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CALLUS INITIATION AND PLANT ORGAN REGENERATION OF *ARBUTUS ANDRACHNE* L. FROM LEAVES AND NODES GROWN FROM *IN VITRO* SEED CULTURE

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ABSTRACT

A successful attempt was done to overcome the traditional seed germination barriers of *Arbutus andrachne* L. by applying the tissue culture technique. Seeds were successfully disinfested by NaOCl and ethyl alcohol to produce healthy cultures. Treating with GA_3 raised the germination rate from only 17% (control) to 45.7 % and 54.28% by adding 1 mg. l^{-1} GA_3 and 2.5 mg. l^{-1} BA with and without cold pretreatment. As well as, the above GA_3 treatment was very beneficial in increasing the speed of seed germination. For callus induction, the culture of node explants was superior to the culture of leaf discs by reaching 100% as compared to only 66% of leaves. The cultured on WPM with both BA and TDZ respectively records the best callus induction (100%). The best-combined treatment was the culture of nodes on WPM enriched with 1.0 mg.l^{-1} NAA+ 1.0 mg.l^{-1} BA by producing the highest callus weight reaching 34.250 g. Finally, interesting organogenesis and regeneration were noticed on the induced callus from both leaf discs and nodes. The best shoot and root regeneration were noticed on node explants grown on WPM enriched with 1.0 mg. l^{-1} NAA and 2.0 mg. l^{-1} BA.

***KEYWORDS*:** *Arbutus andrachne* L., Callus, Nodes, Leaves, BA, TDZ, NAA, PPM (Plant preservative mixture)

1. INTRODUCTION

The eastern strawberry (*Arbutus andrachne* L.) is an evergreen shrub or small tree from the *Ericaceae* Family, widespread from the sea of East Mediterranean to the Northern Black Sea area (Edmondson *et al*., 1978). The tree grows in mountainous rocky habitats with alkaline soil. The Kurdistan region of Iraq comprises the richest country flora cover with many species being under extinction threat. In Kurdistan, the species has been only reported in two sites of the Bekher Mountain southern Zakho city (Edmondson *et al*., 1978; Shahbaz, 2010). *Arbutus andrachne* L. is a medicinal plant with a high antioxidant content. According to Sakar *et al.* (1991) and Said *et al*. (2002) in traditional medicine, *A. andrachne* has many benefits in treating wounds, aching joints, blood tonic, and urinary system, and against some cancer types (Al-jabari, 2011). Furthermore, the tree has ornamental and cosmetic value as well as for reforestation, as it resprouts after the fire and is useful in the food industry. The uses of this plant caused higher requests for this species as well as other species (Bertsouklis and Papafotiou, 2009; Shahbaz, 2010; Al-jabari, 2011; Miguel *et al*., 2014). In the Kurdistan Region of Iraq, only a few individuals have been observed in the wild due to the low rate of seed germination and slow growth under natural conditions. Thus, the *A. andrachne* species in Kurdistan is under extinction threat (Shahbaz, 2020). Open-field notifications imply that the *Arbutus* tree in Kurdistan Region prefers highly drained sandy to moderate soils, and rocky slopes of the middle forest zone (Shahbaz, 2010). More productive propagation methods are required to meet the demand for *A. andrachne* (Odeh, 2017). Clonal propagation of *A. andrachne* L. by stem cuttings is difficult. Moreover, Poor and low rates of germination in seeds under normal conditions, low rooting of stem cuttings, and slow growth of the plant are the main problems in the propagation of *A. andrachne* (Karam and Al-Salem, 2001). High efficient micropropagation protocols could facilitate the use of such species (Dalla Guda *et al*., 2010) in their propagation independent of the growing season, and avoiding the collection

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from the wild area, also permitting fast multiplication of shoots from little quantities of inoculated explant materials. The latter would be particularly useful in countries where these species are listed as endangered plants (Satovic, 2004). Several studies were conducted on *in vitro* propagation of *Arbutus andrachne* L. from seeds (Mostafa *et al*., 2010; Al-jabari, 2011; Papafotiou *et al*., 2013; and Odeh, 2017). Most of the authors have found that the use of sodium hypochlorite followed by ethanol was better for sterilizing the seeds of *A. andrachne*.

