RESEARCH ARTICLE

Analyzing the impact of growth hormones on *in vitro* propagation of *Anacyclus pyrethrum*

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Traditional medicine makes use of the wild species *Anacyclus pyrethrum*, also known as *A. pyrethrum*, which belongs to the *Asteraceae* family. In order to commence the cultivation of *Anacyclus pyrethrum*, the study employed 4 to 5 cm immature nodal shoot segments. Antioxidant therapy solved the early issues of leaching and browning when the axillary meristem was activated on MS media with 2.0 mg/L of 6-Benzylaminopurine (BAP), resulting in profuse shoot growth. 0.5 mg/L 6-benzylaminopurine and kinetin amplified cultures were subculturing on Murashige and Skoog (MS) medium. Root induction was achieved on half-strength MS medium with 2.0 mg/L Indole-3-Butyric Acid (IBA) and 0.2% activated charcoal and *ex vitro* rooting with pulse administration of 200.0 mg/L indole-3-butyric acid demonstrated efficacy. The tissue culture approach shown to be reliable and appropriate for large-scale greenhouse cultivation of akarkara plants.

Key Words: *Anacyclus pyrethrum*; *Micropropogation*; *Growth hormone*; *Auxin*

INTRODUCTION

Akarkara is an herbaceous plant from the Compositae (*Asteraceae*) family, is also known as the toothache plant, Brazil cress or para cress. Its unique ability to numb the tongue when its flower buds are consumed has earned it the nickname "toothache plant". Beyond its culinary uses, akarkara is renowned in traditional medicine for its stimulant and diaphoretic properties. The root contains pyrethrin, which is used therapeutically to treat conditions like rheumatism, neuralgia and toothaches. This hardy plant, characterized by its daisy-like flowers and lobed leaves, thrives in arid conditions when grown in well-drained, sandy soil. Although its conservation status is of least concern, sustainable harvesting remains significant.

Akarkara's essential oil and alkaloids, especially spilanthol are known for their virilizing, nervine, heart tonic and antimicrobial properties. Recognized in ayurveda for its diverse medicinal benefits, akarkara is considered an aphrodisiac and a tonic for the male reproductive system. A technique for micropropagation of *Anacyclus pyrethrum* was developed by Singh et al., showing the best response from cotyledonary nodal explants on MS medium with 2.5 µM Kn, yielding an average of 8.88 ± 0.28 shoots per explant [1]. During sub-culturing, the regenerated shoots proliferated and for root induction, elongated shoots exposed to 5 or 10 µM Indole-3-Acetic Acid (IAA) or 5 µM Naphthaleneacetic Acid (NAA) were most successful.

MATERIALS AND METHODS

The study by Singh et al., investigated callogenesis and pellitorine accumulation in *Anacyclus pyrethrum* a medicinal herb [2]. Using cotyledon, hypocotyl and root explants on MS media supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D), they found that lower 2,4-D concentrations favored root and hypocotyl callus formation, while higher concentrations favored cotyledon callus formation. The weight of harvested calli increased linearly over time. Between days 30 and 75, there was a significant increase in pellitorine accumulation, with the highest amount (94.50 µg/g dry weight) observed in cotyledon calli on day 75. The antioxidant activity of the pellitorine produced from *A. pyrethrum* callus was assessed using the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) method and an efficient procedure for large-scale production was established [3,4].

Nodal shoot segments of *Anacyclus pyrethrum*, sourced from greenhouse or nursery plants, were used as explants for culture initiation. After surface sterilization with bavistin, antibiotics and $HgCl_2$, the explants were treated with a chilled antioxidant solution. Various concentrations of kinetin or BAP were applied on MS media to promote the production of multiple shoots and the multiplication of axillary buds [5]. Culture multiplication required repetitive transfer and subculturing on media supplemented with ascorbic acid and activated charcoal. Different concentrations of BAP were tested on Murashige and Skoog (MS), Modified Murashige and Skoog (MMS), Woody Plant (WP) and white's media, either alone or in combination with kinetin, IAA or NAA for shoot multiplication [6,7].

In vitro, regenerated shoots were rooted in half-strength MS medium supplemented with various amounts of IBA or NAA and activated charcoal. One method of *ex vitro* rooting involved pulse-treating with IBA, NAA. Subsequently, the plants were transferred to soilrite in bottles for root induction and greenhouse hardening. Once hardened, the plantlets were placed in polybags containing a mixture of vermicompost, black soil and sandy soil [8].

Sections of juvenile nodal shoots, measuring 4 to 5 cm in length, were found suitable for culture initiation. However, explant leaching and browning posed significant challenges to bud break and culture establishment. To mitigate this, the nodal explants were treated with an antioxidant solution for 20 min. On MS medium supplemented with 2.0 mg/L of BAP, 90% of the explants showed bud break and produced 2-3 shoots per node. Conversely, on MS medium with 1.0 mg/L of BAP, the explant's response and shoot development were poor [9]. Higher concentrations (3.0 mg/L) of BAP resulted in bud breaking from the axillary meristem and callus formation at the base. Kinetin treatments also induced bud proliferation, but the growth and number of shoots per explant were considerably lower (Table 1).

