treatment	Contamination (%)
EtOH 70º (30 secs) + NaClO 5% (5 mins)	30.75 ± 2.33^{b}
EtOH 70º (1 min) + NaClO 5% (5 mins)	28.57 ± 3.23^{b}
EtOH 70º (3 mins) + NaClO 5% (5 mins)	22.33 ± 2.58^{b}
EtOH 70º (5 mins) + NaClO 5% (5 mins)	3.7 ± 1.62^{c}

Means followed by the same letters are not significantly different by t-test multiple comparison test at 0.05 probability level.

Compared with previously published formulations for sterilization of asparagus samples, the formulation used in this study has a shorter application time but gives better sterilization efficiency with a lower sample contamination rate [7]. Sterilization time affects the percentage of disease-free and infected samples [8], short sterilization time increases the

percentage of contaminated samples, increasing sterilization time reduces the percentage of contaminated explants, but the percentage of dead samples increases.

3.2 Effect of plant growth regulator on callus induction from A. officinalis

Analysis of the results in Tables 2 and 3 showed that the 2,4-D, NAA, and BAP significantly affected the ability to induce callus formation in A. officinalis after 3 weeks of culture. Specifically, the addition of 2,4-D to the culture medium stimulated the induction of callus from *A. officinalis* trunk segments, of which the highest rate was 82.53 ± 2.99% was achieved at the concentration of 1.0 mg/l (Table 2). The callus-forming feature was obtained in the form of a block, pale yellow, arising at the cross-sectional position first and then gradually moving to the surrounding areas of the explants (figure 1). The addition of BAP combined with NAA to the medium gave a higher rate of callus generation than using 2,4-D. The most effective callus-producing medium for young asparagus samples in this experiment was: MS supplemented with 3% sucrose; 0.7% agar; pH= 5.8 supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA with callus generation rate of 96.82 ± 2.23% (Table 3). Callus obtained a greenish mass arising first at the cross-sectional position and then at the surrounding locations of the explants.

Table 2. Effect of 2,4-D on callus induction.

2,4-D (mg/l)	Callus (%)	Explants color	Rooting after 3 weeks
0	22.22 ± 2.14^{a}	pale yellow	+
0.5	75.0 ± 2.57^{ab}	pale yellow	+
1.0	82.53 ± 2.99^{b}	pale yellow	+
1.5	77.77 ± 2.01^{b}	pale yellow	+
2.0	52.37 ± 2.99^{c}	pale yellow	+

Means followed by the same letters are not significantly different by t-test multiple comparison test at 0.05 probability level.

Table 3. Effect of BAP + NAA on callus induction

BAP (mg/l)	NAA (mg/l)	Callus (%)	Explants shape
0	0	22.22 ± 3.33a	Mass, greenish
0.5	0.25	60.31 ± 1.67b	Mass, greenish
1.0	0.5	96.82 ± 2.23c	Mass, greenish
1.5	0.75	94.44 ± 3.10c	Mass, greenish
2.0	1.0	83.33 ± 2.99d	Mass, greenish

Means followed by the same letters are not significantly different by t-test multiple comparison test at 0.05 probability level.

The ability to regenerate shoots will be maintained longer in callus which is firm and more or less green pigmented [9]. In this experiment, callus regenerated on MS medium supplemented with 2,4-D was mostly pale yellow and porous. Especially from the 3rd week, the callus began to appear with uncertain roots with small size and no sign of shoot formation. Therefore, the callus formed on MS medium supplemented with 2,4-D is not suitable for use in the next stage of shoot development.

The combination of auxin and cytokinin in the culture medium with certain ratios will affect the process of callus formation. Compared with the results of Patel et al. [10] when studying in vitro propagation of Asparagus racemosus, the highest rate of callus regeneration was achieved in the medium supplemented with BAP 0.5 mg/ l and NAA 1 mg/l is 91.67%, in this result the best medium for rapid multiplication of *A. officinalis* callus is BAP 1.0 mg/l

combined with NAA 0.5 mg/l with higher induction rate (96.82%). Similarly, the study of Kim et al [11] also suggested that the combination of PGR would give a higher rate of callus generation, specifically, the callus rate of Asparagus cochinchinensis reached 85% on MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP.

