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IN VITRO REGENERATION, CONSERVATION, AND FIELD EVALUATION OF A MEDICINAL PLANT– GREATER BURDOCK (*ARCTIUM LAPPA* L.)

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Abstract

A suitable micropropagation protocol and *ex vitro* acclimation method have been developed from *in vitro* grown seedling explants through cotyledonary node and leaf explants in consideration of the vegetable and medicinal properties of Greater Burdock. MS medium with 0.5-4.0 µM BAP showed highest percentage of axillary shoot regeneration from the cotyledonary nodal explants. Direct shoot regeneration was achieved by culturing 1.0 cm^2 sections of about 25 days old leaves of *in vitro* grown shoot on MS medium enriched with 4.0 µM BAP and 2.0 µM IBA or NAA after 5 weeks of culture. Within six weeks of incubation on medium enriched with 4.0 M BAP and 2.0 M IBA or NAA, the leaf explants also developed callus from the cut margins. The greatest number of adventitious shoots could then be formed from the leaf-derived callus within 10 weeks of culture on the same media mix. More than 20 shoots were formed per callus clump at the third subculture, which had the highest rate of shoot multiplication. *A. lappa's* shoot and callus were both preserved at 5 ºC in MS medium with 4.0 µM Kn and 2.0 µM IBA, as well as 4.0 µM BAP and 2.0 µM IBA, respectively. The *in vitro* proliferated and elongated shoots were separated from callus clump for rooting. A root-induction MS medium with 6.0 µM IBA or NAA was used to cultivate the microshoots individually. All of the cultured microshoots generated 2-16 roots within 4 weeks of being moved to the rooting medium. Regenerated plantlets were transferred to vermiculite and successfully established in an *ex vivo* environment with a 98% survival rate.

Key words: Adventitious shoot regeneration, acclimatization, medicinal plant, plant growth regulators, vegetable.

Introduction

Arctium lappa L. (Greater burdock) is a member of the composite family and is distributed throughout China and Japan. Due to its resistance for drought, salt, disease, and arid land, this plant may thrive in a variety of ecological settings. Its tender leaves, flowers, and roots can all be consumed. The succulent roots are rich in vitamins, minerals, and trace elements in addition to proteins, amino acids, and other nutrients (Cheng et al. 1994). According to Horita and Suoka (2002), it is now a highly popular vegetable, particularly in Asia. Additionally, the seeds or roots can be used to create wholesome goods with a marketable potential, such as gum, tea, and soft beverages. For treating colds, throat swelling, and measles, *A. lappa* has been widely utilized as a traditional Chinese medicine herb (Wu 1990). Currently, more than 30 distinct substances with biological and pharmacological properties have been isolated from the roots, stems, leaves, and seeds of this plant, including lignans, arctigenin, lappaol, arctieacid, and arctinal (Jiang 2001, Hao et al. 2004). According to reports, these substances can boost immunity; fend off diabetes, constipation, and hypertension (Jiang 2001). In addition, leukemia and acquired immune deficiency syndrome have both been treated with it (Schroder et al. 1990, Hirano et al. 1994). In addition, the compounds have anti-aging, anti-viral, and anti-

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tumor characteristics (Kato et al. 1998, Hirose et al. 2000, Takasaki et al. 2000, Li et al. 2004). *A. lappa* has been the subject of further research in recent years due to its therapeutic and nutritional benefits.

Our research suggests that nursery owners are not motivated to raise seedlings of these unusual plants because of a lack of information. The method of seedling production of Greater burdock is not popular. Therefore, these commercially significant plants are not being used sustainably by the population. As a result, the biodiversity is being harmed, and traders and users of the greater burdock plant are having trouble meeting their needs. There is an urgent need for both rapid production and sustainable use. For a planting program, the traditional method of multiplication is insufficient. Then multiplication using contemporary methods is badly needed. The proposed research uses tissue culture technique to create a system for quickly creating a large number of disease-free plants, regardless of seasonal fluctuations. For developing ex situ conservation, cultivation, and nursery development, these plants will be utilized. Greater burdock plants can be quickly multiplied using tissue culture and other traditional methods, guaranteeing the availability of disease-free plantlets across the nation.

There are numerous reports of *A. lappa* that have been well-documented in the pharmaceutical field (Liu et al. 2005, Li et al. 2008, Lou et al. 2010a,b). There aren't many reports on the tissue culture of this medicinally important plant, as far as we know. He et al. (2006) described a callus induction and high-frequency plant regeneration methodology using hypocotyl and cotyledon explants of *Arctium lappa*. Zebarjadi et al. (2011) created a procedure for Burdock *in vitro* propagation by cell differentiation and multiplication. Wenting et al. (2006) described another method of regeneration of Burdock by several explants. In this paper, we have provided descriptions of a high frequency plant regeneration methodology of *A. lappa* from cotyledonary node and leaf explants, as well as conservation techniques and acclimation methods. The fundamental objective of our research is to develop a unique variety or line that can be grown in Bangladesh's climate using somaclonal variation in tissue culture techniques. After meeting the need on the local market, farmers can grow this essential crop for vegetable and export it, generating a sizable amount of foreign revenue.