For breaking seed dormancy, Al-jabary (2011) treated *A. andrachne* L. seeds with four *in vitro* treatments (control, The pretreatment of 4°C for 24 hours, with soaking in 5.0 mg. l^{-1} gibberellic acid solution for 24 hours under room temperature, stratification with immersion in GA_3 solution). Then seeds were cultured on (water agar, MS, B5, and WPM) media and used as plant material. Odeh (2017) obtained the highest germination percentage when seeds were stored at 4°C and soaked in 5.0 mg/l^{-1} GA_3 solution for a week and cultured on WPM. A higher germination percentage (100%) was achieved when 2.0 mg. l^{-1} GA_3 was added to the culture medium as compared to 48% when 1.0 mg. l^{-1} GA_3 was used (Mostafa *et al*., 2010) Also, no seeds were germinated on full-strength or ½ MS medium free of hormones or with 2.0 mg. l^{-1} GA_3, 1.0 mg l-1 6-benzyladenine (BA), and 0.5 mg l-1 NAA.

The plant age is very important as observed from the tree where callus usually could only be induced from juvenile tissue and not from mature trees. Furthermore, the growth regulators are of high importance especially the balance between auxins and cytokinins which was crucial in initiating and maintaining callus cultures (Allan, 1991). Al-jabary (2011) obtained the highest callus induction from seeds, *in vitro* cotyledons, and roots of *A. andrachne*, on a B5 growth medium enriched with 1.0 mg.l^{-1} 2,4-D under full darkness. According to Aljabari *et al*. (2014), callus tissue was initiated from seeds on the B5 medium enriched with vitamins and 1.0 mg.l^{-1} 2,4-D. Other explants, such as *in vitro* roots and cotyledons, were grown on the same medium to induce callus under dark conditions. *Arbutus* species like *A. unedo*, the callus was initiated from intermodal segments taken from axillary shoots grown under *in vitro* conditions and inoculated on MS medium enriched with different concentrations of plant growth regulators (El-Mahrouk *et al*., 2010). Furthermore, Giordani *et al*, (2005) used Leaves, internodes, and root of *A. unedo* L. from *in vitro* cultivated seedlings, the highest callus production was observed from internodes cultured on ½ N-MS medium with 1.14 µM IAA and 5 µM zeatin. However, there is not too much data on the callus induction of *A. andrachne* (Al-jabary, 2011). The aim of this study is to preserve this endangered important local tree from disappearing in the Kurdistan region through the micropropagation technique. Also, developing a reliable and successful micropropagation protocol from seeds, and Inducing callus on different explants taken from *in vitro* grown plantlets with the possibility of plant regeneration from callus.

2. MATERIALS AND METHODS

The seeds of *A. andrachne* L. were taken in November from a mature wild-grown tree located at 1.5 km westers Hasanava town (N:37°5′36.398″, E:42°38′40.801″) 40 km North-west Duhok city (Shahbaz, 2010). Seeds were extracted manually and kept at room temperature until been used for *in vitro* culture. To overcome seed dormancy, seeds were soaked in GA_3 solution (100 ppm) and cold stratification at 6°C for 5 days. The treated seeds were rinsed under running tap water for 30 minutes with adding 2 drops of detergent every 5 minutes. Seeds were dipped in 1.25% (25 % v/v) sodium hypochlorite for five minutes with continuous shaking. The seeds were washed 3 times with sterilized distilled water for 3 minutes each. Finally, the seeds were soaked in ethanol (70%) for 30 seconds and washed 3 times with sterilized distilled water every three minutes with continuous shaking under a laminar air-flow cabinet. The sterilized seeds were cultured in vessels containing 25 ml PGRs-free WPM, supplemented with 1 mgl^{-1} GA_3 , 1 mg.l^{-1} GA_3 plus 2.5 mg.l^{-1} BA, and 1 ml.l^{-1} PPM (active ingredients are 5-chloro 2-methyl 3 (2H) isothiazolone and 2-methyl-3 (2H) isothiazolone). The seed cultures were maintained in the incubation area with photoperiod of 16 hrs of light and 8 hrs of darkness and light intensity of 1000- 2000 lux. Seeds taken at the beginning of November were pretreated before culture to break physiological dormancy and induce germination in two ways:

1. Seeds were soaked in GA_3 solution (100 ppm) and cold stratification at 6°C for 5 days and sterilized with NaOCl (1.25% , 25 % v: v) for 5 minutes and Ethanol 70% for 30 seconds.
2. Cold pretreatment for 24 hours then the seeds were soaked in GA_3 solution (100 ppm) for 48 hours at room temperature and sterilized with NaOCl (2.5%, 50% v:v) for 15 minutes and Ethanol 70% for 30 seconds.

After that, the seeds were surface sterilized and cultured in glass vessels containing 25 ml PGR-free WPM, or supplemented with 1 $mg.l^{-1}$ GA_3 and 2.5 $mg.l^{-1}$ BA plus 1 mg. l^{-1} GA_3, also adding 1 $ml.l^{-1}$ PPM.

For the induction of callus, leaf discs and nodes of about 0.5 cm in length were taken from 6-8 weeks *in vitro* produced seedlings, then cultured on WPM supplemented with different concentrations of NAA (0.5, and 1.0 $mg.l^{-1}$) with TDZ (1.0, 2.0 and 3.0 $mg.l^{-1}$) and NAA (0.5 and 1.0 mg. l^{-1}) with BA (1.0, 2.0 and 3.0 $mg.l^{-1}$) with 3 explants per replicate (4 replicate/ treatment) to induced callus under light conditions. After six weeks, the percentage of callus induction and fresh weight of the callus were measured. For organogenesis, several combinations of growth regulators were tested to enhance regeneration from callus.

The treatments were set up in a Completely Randomized Design (CRD) with seven replications per treatment and five seeds per vessel (105 seeds). The rate and speed of germination were recorded after 4 weeks of culture. After that, the produced microshoots were subcultured several times on the same medium and the explants were used for the later stage of the micropropagation experiment.

Data from the treatments were analyzed by SAS program (SAS, 2017). The ANOVA and Duncan tests were used to compare treatment groups and whether they showed any statistical significance difference which was set at α=0.05 (SAS, 2017).

3.RESULTS AND DISCUSSION

In vitro seed germination was very successful after treating the seeds with GA_3 solution (100 ppm) and stratification treatment for 5 days in the refrigerator which was in accordance with Al-jabary (2011) and Odeh (2017). Healthy sterile cultures through the treatment were achieved with 1.25% (25 % v/v) sodium hypochlorite for 5 minutes and soaked in 70% ethanol for 30 seconds. 100% survival cultures were obtained from the cultured seeds (Figure 1). These results are in agreement with the findings of Al-jabary (2011). The number of germinated seeds was counted every day for 30 days. Germination percentage and rate were determined according to each pretreatment. The below formula was used for calculating the germination rate according to (AL Sakran *et al*, 2020):

GR= (germinated seeds number/ total number of seeds) ×100/

It can be noticed from Table (1) that treating with GA_3 raised the germination rate from only 17 % (control) to 45.7 % and 54.28 % by adding 1 mg. l^{-1} GA_3 and 2.5 $mg.l^{-1}$ BA with and without cold pretreatment. As well as, the above GA_3 treatment was very beneficial in increasing the speed of seed germination as shown below, and was calculated according to ISTA. (1999):

Speed of germination = X_1/N_1+ X_2/N_2+…X_n/N_n

X = number of germinated seeds
N= number of days.

