INTERNATIONAL MULTIDISCIPLINARY JOURNAL FOR RESEARCH & DEVELOPMENT
SJIF 2019: 5.222 2020: 5.552 2021: 5.637 2022:5.479 2023:6.563 2024: 7,805
eISSN :2394-6334 https://www.ijmrd.in/index.php/imjrd Volume 12, issue 05 (2025)

IN VITRO MICROPROPAGATION OF AJUGA TURKESTANICA (REGEL) BRIQ.: A PATHWAY TO PATHOGEN-FREE SEEDLINGS

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ABSTRACT: In recent years, the application of in vitro techniques has become a cornerstone in the conservation and propagation of plant biodiversity. This study focuses on the micropropagation of Ajuga Turkestanica, a medicinal plant species, under in vitro conditions. The research aimed to identify the optimal nutrient media and growth regulators for the efficient propagation of pathogen-free seedlings. The results demonstrated that the B5 nutrient medium supplemented with 0.1 µM BAP (6-Benzylaminopurine) was the most effective for the proliferation of Ajuga Turkestanica. Additionally, the BDS medium enriched with 5.0 µM BAP and 2.0 µM NAA (Naphthalene Acetic Acid) showed high efficiency in promoting shoot regeneration. The study also highlighted the importance of cytokinins, particularly BAP, over other growth regulators like TDZ (Thidiazuron) and Kinetin in the multiplication phase.

Keywords: Ajuga Turkestanica, in vitro, micropropagation, callus, MS medium, phytohormones.

INTRODUCTION

The rapid decline in biodiversity due to ecological changes, anthropogenic activities, and overexploitation of natural resources has necessitated the development of advanced conservation strategies [1]. Among these, in vitro micropropagation has emerged as a powerful tool for the preservation and propagation of endangered plant species [2]. This technique not only allows for the rapid multiplication of plants but also ensures the production of pathogen-free seedlings, which is crucial for the restoration of natural populations [3].

The genus Ajuga, particularly Ajuga Turkestanica, is of significant interest due to its medicinal properties. However, the natural populations of this species are under threat due to overharvesting and habitat destruction [4]. Therefore, the development of efficient in vitro propagation protocols is essential for its conservation and sustainable utilization [5].

This study aims to explore the morphogenetic responses of Ajuga Turkestanica to various growth regulators and nutrient media, with the ultimate goal of establishing an optimal protocol for its micropropagation.

MATERIALS AND METHODS

Plant Material and Sterilization

The study utilized Ajuga Turkestanica explants collected from greenhouse-grown plants. The explants, consisting of shoot tips and nodal segments, were carefully excised and washed under running tap water for 30 minutes to remove surface contaminants. For sterilization, the explants were treated with 70% ethanol for 30 seconds, followed by immersion in a solution of 0.1% mercuric chloride ($HgCl_2$) and 0.1% Tween 80 for 30 minutes. After sterilization, the explants were rinsed three times with sterile distilled water to remove any traces of the sterilizing agents.

This protocol resulted in a high survival rate of 87-96%, with minimal contamination observed during the initial culture phase [6].

Nutrient Media and Growth Regulators

The explants were cultured on two primary nutrient media: MS (Murashige and Skoog) [7] and VM (Van der Meer) [8]. Both media were supplemented with different concentrations of growth regulators, including BAP (6-Benzylaminopurine), Kinetin, and NAA (Naphthalene Acetic Acid). The pH of the media was adjusted to 5.8 using 1N NaOH or HCl before autoclaving at 121^0C for 15 minutes. The media were solidified with 0.8% agar (w/v) and dispensed into sterile culture vessels.

Experimental Design

The study was designed to evaluate the effects of different growth regulator combinations on the morphogenetic potential of Ajuga Turkestanica. A total of 10 different media formulations were prepared, each with varying concentrations of BAP (0.1–5.0 µM) and NAA (0.02–2.0 µM). The explants were cultured in these media under controlled environmental conditions, with a 16-hour photoperiod provided by cool-white fluorescent lamps (40 µmol m^{-2} s^{-1}) and a temperature maintained at 25 ± 2^0C. Each treatment consisted of 50 explants, replicated three times to ensure statistical reliability [9].

Data Collection and Analysis

The regeneration frequency (percentage of explants forming shoots), shoot proliferation (number of shoots per explant), and callus formation (percentage of explants forming callus) were recorded over a period of 8 weeks. Data were analyzed using one-way ANOVA, and mean values were compared using Duncan's Multiple Range Test (DMRT) at a significance level of $p<0.05$. Statistical analyses were performed using SPSS software (version 25.0) [10].

RESULTS

The results of the study are presented in detail, with a focus on the effects of different growth regulators and nutrient media on the shoot regeneration, callus formation, and rooting of Ajuga Turkestanica. The findings are supported by tables and figures to provide a clear and thorough explanation of the outcomes.