Materials and Methods

Arctium lappa seeds were collected from Japan via e-Bay and used as research materials. To reduce the amount of surface bacteria before enriching aseptic seedlings, seeds were thoroughly rinsed under running tap water for ten minutes. For the purpose of surface sterilization, the seeds were treated as follows: first, with 1% savlon for 10 minutes while being continuously shaken; second, with uninterrupted tap water to remove any savlon-foam residue; and third, sensibly washing 2-3 times with distilled water. The seeds were then transferred to a sterilized 250 ml conical flask in a laminar air flow cabinet. They were subsequently immersed for varying lengths of time in 0.1% $HgCl_2$ after being rinsed with 80% ethanol. Without the use of any plant growth regulators, seeds were embedded on the surface of the semisolid MS (Murashige and Skoog, 1962) medium after being washed with double sterile distilled water. After 20–30 days of culture, the aseptic seedlings had grown to a height of 5–6 cm. The aseptically grown seedlings that were six weeks old were processed into explants that included a cotyledonary node and an *in vitro* generated leaf. Cotyledonary nodes were subsequently grown on agar-gelled MS medium supplemented with 0.5-6.0 µM BAP, Kn or CPU for axillary shoots. For the purpose of producing direct adventitious shoots, leaf explants were also cultured on MS media with 2.0-6.0 µM BAP, either alone or in combination with 1.0-4.0 µM IBA or NAA. In addition, leaf-derived callus was cultured on MS containing 2.0-6.0 µM BAP or Kn together with 1.0-4.0 µM IBA or NAA for restoring adventitious shoots. Micro-shoots were created from shoot clusters that had been produced *in vitro* and were rooted in MS media at both full and half strength with 4.0 to 8.0 µM of NAA, IBA,

or IAA. All media were stimulated with 3% sucrose, solidified with 0.8% agar (Hi-media, India), adjusted to pH 5.7 ± 0.1, and steam sterilized for 20 minutes at 121°C under 1.2 kg/cm_2 pressure. The cultures were grown at 25 ± 1°C with a 16-hour photoperiod under a cool-white fluorescence tube lamp with a 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity. Fifty well-rooted plantlets were removed from the test tubes, and the roots were cleaned with tap water. Then it was moved into a little plastic pot, stuffed with a variety of substrates, including garden soil, garden soil:sand:compost (2:1:1), and verimicompost, and covered with a transparent plastic tent. The plants were kept in regulated environments in culture rooms, and the substrate was completely moistened for 30 days with irrigation. The plastic pots were then moved to a shaded area with frequent irrigation in an outdoor setting. The potted plants were moved to beds with a 20:1 (v/v) ratio of garden soil and cattle dung after 15 days, and they were frequently watered.

For *in vitro* conservation of shoot cultures, the proliferated shoots (length of 1.5 cm) were individually transferred to fresh multiplication medium (MS + 4.0 µM Kn + 2.0 µM IBA) and then incubated at 5ºC in the dark. Twenty cultures were taken after 1, 8, 12 and 20 months and survival percentages, healthy shoot percentage, number of proliferated shoots and height of shoots were recorded. The cultures were transferred to fresh medium and placed under standard culture room conditions for four weeks and then survival percentage were assessed (survival on recovery conditions). For cold storage of callus cultures, equal inoculum (about 250 mg) of the proliferated callus were transferred to callus induction medium (MS + 4.0 µM BAP + 2.0 µM IBA) and then they were incubated at 5.0ºC in the dark. Eighty replicated were used in this experiment. After 1, 8, 12 and 20 months, fresh mass, dry mass, dry mass/fresh mass and percentages of embryogenic cultures were recorded from twenty replicates of each treatment. To assess the importance of increased sugar content and osmotic tress in the long-term storage, shoots (1.5 cm) were individually cultured on multiplication medium supplemented with 40 g/l mannitol. Survival percentages, healthy shoots percentages, number of proliferated shoots, sand height of the shoots were recorded after 1, 8, 12, and 20 months of incubation at normal conditions. Each treat consists of twenty replicates. After shifting the cultures to mannitol-free medium, it took four weeks to report the survival percentages. Every experiment followed a completely random design, and the data collected was statistically analyzed using standard error (SE) in accordance with the technique outlined by Snedecor and Cochran (1967).

