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Optimizing Phytohormone Combinations in Murashige and Skoog Media for Enhanced Micropropagation of *Ajuga bracteosa*

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Abstract

Identification and conservation of endangered plant species are essential for safeguarding the flora of the region. *Ajuga bracteosa*(*A. bracteosa*), found in Pakistan's North West, holds immense medicinal potential due to its diverse active ingredients. However, unskilled overharvesting of this plant by uprooting entire herbs has drastically reduced its propagules, worsening its endangered status. The current study was conducted to optimize phytohormone levels in Murashige and Skoog media for micropropagation of *A. bracteosa*. In vitro micropropagation is an effective conservation technique for *A. bracteosa* due to its ability to preserve genetic diversity, enable rapid multiplication, produce disease-free plants, and facilitate long-term storage and utility in medicine. The study was divided into two experiments. In the first experiment, shoot organogenesis was studied in callus explants in the media containing 6-Benzylaminopurine (BAP) alone and combined with Auxins or Gibberellic Acid (GA_3). In the second experiment, these shoots were studied for root organogenesis in the media containing auxins Indole-3-acetic acid (IAA), Indole-3- butyric acid (IBA) and Naphthaleneacetic acid (NAA) at two levels (0.5 and 1.0 mg L^{-1}). In the first experiment, the medium containing BAP+NAA (3+1 mg L^{-1}) induced early and 100% shoots and 13.8 shoot explants^{-1} with 4.1 cm length. In the second experiment, NAA (0.5 mg L^{-1}) induced the roots early and 100% with 7.2 roots shoot^{-1} and 3.6 cm length. The results of the present study suggest that, for better regeneration of shoots, BAP+NAA (3+1 mg L^{-1}) should be used in the medium, while NAA (0.5 mg L^{-1}) is required to be added to the medium for better root regeneration.

Keywords: *Ajuga bracteosa*, medicinal plants, endangered species, micropropagation

Highlight:

- Micropropagation (*Ajuga bracteosa)* is a practical approach to conserving endangered plant species.
- In combination, growth regulators exhibit better results for shoots and roots development.
- In medicinal plants, micropropagation may induce the production of required phytochemicals by abiotic stress.

1.0. Introduction

Ajuga bracteosa Wall. Ex Benth is a vital medicinal plant belonging to the Lamiaceae family. In English, it is called "Bungle" or "Jan-i-adam" in Kashmir, while in the local language, it is known as "Kori Booti". It is distributed from Kashmir to Bhutan, Pakistan, Afghanistan, China, Malaysia, the Western Himalayas and some parts of India. It is mainly found in the northern hilly areas of Pakistan (Arfan et al., 1996; Khare,2007; Pawar et al., 2007). It is an erect, low-growing perennial herb covered with fine hairs and grows to 50 cm in height (Chauhan, 1999; Chandel & Bagai, 2010). *A. bracteosa* contains iridoid glycosides, neo-clerodane diterpenoids, withanolides and phytoecdysteroids. It is also a rich source of tannins, phenolic compounds and flavonoids, which are natural antioxidants (Israili & Lyoussi, 2009). Its roots contain comparatively more considerable amounts of chromium (20 mg per 100 gm) and potassium (159 mg per 100 gm) than sodium, which play an essential role in controlling diabetes and hypertension(Chao et al. 2012). People use it as a traditional medicine for curing headaches, measles, pimples, burns, boils and stomach acidity, malarial fever, diarrhea and dysentery (Verma et al., 2012; Johnson, 1999; Sharma et al.,2004). It is effective in curing sore throat, hypertension, and jaundice, and its juice can work as a blood purifier (Hamayun et al., 2006). Anti-cancerous and anti-inflammatory properties have also been identified for *A. bracteosa* (Singh et al. 2006; Upadhyay et al. 2011). It is more effective in arthritis than standard aspirin (Kaithwas et al. 2012). Almost 85% of medicinal plants are still collected from their wild habitat as there is no such reliable programme for their cultivation (Hamayun et al. 2006). Chaudhary et al. (2000) reported that 500 families are involved in collecting medicinal plants in District Swat, Pakistan, and around 5000 tons of medicinal plants are collected annually. Their collection reaches to maximum during the spring and summer seasons. The unplanned collection of *A. bracteosa* from their wild habitat by digging out the whole herb results in the loss of their propagules. In addition, the indiscriminate use of this vital plant and unrestricted exploitation has made it an endangered plant species (Kaul et al., 2013). Alam & Ali (2009) identified several severe threats to the biodiversity of Pakistan. (Ahmad et al.,2012) and Tali et