Effect of Growth Regulators on Shoot Regeneration

The results indicated that the B5 medium supplemented with 0.1 µM BAP was the most effective for shoot regeneration, with a regeneration frequency of 56.5% and an average of 5.0 ± 1.5 shoots per explant (Table 1). The BDS medium enriched with 5.0 µM BAP and 2.0 µM NAA also showed high efficiency, with a regeneration frequency of 66.2% and an average of 4.0±0.8 shoots per explant. In contrast, the MS and VM media showed lower regeneration frequencies and fewer shoots per explant, indicating that the B5 and BDS media were more suitable for the micropropagation of Ajuga Turkestanica.

Table 1. Effect of Different Growth Regulator Combinations on Shoot Regeneration

Medium	BAP (µM)	NAA (µM)	Regeneration Frequency (%)	Average Shoots per Explant
B5	0.1	0.02	56.5	5.0±1.5
BDS	5.0	2.0	66.2	4.0±0.8
MS	2.0	0.1	49.4	2.9±0.7
VM	2.0	0.1	41.8	2.9±0.5

Callus Formation and Indirect Regeneration

The study also observed callus formation in response to low concentrations of BAP (0.5 µM) and TDZ (0.5 µM). However, the regeneration frequency from callus was relatively low, with only 28% of the callus-derived shoots developing into viable plantlets (Table 2). This suggests that direct organogenesis is more efficient for the micropropagation of Ajuga Turkestanica compared to indirect regeneration via callus.

Table 2. Callus Formation and Indirect Regeneration

Growth Regulator	Concentration (µM)	Callus Formation (%)	Regeneration Frequency (%)
BAP	0.5	37.0	32.0
TDZ	0.5	38.0	28.0

Rooting and Acclimatization

For rooting, the regenerated shoots were transferred to a hormone-free BDS medium, where they developed roots within 4-5 weeks. The rooted plantlets were then acclimatized in a greenhouse environment. The acclimatization process involved transferring the plantlets to a substrate composed of a mixture of sand and peat (1:1 v/v) under high humidity conditions (80-90%) for the first two weeks, followed by gradual exposure to ambient conditions. The survival rate of the acclimatized plantlets was 85%, indicating successful adaptation to ex vitro conditions.

Discussion

The results demonstrate that the B5 medium supplemented with 0.1 µM BAP is highly effective for shoot regeneration in Ajuga Turkestanica, with a high regeneration frequency and a significant number of shoots per explant. The BDS medium enriched with 5.0 µM BAP and 2.0 µM NAA also showed promising results, particularly in terms of regeneration frequency. These findings are consistent with previous studies that have highlighted the importance of cytokinins, particularly BAP, in promoting shoot proliferation in in vitro cultures [11, 12].

Callus formation was observed in response to low concentrations of BAP and TDZ, but the regeneration frequency from callus was relatively low. This suggests that direct organogenesis is a more efficient pathway for the micropropagation of Ajuga Turkestanica, as it bypasses the callus phase and directly induces shoot formation from explants. This is in line with studies on other

medicinal plants, where direct regeneration has been shown to be more reliable and efficient [13, 14].

The successful rooting and acclimatization of the regenerated plantlets further validate the effectiveness of the in vitro propagation protocol. The high survival rate of **85%** indicates that the plantlets were well-adapted to ex vitro conditions, which is crucial for the large-scale production and conservation of Ajuga Turkestanica.

Conclusion

In recent years, the application of in vitro techniques has become a cornerstone in the conservation and propagation of plant biodiversity. This study focuses on the micropropagation of Ajuga Turkestanica, a medicinal plant species of significant interest due to its therapeutic properties, under in vitro conditions. The research aimed to identify the optimal nutrient media and growth regulators for the efficient propagation of pathogen-free seedlings.

The results demonstrated that the B5 nutrient medium supplemented with 0.1 µM BAP (6-Benzylaminopurine) was the most effective for shoot proliferation, achieving a regeneration frequency of 56.5% and producing an average of 5.0 ± 1.5 shoots per explant. Additionally, the BDS medium enriched with 5.0 µM BAP and 2.0 µM NAA (Naphthalene Acetic Acid) showed high efficiency in promoting shoot regeneration, with a regeneration frequency of 66.2% and an average of 4.0±0.8 shoots per explant.

The study also highlighted the importance of cytokinins, particularly BAP, over other growth regulators like TDZ (Thidiazuron) and Kinetin in the multiplication phase. Callus formation was observed in response to low concentrations of BAP and TDZ, but the regeneration frequency from callus was relatively low (28%), indicating that direct organogenesis is a more efficient pathway for micropropagation.

For rooting, the regenerated shoots were transferred to a hormone-free BDS medium, where they developed roots within 4-5 weeks. The rooted plantlets were successfully acclimatized in a greenhouse, with a survival rate of 85%.

This study establishes an efficient protocol for the in vitro micropropagation of Ajuga Turkestanica, ensuring the rapid production of pathogen-free seedlings and providing a reliable method for the conservation and sustainable utilization of this valuable medicinal plant.

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