Results and Discussion

Axillary shoot proliferation

For the purpose of promoting the growth of axillary shoots, the explants carrying pre-formed meristems, such as cotyledonary nodal segments, were cultured on MS medium supplemented with various concentrations of cytokinins. The results of the initial experiment made it clear that nodal segments of seedlings made the best explants and that MS medium was the best medium for proliferating *A. lappa* shoots *in vitro*. In this study, the best cytokinin type and concentration for maximizing shoot proliferation were determined by cultivating cotyledonary nodal segments on MS medium supplemented with BAP, Kn, and CPPU at concentrations of 0.5, 1.0, 2.0, 4.0 and 6.0 µM (Table 1). After six weeks of culture, the medium treated with BAP produced the best results in this experiment among the three types of cytokinin (Fig. 1A). The best results were obtained with a BAP concentration of 2.0 µM, with a shoot formation of 97.5 ± 1.5%. The most shoots per explant, 8.6 ± 0.4, were produced by cultivated explants. On the other hand, across all of the treatments, the 1.0 µM BAP-containing media produced shoots with the longest average length of 6.2 ± 0.1 cm per explant. Similar to Kn and CPPU also generated axillary shoots from cultured explants. In this experiment, the medium

fortified with 2.0 μM Kn produced the highest frequency 73.8 ± 2.3% of shoot proliferation and the highest number 4.9 ± 0.4 of shoots per explants. The lowest result, however, was seen on the 6.0 μM Kn-containing medium, where only 41.3 ± 1.9% of explants showed proliferation with 1.0 ± 0.2 shoots per culture. Additionally, when the explants were cultured on CPPU-based medium, maximum frequency 82.5 ± 1.8% of the explants demonstrated shoot proliferation, and the greatest number of total shoots per explant was 5.2 ± 0.2 at 1.0 μM, whereas the other two cytokinins (BAP and Kn) demonstrated that maximum frequency and number of total shoots per explant were the highest at 2.0 μM. At 6.0 μM CPPU, one shoot only was noted. Based on the experiment's findings, it was clear that the concentrations of various cytokinins had a significant impact on how shoots differentiated. Compared to Kn and CPPU, BAP was a more potent cytokinin in terms of proliferation efficiency. BAP was shown to be most effective at concentrations between 0.5 and 2.0 μM for shoot proliferation of *A. lappa*. Similar findings were also found from different experiments into axillary shoot propagation in a variety of medicinal plants, including and *Acacia mangium* (Shahinozzaman et al. 2012), *Orthosiphon stamineus* (Lai-Keng and Leng 2004), *Citrus grandis* (Begum et al. 2004), *Dorystoechas hastata* (Bengi et al. 2010), *Mentha piperita* (Sunandakumari et al. 2004), *Orthosiphon spiralis* (Elangomathavan et al. 2003), *Adhatoda vasica* (Azad et al. 2003), *Osmium sanctum* (Begum et al. 2000), *Centella asiatica* (Tiwari et al. 2000), *Melissa officinalis* (Tavares et al. 1996), and many other plants.

Table 1. Effects of cytokinins on regeneration of shoots from the cotyledonary nodal explants of *in vitro* grown seedlings on MS medium after 8 weeks of culture.

Growth regulator	Concentration (μM)	Shoot formation (%)	Total number of shoot / explants	Average length of shoot / explant (cm)
BAP	0.5	91.3 ± 1.4^{c}	3.2 ± 0.1^{e}	5.1 ± 0.2^{b}
	1.0	95.0 ± 1.6^{b}	6.7 ± 0.2^{b}	6.2 ± 0.1^{a}
	2.0	97.5 ± 1.5^{a}	8.6 ± 0.4^{a}	5.4 ± 0.5^{b}
	4.0	82.5 ± .6^{d}	6.3 ± 0.5^{b}	4.0 ± 0.4^{c}
	6.0	56.3 ± 2.1^{g}	1.8 ± 0.1^{f}	3.2 ± 0.5^{c}
Kn	0.5	57.5 ± 2.2^{g}	1.9 ± 0.2^{f}	2.2 ± 0.3^{c}
	1.0	67.5 ± 1.5^{f}	3.4 ± 0.5^{e}	2.8 ± 0.5^{c}
	2.0	73.8 ± 2.3^{e}	4.9 ± 0.4^{c}	3.0 ± 0.7^{c}
	4.0	60.0 ± 1.8^{g}	1.8 ± 0.1^{f}	2.3 ± 0.5^{c}
	6.0	41.3 ± 1.9^{i}	1.0 ± 0.2^{f}	1.9 ± 0.4^{c}
CPPU	0.5	67.5 ± 1.5^{f}	1.6 ± 0.3^{f}	2.8 ± 0.5^{c}
	1.0	82.5 ± 1.8^{d}	5.2 ± 0.2^{c}	3.8 ± 0.5^{c}
	2.0	72.5 ± 1.7^{e}	4.1 ± 0.1^{d}	3.3 ± 0.2^{c}
	4.0	67.5 ± 2.1^{f}	4.1 ± 0.5^{d}	2.9 ± 0.5^{c}
	6.0	50.0 ± 1.4^{h}	1.0 ± 0.1^{f}	2.3 ± 0.1^{c}

Values indicate the mean standard deviation of 20 replicates for each treatment over the course of 4 different studies. Duncan's multiple comparison tests at the 0.05 probability level do not find a difference between means that are preceded by the same letter.

Adventitious shoot proliferation

Seedling-derived leaf explants were grown on MS media supplemented with different concentrations of BAP alone or in combination with IBA or NAA in order to promote direct adventitious shoot regeneration. Data on the percentage of shoot formation, the total number of shoots per culture, the number of usable shoots per culture, the average length of shoots per culture, and the intensity of callus growth from various treatments were recorded after 10 weeks of culture initiation.