al.(2016) also reported the vulnerable status of *A. bracteosa* towards its extinction. Therefore, conserving this plant species and ensuring its existence and availability is indispensable to meet the growing demands of the people and medicine industries. Micropropagation offers a potential alternative for the availability of many true-to-type plants throughout the year, irrespective of the season. Therefore, this study was planned to conserve *A. bracteosa*, and an efficient and reliable micropropagation protocol was investigated and optimized.

2.0. Materials and Methods

2.1. Plant collection and culturing for callus

The *Ajuga bracteosa* plants were collected from District Swat (Miadam) and were brought to the Department of Plant Breeding and Genetics, The University of Agriculture Peshawar, Pakistan. Their leaves were washed with running tap water for 5 minutes and then dipped in distilled water containing bleach for 10 minutes; after that, these were treated with mercuric chloride (0.1% w/v) for 2 minutes. Finally, the leaves were rinsed 5 times with sterile water. The disinfected leaves were cultured on MS medium supplemented with 2,4-D+BAP (2+1 mg L^{-1}) for mass production of callus. The developed callus was then used as an explant to establish a protocol for the micropropagation of *Ajuga bracteosa*.

2.2. Media preparation for regeneration of shoots and roots

The Murashige and Skoog (MS) powdered media was used for both shoot and root experiments and was supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} agar. For shoot induction, the media was fortified with three concentrations of BAP (1, 2 and 3 mg L^{-1}) alone and in combination with GA_3, 2,4-D, IAA, NAA and IBA each @ 1 mg L^{-1} while one simple MS medium without PGRs was kept as a control treatment for comparison.

The root-inducing MS media was augmented with IAA, NAA and IBA separately, each at two levels (0.5 and 1.0 mg L^{-1}) (Kaul et al., 2013; Gaspar et al., 1996). while one medium without PGR was kept as a control for comparison. For both shoot and root media, the pH of the media was adjusted at 5.6 to 5.7 by using 0.1 N NaOH or 0.1 N HCl before adding agar. All the media were autoclaved (St. Francis, Model No: STA-400) at 121 ºC with 15 psi for 20 minutes.

2.3. Aseptic culturing of explants

Aseptic culturing of the explants was carried out by using a laminar flow unit (LFU). Before starting culturing, the LFU was sterilized with 70 % ethanol to make an aseptic condition inside the LFU. All the media and culturing tools were placed inside the LFU, and UV light was turned on for 20 minutes. Culturing was carried out using sterilized blades, forceps and petri dishes. The callus explants were inoculated on the media in the presence of a spirit lamp flame. Callus was inoculated in the food jar for shoot induction, while after 6 weeks, the developed shoots were inoculated on the media in the test tubes for root induction. The inoculated jars or test tubes were placed under a controlled temperature at 25±2 ºC. In both experiments, the cultured explants were exposed to 16 hours of light using simple white fluorescent tubes and 8 hours of dark.

2.4. Statistical analysis

Data were recorded after 6 weeks of culturing for both shoot and root experiments. Both experiments were carried out using a Completely Randomized Design with three replications. The data were analyzed in one way using Statistix 8.1. Multiple means comparison was done by using the least significant difference test.