Table (1): Seed germination experiment and speed of germination of *Arbutus andrachne* L.

Treatments	Rate of Germination (%)	Speed of Germination (Days)
Control	17.00 c	0.461 b
GA_3 (1 mg. l^{-1})	45.70 b	1.510 a
GA_3 (1 mg. l^{-1}) + BA (2.5 mg. l^{-1})	54.28 a	1.592 a

Table (2) declares that a 100% of survival rate was achieved for both treatments of cold stores and gibberellic acid treatments with no contaminated cultures. The seed germination rate was only 25% in both cases. For callus induction, it was very clear that the culture of node explants was superior upon the culture of leaf discs (Table 3) by reaching 100% as compared to only 66% in the case of leaves. Similar results were found by Giordani *et al*, (2005) who reported that the highest callus production was observed from internodes compared to leaves. The best callus induction (100%) was recorded for nodes cultured on WPM with both BA and TDZ respectively (Figure 2).

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Al-jabari (2011) and Odeh (2017) reported that seeds of *Arbutus andrachne* L. were sterilized very well by immersing in 20% NaOCl followed by dipping in ethanol (70%) for thirty seconds. Furthermore, Mostafa *et al*., (2010) obtained the best results when they used 4% sodium hypochlorite for 10 min followed by 70% ethanol for 30 s for sterilizing the seeds of *A. andrachne*. According to Kose (1998), Karam and Al-Salem (2001), Olmez *et al*. (2007), Tilki and Guner (2007), and Mostafa *et al*. (2010) dormancy was reported in seeds of *Arbutus* andrachne, which was broken by GA3 and cold stratification pretreatment.