Results on the morphogenic responses of the cultured explants are shown in Table 2. BAP alone in the medium failed to create any morphogenic or non-morphogenic callus, but it did induce small percentages (10–30%) of adventitious shoots. The medium containing 4.0 µM BAP and 2.0 µM IBA produced the largest percentage of shoots from leaf explants (75.0%) without any callus developing out of the three BAP-IBA combinations (Fig. 1B). In this combination total number of shoots per-culture 5.5 ± 0.5, number of usable shoots per-culture 4.1 ± 0.6, and average length of shoot per-culture 7.2 ± 0.9 cm were obtained from the same explants. On the other hand, among the BAP with NAA combination, the medium containing 4.0 µM BAP and 2.0 µM NAA showed the highest frequency of 60.0 percentage direct shoot bud formation, with the maximum number of 3.8 ± 0.3 shoots per cultures, 3.1 ± 0.1 usable shoots per cultures, and 5.3 ± 0.8 cm average length of shoots per cultures recorded from leaf explants. A significant amount of callusing was created in this experiment when 6.0 µM of BAP was combined with 4.0 µM of IBA or NAA. The culture conditions and types of explants had a substantial impact on whether morphogenic or non-morphogenic callus development occurred.

Table 2. Effects of various cytokinin concentrations alone and in conjunction with auxins on the induction of adventitious shoots from leaf explants.

Growth regulators (µM)	Shoot formation (%)	No. of total shoot / culture	No. of usable shoot / culture	Average length of shoot / culture (cm)	*Intensity of callus growth
BAP					
2.0	10.0	1.1 ± 0.4^{g}	1.0 ± 0.5^{d}	5.1 ± 1.2^{c}	-
4.0	30.0	2.5 ± 0.2^{d}	2.1 ± 0.3^{c}	4.5 ± 0.9^{c}	-
6.0	20.0	1.3 ± 0.5^{f}	1.2 ± 0.4^{d}	3.5 ± 0.5^{d}	-
BA + IBA					
2.0 + 1.0	45.0	4.6 ± 0.4^{b}	3.6 ± 0.7^{b}	6.1 ± 1.1^{b}	-
4.0 + 2.0	75.0	5.5 ± 0.5^{a}	4.1 ± 0.6^{a}	7.2 ± 0.9^{a}	+
6.0 + 4.0	50.0	3.5 ± 0.2^{c}	2.8 ± 0.4^{b}	4.8 ± 0.6^{c}	++
BA + NAA					
2.0 + 1.0	25.0	1.9 ± 0.5^{e}	1.3 ± 0.6^{d}	3.2 ± 1.3^{d}	-
4.0 + 2.0	60.0	3.8 ± 0.3^{c}	3.1 ± 0.1^{b}	5.3 ± 0.8^{c}	++
6.0 + 4.0	35.0	2.7 ± 0.2^{d}	2.2 ± 0.5^{c}	3.8 ± 0.3^{d}	+++

*Intensity of callusing: massive (+++); considerable (++); poor (+) and no callusing (-). Values indicate means with a standard error of 20 explants for each treatment. By Duncan's Multiple Range Test at 0.05% probability level, means that are denoted by the same letters are not statistically distinct.