TABLE 1

Impact of BAP and kinetin concentrations on bud break and multiple shoot induction from *Anacyclus pyrethrum* nodal explants on MS+additives

Concentration of cytokine (mg/L)	Response (%)	Shoot no. ± SD	Shoot length (cm) ± SD
BAP			
0.5	45	1.35 ± 0.5	0.95 ± 0.28
1	66	1.4 ± 0.55	1.08 ± 0.3
2	95	1.8 ± 0.5	1.2 ± 0.19
3	45	1.5 ± 0.3	1.3 ± 0.25
Kinetin			
0.5	-	-	-

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1	25	0.75 ± 0.51	0.47 ± 0.15
2	55	1.4 ± 0.55	1.2 ± 0.2
3	25	1.2 ± 0.59	1.33 ± 0.18

Note: BAP: 6-Benzylaminopurine; MS: Murashige and Skoog; SD: Standard Deviation.

RESULTS AND DISCUSSION

Multiplication and maintenance of cultures

Repeated transfers of the mother plant for two to three cycles resulted in the amplification of *Anacyclus pyrethrum* shoots (Table 2). Shoot multiplication was achieved on MS medium supplemented with 0.5 mg/L of both kinetin and BAP, along with 100 mg/L ascorbic acid and 0.1% activated charcoal. The inclusion of activated charcoal was significant for proper culture growth, as it mitigated the phenol leaching at the culture bases, which otherwise slowed the multiplication rate [10,11]. Approximately 12-13 shoots were regenerated on the shoot multiplication medium with sub culturing every 20 to 25 days (Table 3).

TABLE 2

Production of shoots with repeated transfers of the *Anacyclus pyrethrum* mother explant on MS+0.5 mg/L BAP+additives

Passages	Shoot no. ± SD	Shoot length (cm) ± SD
I	2.6 ± 0.4	1.95 ± 0.4
II	6.9 ± 1.2	3.1 ± 0.3
III	9.1 ± 0.7	2.7 ± 0.3
IV	5.1 ± 0.8	2.1 ± 0.2

Note: BAP: 6-Benzylaminopurine; MS: Murashige and Skoog; SD: Standard Deviation.

TABLE 3

Impact of different growth regulator combinations and doses on *Anacyclus pyrethrum* shoot multiplication on MS+additives

Concentration of cytokine (mg/L)	Shoot no. ± SD	Shoots length (cm) ± SD
BAP		
1	3.91 ± 0.91	2.63 ± 0.77
0.5	4.8 ± 1.25	3.31 ± 0.89
0.25	3.31 ± 0.95	1.89 ± 0.47
BAP 0.5+kinetin		
1	7.81 ± 0.69	3.22 ± 0.45
0.5	12.72 ± 0.91	3.91 ± 0.37
0.25	9.86 ± 0.71	1.35 ± 0.37
BAP 0.5+NAA		
1	6.43 ± 0.38	1.6 ± 0.98
0.5	6.71 ± 0.23	1.3 ± 0.65
0.25	7.91 ± 0.75	0.81 ± 0.28
BAP 0.5+IAA		
1	5.89 ± 0.91	1.31 ± 0.47
0.5	7.89 ± 0.79	1.33 ± 0.77
0.25	5.45 ± 0.67	1.29 ± 0.29

Note: BAP: 6-Benzylaminopurine; MS: Murashige and Skoog; SD: Standard Deviation; NAA: Naphthaleneacetic Acid; IAA: Indole-3-Acetic Acid.

Using BAP alone at various concentrations on MS medium resulted in the differentiation of only 3-5 shoots. Compared to kinetin, combining NAA or IAA with 0.5 mg/L of BAP was less effective, yielding fewer and shorter shoots. Of all the media types tested, MS medium proved to be the most suitable for culture proliferation. Cultures on MMS medium began to yellow, while those on WP and white's media showed very low rates of shoot growth and multiplication (Table 4).

TABLE 4

Effects of different culture media types on *Anacyclus pyrethrum* shoot multiplication (each medium treated with 0.5 mg/L of BAP and kinetin)

Type of media	Shoot no. ± SD	Shoot length (cm) ± SD
MS	12.7 + 0.86	3.45 + 0.17
MMS	9.71 ± 0.97	2.98 ± 0.18

Note: BAP: 6-Benzylaminopurine; MS: Murashige and Skoog; MMS: Modified Murashige and Skoog; SD: Standard Deviation.

After three to four cycles of maintaining shoot cultures on fresh media, tuber development occurred at the nodes of regenerated shoots. Initially, the nodes formed green, tumor-like structures that eventually turned brown and exhibited root differentiation with abundant root hair development. These tubers later produced shoots with well-developed roots [12-14].

Rooting of *in vitro* produced shoots

Shoots planted on half strength MS medium supplemented with 2.0 mg/L of IBA and 0.2% of activated charcoal were successfully produced *in vitro*. As shown in Table 5, a rooting percentage of 90%-95% was achieved. In contrast, NAA was found to be less effective at inducing roots from *Anacyclus pyrethrum* shoots.