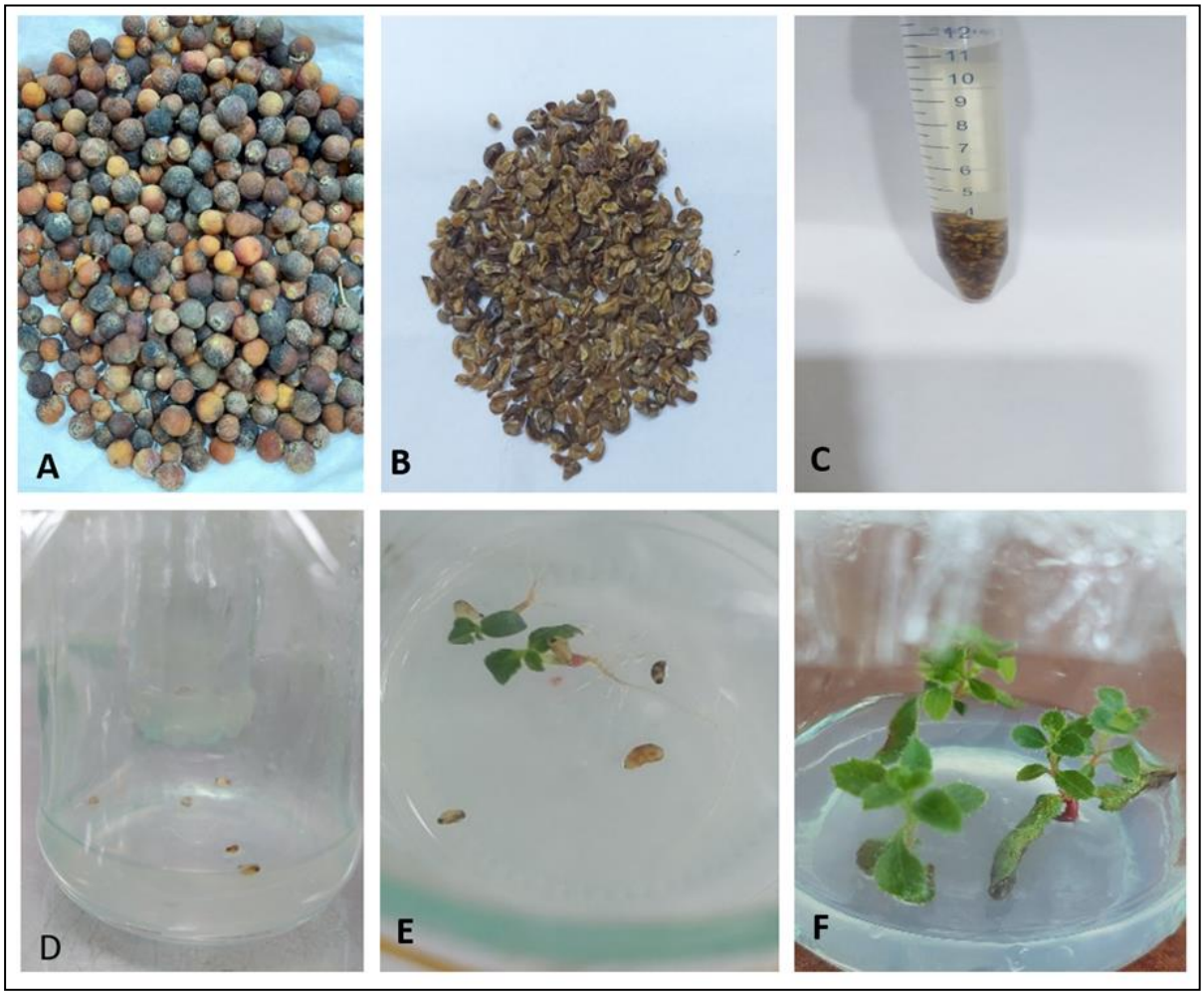

Fig. (1): Seed germination experiment of *Arbutus. andrachne* L. A. Collected fruits before excising the seeds. B. Seeds excised for culture. C. Treatment with GA3. D. Inoculated seeds in WPM culture. E. Initially germinated seeds grown on WPM after 4 weeks in culture. F. Subcultured seedlings after 4 weeks of culture.

Table (2): Survival rates of seeds of *Arbutus andrachne* L. after the disinfestation process.

Pre-Treatment for breaking dormancy		Germination rate (%)	Contamination%	Survival % after 60 days
Cold store (6 °C/ 120 hours) + GA_3 (100 ppm/ 120 hours)	NaOCl (1.25% for 5 min)+ Alcohol (70% for 30 sec)	25	0.0	100
Cold store (6 °C/ 24 hours) GA_3 (100 ppm/48 hours	NaOCl (2.5% for 15 min)+ Alcohol (70% for 30 sec)	25	0.0	100

Table (3): Callus induction on leaf discs and nodes of *Arbutus andrachne* L. as a response to different NAA, BA, and TDZ concentrations grown on WPM after 6 weeks in culture.

Explants	NAA ($mg.l^{-1}$)	Cytokinins ($mg.l^{-1}$)	Callus Induction Rate (%)	Means of explants	Remarks
Leaf discs	0.5	TDZ 1.0	41.66	34.70	Red green
		TDZ 2.0	33.30		Red green
		TDZ 3.0	66.66		Red green
		BA 1.0	33.30		Green
		BA 2.0	08.30		Green
		BA 3.0	41.60		Red green
	1.0	TDZ 1.0	08.33		Red green
		TDZ 2.0	08.33		green
		TDZ 3.0	50.00		Red green
		BA 1.0	33.30		Red green
		BA 2.0	66.60		Red green
		BA 3.0	25.00		Red green
Nodes	0.5	TDZ 1.0	100	95.13	Red green
		TDZ 2.0	100		Red green
		TDZ 3.0	100		Red green
		BA 1.0	83.30		Red green
		BA 2.0	100		Red green
		BA 3.0	91.60		Red green
	1.0	TDZ 1.0	100		Red green
		TDZ 2.0	91.66		Red green
		TDZ 3.0	100		Red green
		BA 1.0	100		Red green
		BA 2.0	75.00		Red green
		BA 3.0	100		Red green