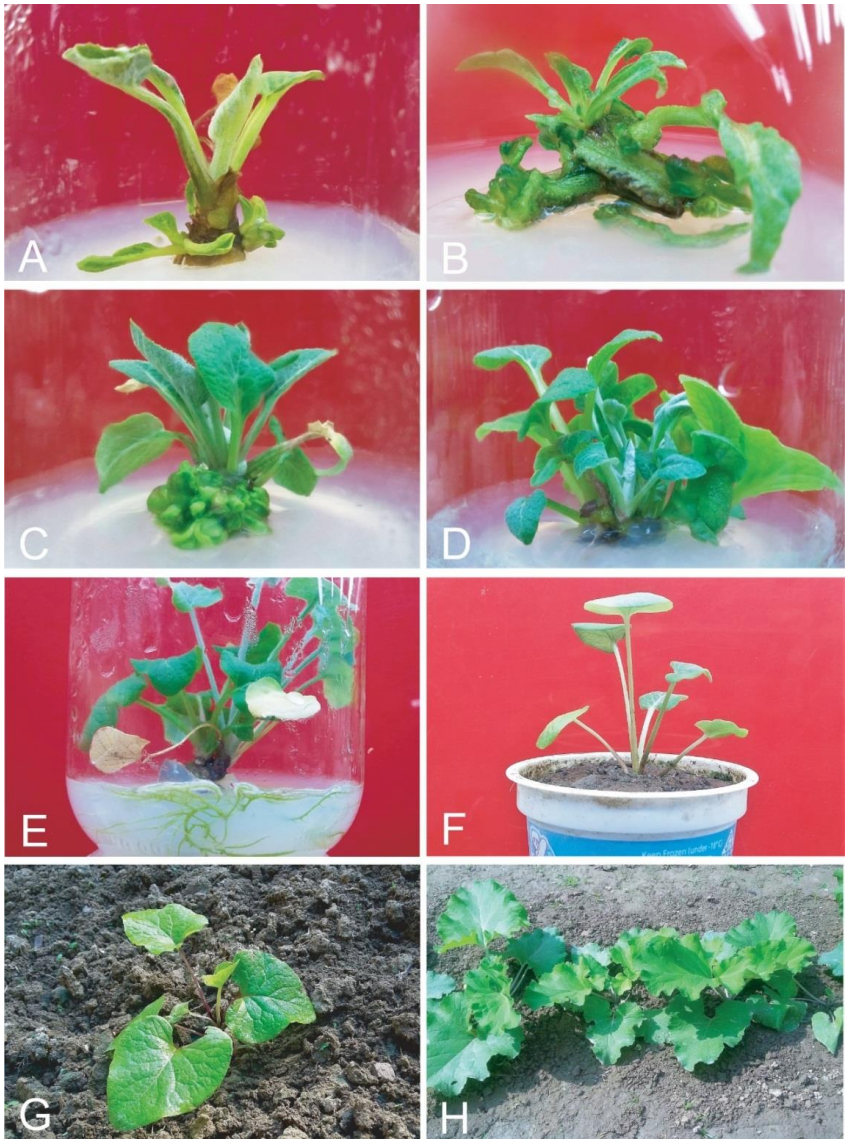

Fig. 1 (A-H): Plant regeneration and *ex vitro* acclimatization of *A. lappa*. A: On MS medium supplemented with 2.0 µM BAP, an axillary shoot formed from the cotyledonary node. B: After 6 weeks of culture incubation, a direct adventitious shoot developed from the cut margin of leaf explants on MS medium containing 4.0 µM BAP and 2.0 µM IBA. C-D: After 6 weeks of culture incubation on 4.0 µM BAP and 2.0 µM IBA supplemented MS medium, adventitious shoot development from leaf derived callus is shown (C), and elongation and multiplication of shoots are shown (D) after 10 weeks. E: Rooting of the *in vitro* proliferated shoots in MS media containing 6.0 µM IBA. F: Regenerated plantlets acclimatized in *in vivo* condition on a soil mixture of garden soil: sand: compost (2:1:1) after 3 weeks of translation. G-H: *In vivo* acclimatized plantlets were raised under *ex vitro* condition for one month (G), and two months (H), on a soil mixture of garden soil and cattle dung.

Different frequencies of adventitious shoot formation and the variations among them were statistically significant. Among the various treatments, MS medium containing 4.0 µM BAP plus 2.0 µM IBA produced adventitious shoots at high rates, with a frequency of 80.0% from leaf-derived callus explants (Table 3). On MS media supplemented with 4.0 µM BAP and 2.0 µM NAA, significant adventitious shoot development was produced from the same explants of *A. lappa*.

Table 3. Effects of various cytokinin and auxin concentrations and combinations on the production of adventitious shoots from leaf-derived callus.

Growth regulators (µM)	Shoot formation (%)	No. of total shoot / culture	No. of usable shoot / culture	Average length of shoot / culture (cm)	*Intensity of callus growth
BAP + IBA					
2.0 + 1.0	70.0	5.6 ± 0.4^{c}	4.2 ± 0.5^{c}	5.5 ± 1.2^{b}	+
4.0 + 2.0	80.0	10.2 ± 0.5^{a}	8.3 ± 0.3^{a}	6.9 ± 0.8^{a}	++
6.0 + 4.0	55.0	4.2 ± 0.2^{d}	3.9 ± 0.1^{c}	4.1 ± 0.4^{d}	++
BAP + NAA					
2.0 + 1.0	50.0	4.7 ± 0.5^{d}	4.0 ± 0.9^{c}	3.5 ± 1.2^{e}	+
4.0 + 2.0	75.0	7.6 ± 0.3^{b}	6.4 ± 0.7^{b}	5.4 ± 0.7^{b}	++
6.0 + 4.0	35.0	3.5 ± 0.2^{d}	3.1 ± 0.8^{d}	3.2 ± 0.3^{e}	+++
Kn + IBA					
2.0 + 1.0	35.0	2.9 ± 0.4^{d}	2.1 ± 0.9^{e}	4.6 ± 0.7^{c}	+
4.0 + 2.0	55.0	3.8 ± 0.5^{d}	3.2 ± 0.2^{d}	5.9 ± 0.5^{b}	+
6.0 + 4.0	15.0	2.1 ± 0.2^{d}	1.9 ± 0.3^{e}	3.5 ± 0.3^{c}	++
Kn + NAA					
2.0 + 1.0	10.0	2.2 ± 0.5^{d}	2.1 ± 0.4^{e}	3.1± 0.6^{e}	+
4.0 + 2.0	35.0	3.1 ± 0.3^{d}	2.8 ± 0.6^{d}	4.4 ± 0.5^{d}	++
6.0 + 4.0	0.0	-	-	-	++

*Intensity of callusing: (+) slight callusing, (++) considerable callusing, (+++) intensive callusing and (-) no callusing. Values indicate means with a standard error of 20 explants for each treatment. By Duncan's Multiple Range Test at 0.05% probability level, means that are denoted by the same letters are not statistically distinct.