3.0 Results and Discussion

3.1. Optimization of phytohormones for maximum shoot regeneration

Callus cultured on media supplemented with various PGR treatments showed varying responses for shoot regeneration. The PGR supplementation was crucial for shoot regeneration of *A. bracteosa*, because the control treatment (medium without PGRs) could not induce shoot regeneration in the explant. The results indicated that the shoot induction and growth response depended on BAP concentration. The response was enhanced as the BAP concentration was increased alone or in combination with GA_3/Auxins. Furthermore, the combination of NAA was best with BAP compared to GA_3 and other auxins. The earliest shoots after 9.5 days with 100% induction were observed in the medium supplemented with BAP+NAA (3+1 mg L^{-1}) and produced 13.8 shoots $explant^{-1}$ with 4.1 cm length. IBA and 2,4-D, both with BAP showed no response for shoot induction, and BAP 1 mg L^{-1} alone and in combination with NAA also failed to regenerate any shoot (Fig. 1).

Cell growth, differentiation and organogenesis in tissue culture are controlled by the interaction of auxins and cytokinins (Skoog & Miller, 1957). The balance within the ratio of auxins and cytokinins is a fundamental factor in in vitro organogenesis. Generally, callus exposed to higher cytokinins concentration than auxins results in shoot regeneration (Beale & Sponsel 1993). The optimum hormone levels depend on the type of explants, culturing conditions and the type of plant hormone used (Skoog & Miller 1957). Besides a direct effect on cellular mechanisms, exogenously applied plant growth regulators affect the activation, synthesis, destruction, transport and even the sensitivity of the cells to the endogenous growth substances (Table 1) (Davies,1995; Yip & Yang, 1986). Exogenous cytokinins are reported to inhibit the enzymes that cause the conjugation of IAA into an inactive state (Gordon et al., 2009). Auxins enhance the expression of cytokinins

receptors, and cytokinins then combine with its receptors to induce regulatory proteins, which are responsible for shoot induction (Jones et al. 2010; Karhu 1997). In the current study, the endogenous auxins and cytokinins in the *A. bracteosa* callus were insufficient to trigger the process of shoot induction because there were no shoots found in the control medium (medium without PGRs) and (Kaul et al. 2013) reported the same findings. However, shoot induction and growth enhanced with increasing the exogenously applied BAP concentration alone and in combination with GA_3/auxins, and it was in accordance with the results obtained by (Kaul et al. 2013) and also reported by Verstraeten et al. (2013). Exogenous application of cytokinins not only regulates the uptake of carbohydrates but also enhances the utilization of carbohydrates in *in-vitro* plantlets (Delbarre et al. 1996), and therefore, with the increase of BAP concentration either alone or with GA_3/auxins, the shoot growth increased and reached to maximum when BAP was augmented with NAA in the medium. The number of shoots was also higher when NAA was added along with BAP in the medium, and the same results have also been reported by (Gaspar et al.1996). The response of auxins to morphogenesis depends on their metabolism, conjugation with other materials, and transport. All these factors determine the active auxins concentration reaching the target cells (Petrasek et al. 2006). The present best results for NAA with BAP might be due to the reason that NAA enters quickly into the cells by passive transport and ensures an increased level of NAA within the target cells (Haissig et al. 1992; Fogaca & Fett-Neto 2005) and, therefore, it might have reached to a maximum number of cells and have expressed more cytokinins receptors for triggering the process of shoot induction. The failure of IBA and 2,4-D in combination with BAP to induce shoots might be the reason that both IBA and 2,4-D could have no binding affinity for auxins receptors and, therefore, failed to activate cytokinins receptors for initiating the process of shoot induction.