TABLE 5

Impact of auxins (IBA and NAA) on root induction from *Anacyclus pyrethrum* shoots that have been *in vitro* regenerated on half strength MS medium

Concentration in (mg/L)	Response (%)	Root no. ± SD	Root length (cm) ± SD
IBA			
1	81	1.9 ± 0.55	0.9 ± 0.60
1.5	87	2.9 ± 0.45	1.2 ± 0.15
2	93	3.3 ± 0.92	2.7 ± 0.2
3	73	1.9 ± 0.77	1.2 ± 0.2
NAA			
1	61	1.5 ± 0.58	0.96 ± 0.9
1.5	66	1.8 ± 0.77	0.97 ± 0.11
2	73	2.7 ± 0.58	1.7 ± 0.12
5	55	1.9 ± 0.49	0.77 ± 0.71

Note: MS: Murashige and Skoog, SD: Standard Deviation; NAA: Naphthaleneacetic Acid; IBA: Indole-3-Butyric Acid.

Ex vitro rooting of shoots

By pulsing 200 mg/L of IBA over the course of three minutes, shoots grown *in vitro* became rooted. Three to four roots were formed from each of the 90%-95% of the pulse-treated shoots that rooted (Table 6). Moreover, NAA worked well for roots at all concentrations.

TABLE 6

Impact of pulse treatments with IBA or NAA concentrations on the *ex vitro* rooting of *Anacyclus pyrethrum* regenerated shoots

Concentration of auxin	Response (%)	Root no. ± SD	Root length in (cm) ± SD
IBA			
100	65	1.9 ± 0.65	2.5 ± 0.17
200	95	3.1 ± 0.99	3.5 ± 0.14
500	75	1.9 ± 0.49	2.9 ± 0.15
NAA			
100	75	1.6 ± 0.59	1.9 ± 0.15
200	81	2.7 ± 0.69	2.5 ± 0.22
500	62	2.9 ± 0.79	1.5 ± 0.31

Note: SD: Standard Deviation; NAA: Naphthaleneacetic Acid; IBA: Indole-3-Butyric Acid.

Observation

Juvenile nodal shoot segments of *Anacyclus pyrethrum* were effectively used to initiate cultures, leading to the development of multiple shoots through axillary meristem activation on MS medium supplemented with 2.0 mg/L BAP. More than 90% of the nodal explants produced 2-3 shoots [15,16]. Initial issues with leaching and browning were mitigated using antioxidant treatments. Compounds such as ascorbic acid and sodium hydrosulphite were successful in preventing darkening. Other effective agents include cystine, Diethyldithiocarbonate (DTT), sodium bisulphate, citric acid and polyclar [17]. In the plant biotechnology laboratory in Jodhpur, ascorbic acid and citric acid have been utilized to prevent browning and deterioration in cultures of various plant species, including woody plants and trees. Abdelwahd et al., used oxidants and absorbents to reduce the impact of leached phenolics on faba bean regeneration *in vitro* [18].

For *Anacyclus pyrethrum*, nodal explants were cultured and subsequently multiplied through subculturing on MS medium with activated charcoal as an adsorbent and ascorbic acid as an antioxidant. The optimal BAP concentration for shoot multiplication was found to be 0.5 mg/L. Once the axillary meristems are activated, the medium is conditioned for shoot growth, requiring lower levels of cytokinin. This study demonstrated a high rate of shoot multiplication (12-13 shoots per inoculum) for akarkara cultivation.

Root production in adventitious shoots can be influenced by various factors [19]. Heimsch et al., reviewed the organization of the root apical meristem in angiosperms [20]. Micropropagated shoots of *Anacyclus pyrethrum* can be rooted on half-strength MS medium supplemented with 2.0 mg/L IBA and 0.2% activated charcoal. Additionally, a pulse treatment with 200.0 mg/L IBA effectively stimulated rooting *in vitro*. More than 90%-95% of the shoots underwent successful rooting both *in vitro* and *ex vitro*. IBA was identified as the most effective auxin for root induction in akarkara. The micropropagated plants were then acclimated and hardened in a greenhouse. This tissue culture method is proven to be repeatable and suitable for large-scale plant production.

CONCLUSION

Recently sprouted shoots were utilized as explants, with nodal shoot segments proving effective for culture initiation. Multiple shoots were generated by activating the axillary meristem on MS medium supplemented with 2.0 mg/L BAP. Initial issues with browning and leaching were managed through antioxidant treatment. The cultures were subsequently proliferated by subculturing on MS medium enhanced with ascorbic acid and activated charcoal. For optimal shoot multiplication, a lower concentration of BAP (0.5 mg/L) was found to be ideal. Rooting was successfully induced on half-strength MS medium containing 2.0 mg/L IBA and 0.2% activated charcoal. A pulse treatment with 200.0 mg/L IBA resulted in over 90%-95% of the shoots developing roots both *in vitro* and *ex vitro*. The micropropagated akarkara plants were effectively hardened and acclimated in a greenhouse.

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