The lowest frequencies of adventitious shoot formation from leaf-derived callus were observed in the MS medium supplemented with 6.0 µM Kn and 4.0 µM IBA or NAA. Among other *in vitro* parameters, one of the most crucial parts of successful micropropagation is determining the best types and quantities of plant growth regulators to use as medium constituents (Shimizu-Sato et al. 2009). Auxin and cytokinin combinations in excess concentrations produced large amounts of either morphogenic or non-morphogenic callus but not excessive frequencies of adventitious shoots. In this experiment, it was observed that the ideal media combinations for the proliferation of the adventitious shoots from leaf-derived callus were 4.0 µM BAP

as cytokinin and 2.0 µM IBA as auxin (Fig. 1C-D). Hesami et al. (2018) found that direct shoot regeneration and the development of morphogenic and non-morphogenic calluses from *Ficus religiosa* seedling-derived petiole segments depend on the auxin-cytokinin ratio. According to their findings, a 10:1 ratio between BAP and IBA produced the maximum 93% of shoot regeneration. Similar findings were made with *Cassia angustifolia* (Siddque et al. 2015). On the other hand, according to Khan et al. (2015), grapes grew the most calluses when auxin and cytokinin were mixed in a 10:2 ratio. Depending on the type and concentration of plant growth regulators used, leaf explants responded differently to callus induction and concurrent shoot regeneration. Callus began to develop on the cut margins and eventually covered the whole surface of the responsive explants grown on medium with plant growth regulators after an 8-week culture period.

Formation of adventitious root

The successful establishment of the proliferating shoots in the soil is necessary for meaningful *in vitro* dispersion. Because of this, microshoots from the *in vitro* multiplied shoot clusters were individually rooted on MS and ½MS medium, along with different auxin (IBA, NAA, and IAA) absorptions (4.0-8.0 µM). Our research showed that MS was the most effective medium for *A. lappa's* root production out of the two strengths of MS medium (Table 4). The highest rate of root production (100%) was observed in MS medium supplemented with 6.0 µM IBA, whereas 95% of roots were formed in MS medium containing 4.0 µM NAA (Fig. 1E). On the other hand, 6.0 µM IBA containing ½MS medium showed the highest root development rate of 80%. The highest number of roots, 7.2 ± 0.3, and the longest root per shoot, 16.6 ± 0.2 cm, were found in MS with 6.0 µM IBA, but in ½MS medium, they were 4.7 ± 0.4 and 11.8 ± 0.4 cm, respectively, with the same IBA concentration. Microshoots developed small percentages of roots when they were educated on MS or ½MS medium combined with 8.0 µM IBA or NAA. Furthermore, both in MS and ½MS medium, deformity and delayed root growth were seen at high IBA or NAA complement absorption levels.

The proportion of root initiation, number of roots per shoot, length, and timing of root formation were all clearly influenced by the concentrations and types of auxins as well as the basal medium. Among the various absorptions of IBA, NAA, or IAA-complemented MS that were investigated, the rooting response of microshoots in IBA-containing medium was superior to that in NAA-containing medium, however the least rooting response was seen with all IAA concentrations examined. The findings are in agreement with those of *Ruta graveolens* (Ahmad et al. 2010), *Citrulus lanatus* (Khatun et al. 2010), *Passiflora foetida* (Shekhawat et al. 2015), and other plant species. According to the results of our tests, MS medium exhibited better rooting than ½MS among the two strengths of MS medium. The root produced in ½MS with IBA in various concentrations was noticeably superior to that produced in MS from multiplied shoots of *Gleditsia capsica, Caesalpinia bonduc, Stevia rebaudiana*, and *Psidium guajava,* according to Zarinjoei et al. (2014), Cheruvathur et al. (2010), Janarthanam et al. (2009), and Jaiswal and Amin (1987), respectively. In addition, Cheruvathur et al. (2010) discovered that when *Caesalpinia bonduc* shoots were grown on ½MS with 6.0 mg/l IBA, the extreme frequency of the culture that formed roots was 100%. Well-rooted plantlets were transferred to tiny plastic pots filled with garden soil, compost, and sand in a 2:1:1 ratio and kept in the growth room's humid *ex vitro* environment (Fig. 1F). The plantlets adapted more successfully in *ex vivo* if they were kept in the growing room for 30

days before being transferred to outside circumstances. In *ex vitro* settings, 98% of transplanted plantlets survived and adjusted satisfactorily after 25 days (Fig. 1G).

Table 4. Effects of different auxin concentrations on the growth of adventitious roots from *A. lappa* microcuttings in MS and ½MS medium.