(a) **(b)** **(c)**

Figure 1. a). **The produced callus of *A. bracteosa* used as an explant, b). The shoots developed during the first phase of the experiment and c). The inoculation of shoots in the rooting media of the second phase of the experiment**

MS+PGRs	mg L^{-}	Days to shoot induction	Percent shoot induction (%)	Shoots explant^{-1}	Shoot length (cm)
BAP	1.0	---	---	---	---
BAP	2.0	13.2 ± 0.65 B	66.6 ± 0.00 C	4.5 ± 0.19 F	3.4 ± 0.22 ABC
BAP	3.0	10.4 ± 0.42 C	94.4 ± 5.57 A	10.1 ± 0.35 B	3.8 ± 0.18 AB
BAP+GA_3	1+1	13.3 ± 0.44 B	66.6 ± 0.00 C	5.0 ± 0.18 F	2.3 ± 0.48 C
BAP+GA_3	2+1	10.3 ± 0.47 C	72.2 ± 5.57 BC	5.2 ± 0.22 F	2.5 ± 0.23 BC
BAP+GA_3	3+1	9.7 ± 0.15 C	83.3 ± 0.00 ABC	6.8 ± 0.18 E	2.7 ± 0.27 BC
BAP+2,4-D	1+1	---	---	---	---
BAP+2,4-D	2+1	---	---	---	---
BAP+2,4-D	3+1	---	---	---	---
BAP+IAA	1+1	24.2 ± 0.67 A	72.2 ± 5.57 BC	5.5 ± 0.15 F	2.4 ± 0.18 C
BAP+IAA	2+1	13.8 ± 0.12 B	83.3 ± 0.00 ABC	7.5 ± 0.15 DE	2.9 ± 0.31 ABC
BAP+IAA	3+1	10.3 ± 0.44 C	88.9 ± 5.57 AB	8.4 ± 0.15 CD	3.3 ± 0.19 ABC
BAP+NAA	1+1	---	---	---	---
BAP+NAA	2+1	12.8 ± 0.15 B	94.4 ± 5.57 A	9.2 ± 0.17 BC	3.5 ± 0.12 ABC
BAP+NAA	3+1	9.5 ± 0.39 C	100.0 ± 0.00 A	13.8 ± 0.26 A	4.1 ± 0.09 A
BAP+IBA	1+1	---	---	---	---
BAP+IBA	2+1	---	---	---	---
BAP+IBA	3+1	---	---	---	---

Control	---	---	---	---	---

Values in each column represent means ± SE. Means followed by different letters in each column are significantly different from one another using LSD test ($p \leq 0.001$).

Table 1. Effect of BAP alone and in combination with GA_3/auxins on shoot regeneration of *A. bracteosa*

3.2. Optimization of phytohormones for maximum root regeneration

Shoots developed in the first experiment were transferred to rooting media supplemented with auxins. The results revealed that auxin was vital for root induction of *A. bractesosa* in the medium because the control treatment (medium without auxin) failed to induce any root in the cultured shoots. Among the treatments, the earliest root regeneration was observed after 14.7 days in the medium containing NAA (0.5 mg L^{-1}). Root was induced in all the culture media supplemented with NAA at both concentrations (0.5 mg L^{-1} & 1.0 mg L^{-1}) and IBA only at 0.5 mg L^{-1}. Maximum number of roots 7.2 with 3.6 cm length were observed in the medium augmented with NAA (0.5 mg L^{-1}) (Table 2).

MS+PGRs	mg L^{-1}	Days to root induction	Percent root induction (%)	Roots $shoot^{-1}$	Root length (cm)
IAA	0.5	21.5 ± 0.35 A	93.3 ± 3.33 AB	2.9 ± 0.20 D	1.6 ± 0.21 D
IAA	1.0	17.8 ± 0.15 B	86.7 ± 3.33 B	4.1 ± 0.54 CD	2.5 ± 0.15 C
IBA	0.5	17.2 ± 0.15 B	100.0 ± 0.00 A	5.4 ± 0.09 BC	2.8 ± 0.19 BC
IBA	1.0	17.3 ± 0.32 B	93.3 ± 3.33 AB	4.9 ± 0.12 C	2.6 ± 0.12 BC
NAA	0.5	14.7 ± 0.23 C	100.0 ± 0.00 A	7.2 ± 0.15 A	3.6 ± 0.09 A
NAA	1.0	15.8 ± 0.68 BC	100.0 ± 0.00 A	6.7 ± 0.20 AB	3.3 ± 0.07 AB
Control	---	---	---	---	---

Values in each column represent means ± SE. Means followed by different letters in each column are significantly different from one another using LSD test ($p \leq 0.001$).