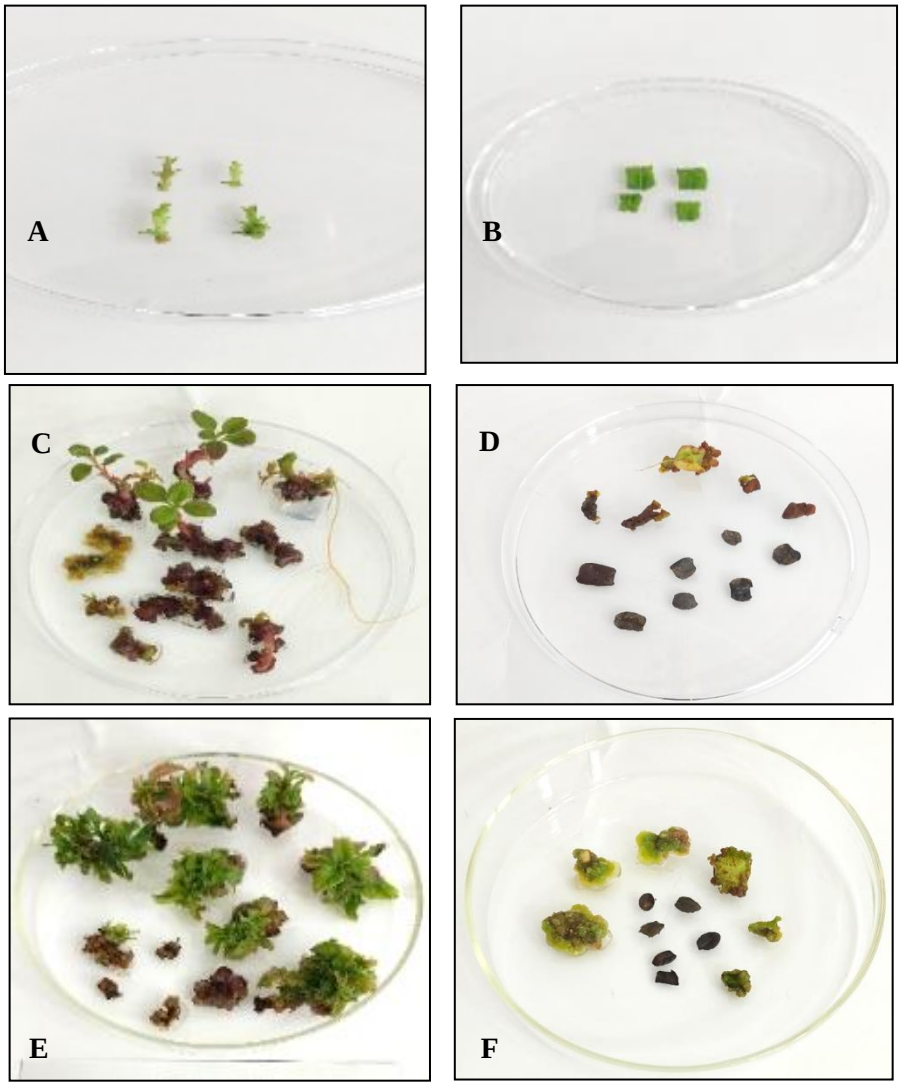

Fig. (2): Callus induction on leaves and nodes explants after 6 weeks on WPM. A. Node explants ready for culture B. Leaf explants ready for culture C. The best callus induction of node explants on WPM enriched with 1.0 mg.l-1 NAA and 1.0 mg.l-1 BA D. The best callus induction on leaf explants on WPM enriched with 1.0 mg.1-1 NAA and 1.0 mg.I-1 BA E. Best callus induction on node explants on WPM enriched with 1.0 mg.l-1 NAA and 1.0 mg.1-1 TDZ F. The best callus induction on leaf explants on WPM enriched with 1.0 mg.l-1 NAA and 1.0 mg.l-1 TD.