Media and types of auxins	Conc. of auxins (µM)	Root formation (%)	No of roots/ rooted cutting	Average length of roots (cm)	Days to root formation	Callus formation at the cutting base
	4.0	95.0	4.5 ± 0.2^{c}	14.5 ± 0.1^{b}	7-10	-
MS + IBA	6.0	100.0	7.2 ± 0.3^{a}	16.6 ± 0.2^{a}	8-12	-
	8.0	65.0	3.5 ± 0.1^{c}	9.5 ± 0.4^{e}	11-13	++
	4.0	85.0	3.6 ± 0.3^{c}	11.7 ± 0.7^{d}	8-11	-
MS + NAA	6.0	95.0	6.2 ± 0.5^{b}	13.1 ± 0.1^{c}	10-12	+
	8.0	55.0	1.9 ± 0.1^{d}	7.5 ± 0.1^{f}	12-16	++
	4.0	30.0	2.8 ± 0.3^{c}	5.6 ± 0.7^{f}	13-15	-
MS + IAA	6.0	55.0	3.8 ± 0.2^{c}	7.7 ± 0.1^{f}	11-16	-
	8.0	60.0	1.2 ± 0.4^{e}	4.2 ± 0.1^{f}	12-15	-
	4.0	75.0	3.9 ± 0.2^{c}	7.3 ± 0.1^{f}	11-16	-
½MS + IBA	6.0	80.0	4.7 ± 0.4^{c}	11.8 ± 0.4^{d}	9-15	+
	8.0	50.0	1.9 ± 0.2^{d}	6.8 ± 0.5^{f}	12-16	++
	4.0	60.0	3.1 ± 0.2^{c}	6.3 ± 0.2^{f}	11-14	-
½MS + NAA	6.0	75.0	4.3 ± 0.3b^{c}	8.3 ± 0.3^{f}	10-16	+
	8.0	45.0	1.9 ± 0.3^{d}	5.1 ± 0.3^{f}	12-18	++
	4.0	20.0	1.2± 0.2^{e}	4.7 ± 0.4^{f}	16-20	-
½MS + IAA	6.0	35.0	2.7 ± 0.4^{c}	6.2 ± 0.3^{f}	14-15	-
	8.0	40.0	1.0 ± 0.1^{e}	2.3 ± 0.5^{g}	13-18	-

*****Intensity of callusing: (+) slight callusing, (++) considerable callusing, and (-) no callusing. Values indicate means with a standard error of 20 explants for each treatment. By Duncan's Multiple Range Test at 0.05% probability level, means that are denoted by the same letters are not statistically distinct.

In vitro conservation

A. lappa shoots held for up to 8 months were entirely alive (100 percent survival), and it was found that the shoots multiplied slowly and grew slowly in height (Table 5). After another 4 months, 80% of the shoots were still alive, but some of them turned white and succulent. After 20 months of storage, 60% of the shoots were still in good condition. When shoots were recultured under typical conditions, this percentage increased. According to Conner and Fallon (1993), mannitol prevents the growth of micropropagated asparagus. Regarding this research, we applied 40 g/l mannitol in our investigation to promote the sluggish development of *A. lappa*. On medium with 40 gm/l mannitol, 80% of shoot cultures remained healthy and green for up to 8

months under standard culture conditions. Normal morphology and a low rate of multiplication were also noted. After one year, however, there was a decrease in shoot viability (with 60% surviving and 40% healthy cultures), and the rate of shoot multiplication also reduced.

Table 5. Survival and proliferation of *A. lappa* shoot cultures during storage at low temperature of 5ºC of osmotic stress induced by 40 g/l mannitol.

Storage duration (month)	Survival (%)		Healthy shoots (%)		No. of proliferated shoots		Height of shoot (cm)		Recovery (cm)	
	5 ºC	Mannitol	5 ºC	Mannitol	5 ºC	Mannitol	5 ºC	Mannitol	5 ºC	Mannitol
1	100.0	100.0	100.0	100.0	2.3±0.1^{c}	2.1±0.2^{c}	1.6±0.3^{d}	1.2±0.3^{d}	100.0	90.0
8	100.0	100.0	100.0	80.0	4.3±0.3^{b}	3.2±0.6^{b}	2.4±0.9^{c}	2.1±0.2^{c}	100.0	80.0
12	80.0	60.0	70.0	40.0	5.4±0.2^{b}	4.1±0.2^{a}	3.5±0.8^{b}	2.9±0.2^{b}	90.0	60.0
20	60.0	20.0	50.0	10.0	6.5±0.5^{a}	4.5±0.1^{a}	4.7±0.4^{a}	3.5±0.7^{a}	70.0	30.0

Values indicate means with a standard error of 20 explants for each treatment. By Duncan's Multiple Range Test at 0.05% probability level, means that are denoted by the same letters are not statistically distinct.