Table 2. Effect of auxins on root regeneration of *A. bracteosa*

Plant growth regulators applied to in vitro plant cultures directly influence endogenous growth substances in terms of their synthesis, destruction, activation, transport, and behavioural response (Davies,1995; Yip & Yang, 1986). Auxins are rooting hormones and have a central role in triggering the process of root formation (Kevers et al., 2009; Kuroha & Satoh, 2007; Geiss et al., 2009). Cytokinins are rooting inhibitors (Leyser 1998; Kepinski, & Leyser 2002) and therefore are not added to rooting media. Auxins bind with its receptors "Transport Inhibitor Response 1" (TIR1) and initiate the degradation of Proteins "AUX/IAA" for releasing auxin response factors (ARFs), which are the key factor for activation of auxin-induced genes for triggering the root induction (Gatineau et al. 1997; Blakesley, & Chaldecott 1993). Exogenously applied auxins increase the concentration of endogenous auxins (Skoog & Miller 1957; Tan et al. 2010). All the cells in their differentiating phase of development require a suitable type of auxins that should be capable of a better response to promoting rhizogenesis (Calderon-Villalobos et al. 2010). The current study observed that the endogenous auxin level in the cultured plantlets was insufficient to induce roots, as no roots were found in the control medium. The application of exogenous auxin to the media is vital for root induction in *A. bracteosa*, and the same findings were reported by Kaul et al. (2013). Among the auxins, NAA showed a better response to rooting. These results might be due to the fact that NAA has the most vigorous root induction activity. It is reported that structurally, NAA binds to the auxins receptors TIR1 (Transport Inhibitor Response 1) in a similar pattern as followed by IAA (Sugiyama 1999; Kollmeier et al. 2000). Taiz & Zeiger (2002) reported the promontory effect of auxins on rooting at low concentrations as compared to high concentration and this was also in accordance to our findings. In addition, Gaspar et al. (1996) also obtained the same results and reported that increasing the concentration of NAA, reduced the quality and number of roots.

Moreover, the response of auxins to morphogenesis depends on their metabolism, conjugation with other materials, and transport. NAA enters the cells very quickly through passive transport compared to other auxins. All these factors determine the active auxins concentration reaching the target cells. Furthermore, changes in endogenous auxins and the receptivity or physiological status of the target cells to it also have an important role in morphogenetic response (Petrasek et al. 2006; Haissig et al. 1992; Fogaca et al. 2005). In the current study, root elongation at low concentrations of NAA and IBA is justified by the findings of (Kollmeier et al. 2000; Taiz & Zeiger 2002) and (Sattar et al. 2021) that the elongation phase of root is sensitive to the concentration of auxins and root growth is promoted at very little concentration while inhibited at high concentration because of ethylene synthesis due to high level of auxins (Sattar et al. 2021; Hamooh et al. 2021).

Conclusion

It is investigated from the experiment that plant growth regulators were significant for both *in vitro* shoot and root development of *Ajuga bracteosa*. The growth of shoots is enhanced by the increase of BAP, either alone or in combination with GA_3/auxins. However, the combination of BAP (3 mg L^{-1}) and NAA (1 mg L^{-1}) showed the best response. The response of root induction and its growth was best in the medium supplemented with NAA at the rate of 0.5 mg L^{-1}. BAP levels need

to be evaluated at higher concentrations in combination with varying levels of NAA for an even better response of shoot organogenesis. Further research is required to study the acclimatization of micropropagated *Ajuga bracteosa*.

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