On the other hand, regarding the weight of callus initiated on leaves and nodes grown on WPM, the nodes exceed the leaves by producing 28.933 g as compared to only 0.333 g for leaf discs. The best-combined treatment of the culture of nodes on WPM enriched with 1.0 $mg.l^{-1}$ NAA+ 1.0 $mg.l^{-1}$ BA by producing the highest callus weight by recording 34.250 g (Table 4). These results indicate the importance of auxins and cytokinins being existence together in the culture medium to induce the callus of explants. Since, they promote cell division and differentiation in plant cells (Al-jabary, 2011). Valuable organogenesis and regeneration were noticed on the induced callus from both leaf discs and nodes. Figure (3) shows that the best shoot and root regeneration was noticed on node explants grown on WPM enriched with 1.0 $mg.l^{-1}$ NAA and 2.0 $mg.l^{-1}$ BA. Whereas the leave discs showed the best regeneration on WPM media supplemented with 1.0 $mg.l^{-1}$ NAA and 2.0 $mg.l^{-1}$ BA (Figure 3, A, B).

Table (4): Callus weight initiated on leaf discs and nodes of *Arbutus andrachne* L. as a response to different NAA, BA, and TDZ concentrations grown on WPM after 6 weeks in culture.

Explants	NAA (mg. l^{-1})	Cytokinins (mg. l^{-1})	Callus Weight (g)	Means of explants	Remarks
Leaf discs	0.5	TDZ 1.0	0.100 e	0.333 b	Red green
		TDZ 2.0	0.000 e		Red green
		TDZ 3.0	0.000 e		Red green
		BA 1.0	0.000 e		Red green
		BA 2.0	0.450 e		Red green
		BA 3.0	1.550 e		Red green
	1.0	TDZ 1.0	0.250 e		Red green
		TDZ 2.0	0.350 e		Red green
		TDZ 3.0	0.300 e		Red green
		BA 1.0	0.125 e		Red green
		BA 2.0	0.150 e		Red green
		BA 3.0	0.725 e		Red green
Nodes	0.5	TDZ 1.0	31.225 b-d	28.933 a	Red green
		TDZ 2.0	30.250 cd		Red green
		TDZ 3.0	31.125 b-d		Red green
		BA 1.0	0.250 e		Red green
		BA 2.0	31.700 bc		Red green
		BA 3.0	32.225 b		Red green
	1.0	TDZ 1.0	31.950 bc		Red green
		TDZ 2.0	29.875 d		Red green
		TDZ 3.0	30.975 b-d		Red green
		BA 1.0	34.250 a		Red green
		BA 2.0	31.575 bc		Red green
		BA 3.0	31.800 bc		Red green