Data on the dynamics of callus growth on mannitol-containing medium showed an increase in fresh mass, dry mass, and the percentage of embryogenic calli up to 8 months (Table 6). After a year of growth, the highest dry and fresh masses were discovered; however, a year of storage resulted in a dramatic drop in the growth parameters. After 18 months of storage, all of the callus cultures were dried and died. Regarding this, a number of publications asserted that high sugar concentrations in culture media boosted the lifespan of *in vitro* stored cultures. On medium containing 30% sucrose and 4% sorbitol, for instance, asparagus shoot cultures were able to survive for 20 months (Fletcher 1994). Cassava tissue cultures lasted longer when kept at -22°C, and simultaneously raised sucrose concentrations up to 40% (Roca et al., 1982). In conclusion, low temperature storage seems to be the most effective method for maintaining *A. lappa's in vitro* germplasm. Utilizing a different sugar concentration would lengthen the time required for reculturing and reduce the necessity for pricey sub-culturing.

In vivo and ex vitro evaluation

The growth and survival rate of *in vitro* plantlets are influenced by a variety of environmental factors. Plants were transplanted into a suitable substrate, such as garden soil, garden-soil: sand: compost (2:1:1), or vermicompost, after the surplus media was removed from the roots. In the beginning, plantlets were given a month to grow in *in vivo* (in a culture chamber) settings. Garden soil plus sand plus compost, as well as vermicompost, had the largest (100) percentage of plantlets that survived and adapted, with 8.3 ± 0.6 and 6.1 ± 0.3 leaves per plant, respectively. However, only garden soil transplanted plantlets were found to have a 75% survival probability of acclimation under *in vivo* conditions.

Table 6. Growth and differentiation of *A. lappa* callus during storage at low temperature of 5ºC of osmotic stress induced by 40 g/l mannitol.

Storage duration (month)	Fresh mass (g)		Dry Mass (mg)		Dry mass/ fresh mass (%)		Embryogenic calli (%)	
	5 ºC	Mannitol	5ºC	Mannitol	5ºC	Mannitol	5ºC	Mannitol
1	1.00±0.1^{c}	0.81±0.1^{b}	110.24±1.1^{b}	95.15±2.1^{b}	0.110	0.117	20.0	30.0
8	1.21±0.9^{b}	1.01±0.6^{a}	130.11±0.2^{a}	112.12±1.9^{a}	0.107	0.111	30.0	40.0
12	1.42±0.4^{a}	0.82±0.9^{b}	115.12±0.7^{b}	76.32±0.2^{c}	0.081	0.093	10.0	20.0
20	1.12±0.7^{b}	0.23±0.2^{c}	80.45±0.8^{c}	42.13±1.1^{d}	0.718	0.183	-	-

Values indicate means with a standard error of 20 explants for each treatment. By Duncan's Multiple Range Test at 0.05% probability level, means that are denoted by the same letters are not statistically distinct.

Plantlets were transplanted under *ex vitro* conditions after *in vivo* acclimatization. Plantlets were raised outdoors for two months on a mixture of garden soil and cattle manure (20:1) and information was recoded (Fig. H). The plantlets that were primarily acclimated on garden soil plus sand and compost had the highest survival rate (95%) whereas plantlets that were initially adapted to vermicompost and garden soil in *in vivo* conditions had survival rates of 80% and 65.0%, respectively. Additionally, the plants' maximum height of 50.4 ± 1.5 cm was observed on garden soil and cattle dung mixture (Table 7).

Table 7. Effects of substrates on the acclimation of *A. lappa* plantlets generated from *in vitro* under *in vivo* and *ex vitro* conditions.

Substrate (*In vivo* condition)	Survival (%)		No. of leaf/ plant		Plant height (cm)/plant
	In vivo	*Ex vitro*	*In vivo*	*Ex vitro*	*Ex vitro*
Garden soil (GS)	75.0	65.0	5.2 ± 0.4^{c}	11.5 ± 0.7^{c}	30.3 ± 1.7^{c}
GS + Sand + Compost (2:1:1)	100.0	95.0	8.3 ± 0.6^{a}	16.9 ± 0.4^{a}	50.4 ± 1.5^{a}
Vermicompost	100.0	80.0	6.1 ± 0.3^{b}	13.4 ± 0.2^{b}	42.5 ± 1.6^{b}

Values indicate means with a standard error of 20 explants for each treatment. By Duncan's Multiple Range Test at 0.05% probability level, means that are denoted by the same letters are not statistically distinct.

This experiment showed that the optimal substrate for primary acclimation was garden soil combined with sand and compost. According to these studies (Atiyeh et al. 2000, Arancon et al. 2004, Azad and Amin 2018), improvements in the physico-chemical properties of the container medium, increases in enzymatic activity, increases in microbial diversity and activity, nutritional factors, and plant growth regulators could all contribute to increases in plant growth when garden soil plus sand plus compost is used as the potting medium. Results from this experiment showed that adding sand and compost had a significant impact on growth parameters including survival rate and number of leaves. Vermicompost, as demonstrated by Mishra et al. (2005), had positive benefits on the growth and yield of rice, particularly by significantly increasing

numerous growth metrics such seed germination, chlorophyll content, and yield. Similar findings were made by Najar et al. (2015), who noted that the yields and plant growth of brinjal were much higher in field soils supplemented with compost than in untreated plots. Plantlets were retained in a constrained habitat for a brief period of time before they gradually adapted in a typical environment with dry air, high candlepower, and temperature changes.

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