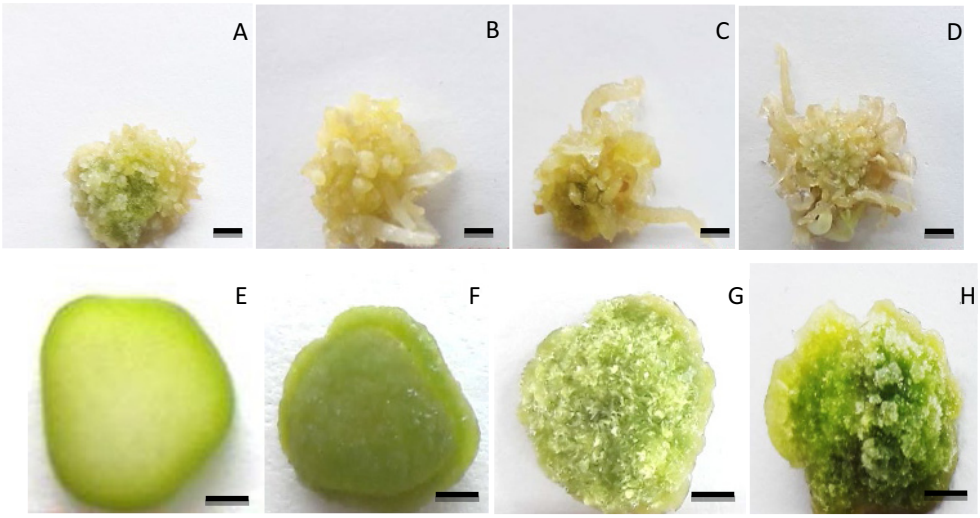

Fig. 1. Callus grown on MS medium supplemented with PGR, 5mm scale horizontal bar. *[A]-[D] 2,4-D with concentrations of 0.5 mg/l, respectively; 1.0 mg/l; 1.5 mg/l and 2.0 mg/l; [E]-[F] 1.0 mg/l BAP and 0.5 mg/l NAA after 0, 2, 3, 4 weeks of culture.*

3.3 Effect of PGR on shoot production from A. officinalis

According to the results of this study, the culture medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA gave the highest percentage of shoot formation, accounting for 87.20 ± 2.42% (figure 2). The obtained shoots were green, in the form of young shoots. As for the medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA, the average rate of shoot regeneration was 55.55 ± 2.53% (Table 4).

Table 4. Effect of BAP + NAA on shoot production in vitro.

BAP (mg/l)	NAA (mg/l)	Shoot (%)	Rapid shoot multiplication rate (shoots/explants)	Length of shoots/culture (mm)
0	0	0	0	0
0.75	0.25	0.15 ± 1.58[a]	2.69 ± 1.21[a]	1.0 - 2,0
1.0	0.5	5.55 ± 2.53[b]	4.27 ± 1.35[b]	2.0 - 3,0
1.5	0.75	6.18 ± 2.12[c]	5.22 ± 1.55[c]	3.0 – 4.0
2.0	1.0	7.20 ± 2.42[d]	6.39 ± 0.57[d]	3.0 – 5.0
2.5	1.25	1.20 ± 2.12[e]	3.55 ± 0.57[e]	2.0 – 3.0
3.0	1.5	3.80 ± 2.53[a]	1.85 ± 1.02[f]	1.0 – 2.0

Means followed by the same letters are not significantly different by t-test multiple comparison test at 0.05 probability level.

The experimental results showed similarities with the study of Patel et al (2015) on Asparagus racemosus, MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA. gave the highest rate of shoot growth at 83.33%. Meanwhile, the in vitro propagation study of *A. racemosus* by Nabi et al [12] showed that the combination of 2.0 mg/l BAP and 0.5 mg/l NAA gave a high rate of axillary shoot formation.

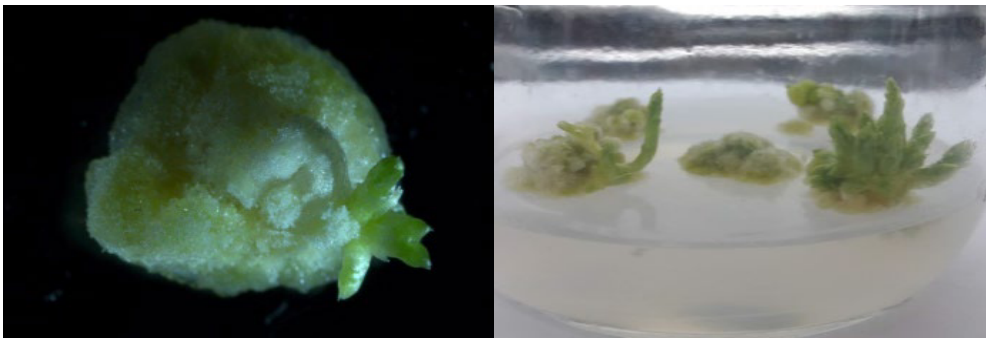

Fig. 2. Shoot regeneration from callus on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA.

3.4 Effect of PGR on the growth of root in vitro

For the formula of adding ¼ MS to the culture medium, *A. officinalis* explants still produced roots at the rate of 30.15 ± 2.51%. The culture medium supplemented with ½ MS and MS gave approximately the same high percentage of rooting with 84.79 ± 2.99 % and 87.3 ± 2.53%. Roots were yellow, clustered, slender and about 25-35 mm long (Table 5).

Table 5. Effect of MS on rooting after 4 weeks of culture.