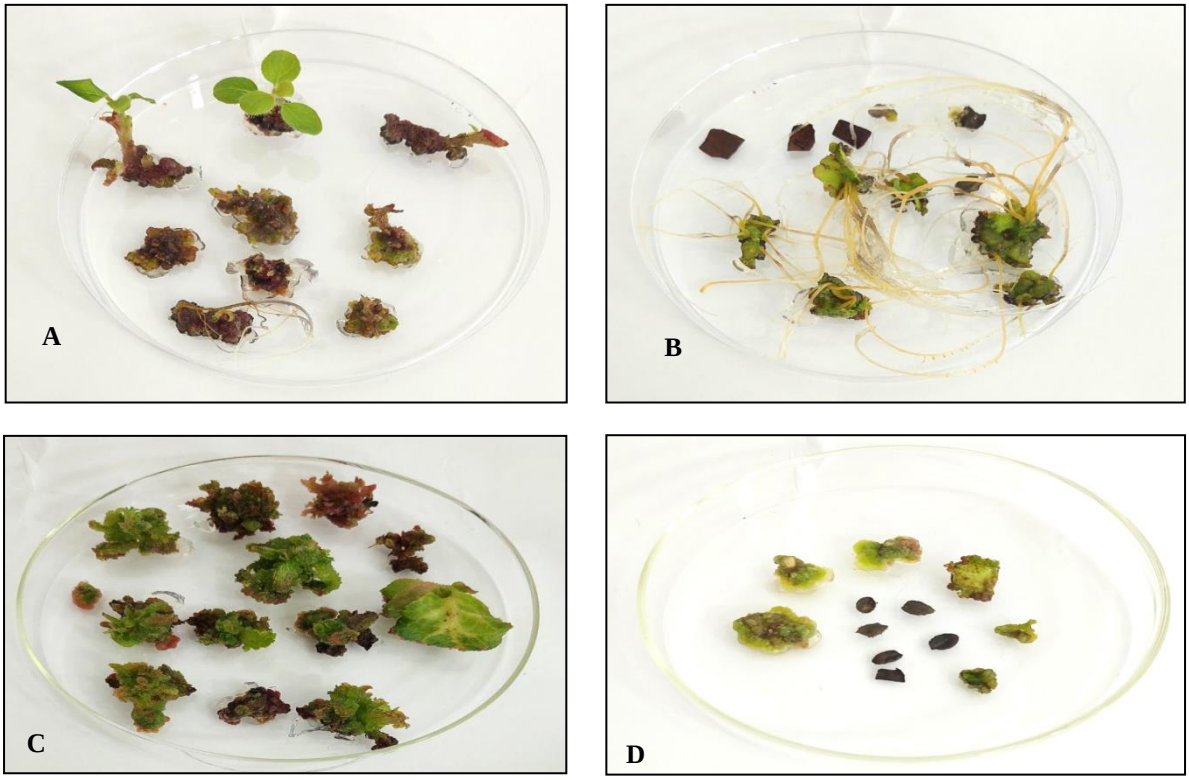

Fig. (3): Plant regeneration on callus of *Arbutus andrachne* L. A. Regenerated shoots and roots on callus initiated on node explants on WPM enriched with 1.0 mg.l-1 NAA and 2.0 mg.l-1 BA B. Regenerated shoots and roots on callus initiated on leaf discs treated with 1.0 mg.1-1 NAA and 2.0 mg.l-1 BA C. Regenerated shoots and roots on callus initiated on node explants on WPM enriched with 1.0 mg.l-1 NAA and 3.0 mg.I-1 TDZ D. Non-regenerated shoots and roots on leaf callus initiated on leaf explants on WPM enriched with 1.0 mg.l-1 NAA and 3.0 mg.1-1 TDZ.

3. CONCLUSION

Arbutus seed germination is usually very poor, this case could be overcome by GA_3 treatment plus cold treatment. The problem of fungal infection could be successfully overcome by PPM (Plant Preservative Mixture) treatment. Moreover, the seed germination could be spend around six weeks in culture. On the other hand, adding BA in addition to GA_3 may increase the rate of healthy and raise the survival rate of plantlets. Adding BA in addition to GA_3 made the plant healthier and raised the survival rate of plantlets. For callus induction, nodal explants showed better performances than leaf disc explants. Regarding the hormonal combination, adding 1.0 mg. l^{-1} of NAA and BA gave the best callus induction on nodal explants grown on WPM. Best plant regeneration on callus was found on nodal explants grown on WPM enriched with 1.0 mg. l^{-1} of NAA and 2.0 mg.l^{-1} of BA. These results confirm that this endangered plant can be multiplied, propagated, and commercially produced via tissue culture technique.

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