MS concentration	Roots (%)	Root multiplication rate (roots/explants)	Length of shoots/culture (mm)
¼	30.15 ± 2.51[a]	5.78 ± 1.06[a]	20 - 25
½	84.79 ± 2.99[b]	6.84 ± 1.55[b]	25 - 35
1	87.30 ± 2.51[b]	7.33 ± 1.01[b]	25 - 35

Means followed by the same letters are not significantly different by t-test multiple comparison test at 0.05 probability level.

The results of rooting in this experiment were relatively consistent with the study of Kim et al. on *A. cochinchinensis*. Kim said that MS and ½ MS medium gave the highest rate of rooting, the roots obtained on this medium were thin and long. The data presented in Table 5 showed that roots were formed in all treatments. *A. officinalis* plants in vitro with thin, weak roots can be a major problem, as not only do they have poor survival rates, but growth in soil-dwelling plants is also very slow [13]. Therefore, it is necessary to add PGR to the basic culture medium to promote the formation of stronger and stronger roots.

The formula adding 0.5 mg/l NAA to MS medium to rooting in vitro had the highest efficiency (74.59 ± 1.13%). For additional remaining cultures: 1.0; 1.5 and 2.0 mg/l NAA both gave rooting results but the mean value was lower. Root explants were yellow, short but sturdy, about 13.0-18.0 mm in length (Table 6).

Table 6. Effect of NAA on rooting after 4 weeks of culture.

NAA (mg/l)	Rooting (%)	Root multiplication rate (roots/explants)	Length of shoots/culture (mm)
0.5	74.59 ± 1.13[a]	5.14 ± 0.51[a]	13 - 15
1.0	66.67 ± 2.91[a]	4.01 ± 0.64[b]	13 - 15
1.5	38.09 ± 2.97[b]	2.23 ± 0.23[c]	15 - 18
2.0	36.05 ± 1.84[b]	2.17 ± 0.4[c]	15 - 18

Means followed by the same letters are not significantly different by t-test multiple comparison test at 0.05 probability level.

NAA was also evaluated as the best PGR for *A. officinalis* roots growth in the study of Pindel A. [14]. But Sallam et al. [15] suggested that NAA was the worst PGR for *A. officinalis* roots formation, with the lowest percentage of roots produced despite the longest root size. Based on Table 5, the rooting rate of the treatments was still not high, besides, the roots were shorter in size than the roots produced only on MS and ½ MS medium (figure 3, 4).

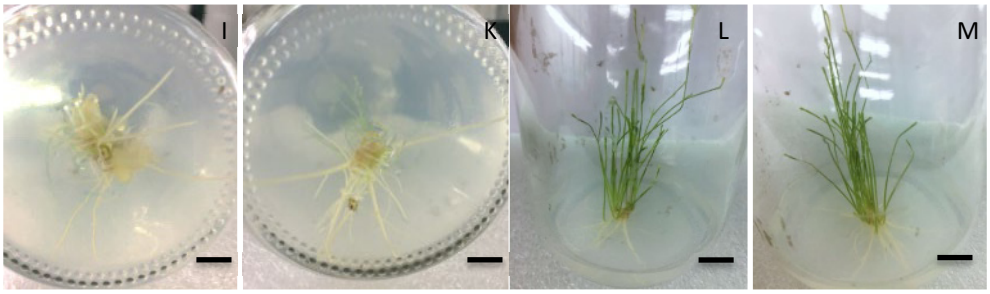

Fig. 3. Rooting after 4 weeks of culture, 10 mm scale bars. *[I], [L] ½ MS; [K], [M] 1 MS.*

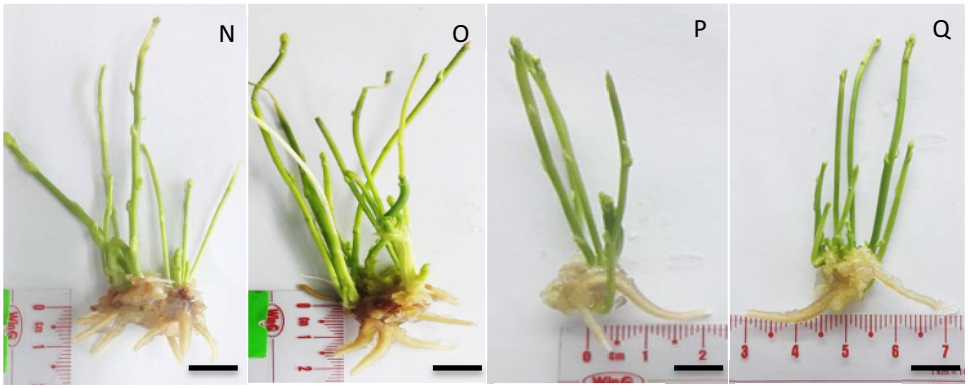

Fig. 4. Rooting on medium supplemented with NAA after 4 weeks of culture, 10 mm horizontal bar. *[N] NAA 0.5 mg/l; [O] NAA 1.0 mg; [P] NAA 1.5 mg/l; [Q] NAA 2.0 mg/l.*

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