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RESEARCH

A rapid *in vitro* propagation protocol of *Bunium persicum* (Boiss.)B.Fedtsch. growing in Kashmir, Himalaya

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ABSTRACT

During the present study an efficient *in vitro* propagation protocol has been developed viz; callus induction, multiple shoot regeneration and rooting of regenerated shoots from hypocotyl explants. Maximum callus production was obtained on MS medium fortified with BAP (2mg/l) after 24 days of inoculation. Multiple shoot regeneration was achieved without sub-culturing of the callus as well as after sub-culturing it on MS medium supplemented with different growth regulators. Maximum shoot regeneration (70%) was achieved without sub-culturing of callus on MS medium augmented with BAP (2mg/l) + NAA (1mg/l) within 18 days. Rooting of regenerated shoots was best achieved on half salt strength MS medium fortified with IBA (1.5mg/l) within 45 days.

Keywords: *Bunium persicum,* hypocotyl, Callus, multiple shoot regeneration, rooting

Abbreviations: BAP; 6-benzyl amino purine, ^{o}C; Degree centigrade, 2,4-D; 2,4 Dichlorophenoxyacetic acid, IAA; Indole 3-acetic acid, IBA; Indole 3-Butyric acid, masl; Meters above sea level, NAA; α – Napthalene acetic acid.

1. INTRODUCTION

Bunium persicum commonly known as “Kala Zira” or “Black cumin” belongs to the family apiaceae. The genus *Bunium* consists of about 166 species (*Vasilava et al., 1985). B. persicum is a native of west Asia and has a limited distribution. The plant is distributed in the mountainous regions of* Iran, Turkmenistan, Tajikistan, Afghanistan, Pakistan and India (Jahansooz *et al*., 2012).

The plant type of *B.persicum* varies from dwarf (30 cm) to tall (80 cm) compact or spreading, moderately to highly branched, tuberous and perennial herb (Panwar ,2000) (Fig. 1a). The leaves are freely, pinnate (2-3), finely dissected and filiform. The flowers are small, white in color with readily symmetrical small sepals, petals and stamens (each five in number), and are present in compact umbels (Fig. 1b). *The economic production of B. persicum is through seeds (schizocarp fruits) that are used as medicine and spices (Khosravi, 1993).* The ripe fruits are used as carminative, lactagogue, diuretic, expectorant, antispasmodic, antiobesity and a valuable spice for flavouring foods (Kala, 2003 and *Sharififar et al., 2010)*. In addition, the essential oil is reported to exhibit significant antioxidative (Shahsavari et al., 2008), antibacterial (Moghtader et al., 2009; Talei and Mosavi, 2009) and antifungal (Sekine et al., 2007) activities.

Over exploitation poor and erratic germination of seeds under natural conditions is one of the major restrictions to the cultivation of this valuable plant species and has been catagorized as a Threatened plant species of Himalayas (Sharma et al., 2010). Thus during the present study an efficient in vitro propagation protocol has been standardized for the mass propagation of this plant species.

Figure 1
a) Plant, b) Inflorescence

2. MATERIALS AND METHODS

Bunium persicum was collected from Dawar-Gurez and Chatterhama-Srinagar (Jammu and Kashmir) at an altitude of 3300 masl and 1894 masl respectively (Fig. 2), and transplanted at Kashmir University Botanical Garden (KUBG). Seeds were collected from plants grown at KUBG and were first thoroughly washed under running tap water in order to remove dirt and dust followed by washing with detergent labolene and surfactant tween-20. Detergent was removed by washing the seeds with double distilled water followed by chemical sterilization of seeds with mercuric chloride 0.1% for 5-10 min. This was followed by washing with autoclaved double distilled water and finally inoculation on sterilized nutrient medium. These seeds germinated after stratification at 4°C in dark for 80-85 days. Hypocotyl explants were excised from 32 days old seeding and inoculated on MS medium.

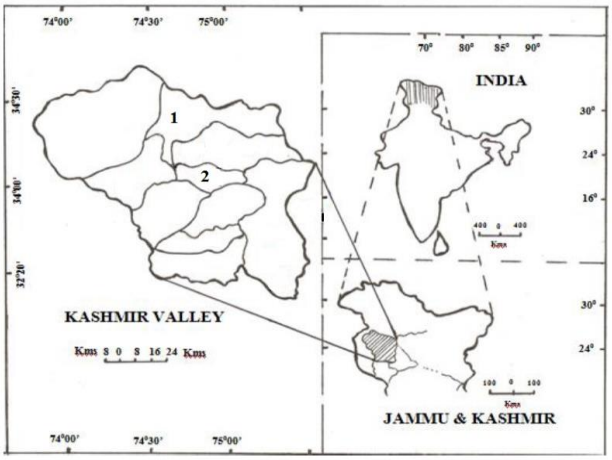

Figure 2
Map showing collection sites of *Bunium persicum*
1) Dawar-Gurez (Bandipora) 2) Chatterhama (Srinagar)

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Medium and Culture conditions

Murashige and Skoog's (MS, 1962) medium, gelled with 8% agar was supplemented with different concentrations of auxins and cytokinins both individually and in combination. Auxins like 2, 4- D; IAA; NAA; IBA and cytokinins like BAP and Kn were used in concentration range of 0.1-5 mg/l. The pH of the media was adjusted to 5.8 before autoclaving at 121°C and 15 lb. The cultures were incubated at 22±4^{0}C and exposed to a regular photoperiod of 24 hours.

3. RESULTS

Callus production

Callus was obtained from hypocotyl explants when MS medium was fortified with auxins (IAA, IBA, NAA, 2,4-D) and cytokinins (BAP and Kn) both individually as well as in combination (Table 1). Among cytokinins BAP and Kn at the concentration of 2mg/l were effective in producing nodular and cream coloured (Fig. 3a & Fig. 3b) callus in 80% and 40% cultures within 24 and 26 days respectively. Among auxins 2,4-D at the concentration of 0.5mg/l was effective in producing nodular and brownish green coloured callus in 60% cultures within 22 days (Fig. 3c). When MS medium was supplemented with BAP in combination with auxins viz; BAP (3mg/l) + IAA (5mg/l) and BAP (2mg/l) + NAA (1mg/l) friable, cream (Fig. 3d) and nodular, green (Fig. 3e) coloured callus was obtained in 70% and 60% cultures within 28 and 24 days respectively. Nodular and brownish coloured callus was also obtained on MS medium fortified with Kn (1mg/l) + IAA (2mg/l) in 40% cultures within 36 days (Fig. 3f).

Table 1

Effect of different hormones on callus production from hypocotyl explants

Treatments	Mean number of days taken for callus production	Amount of callus produced	Texture and colour of callus	Percent culture response
MS Basal	No response	-	-	-
MS + BAP (2mg/l)	24	++++	Nodular, Cream	80
MS + Kn (2mg/l)	26	+++	Nodular, Cream	40
MS + 2,4-D (0.5mg/l)	22	+++	Nodular, Brownish Green	60
MS + BAP (3mg/l) + IAA (5mg/l)	28	++++	Friable, Cream	70
MS + BAP (2mg/l) + NAA (1mg/l)	24	+++	Nodular, Green	60
MS + Kn (1mg/l) + IAA (2mg/l)	36	++++	Nodular, Brownish	40

(10 replicates per treatment)
++++ (High); +++ (Moderate); ++ (Little)

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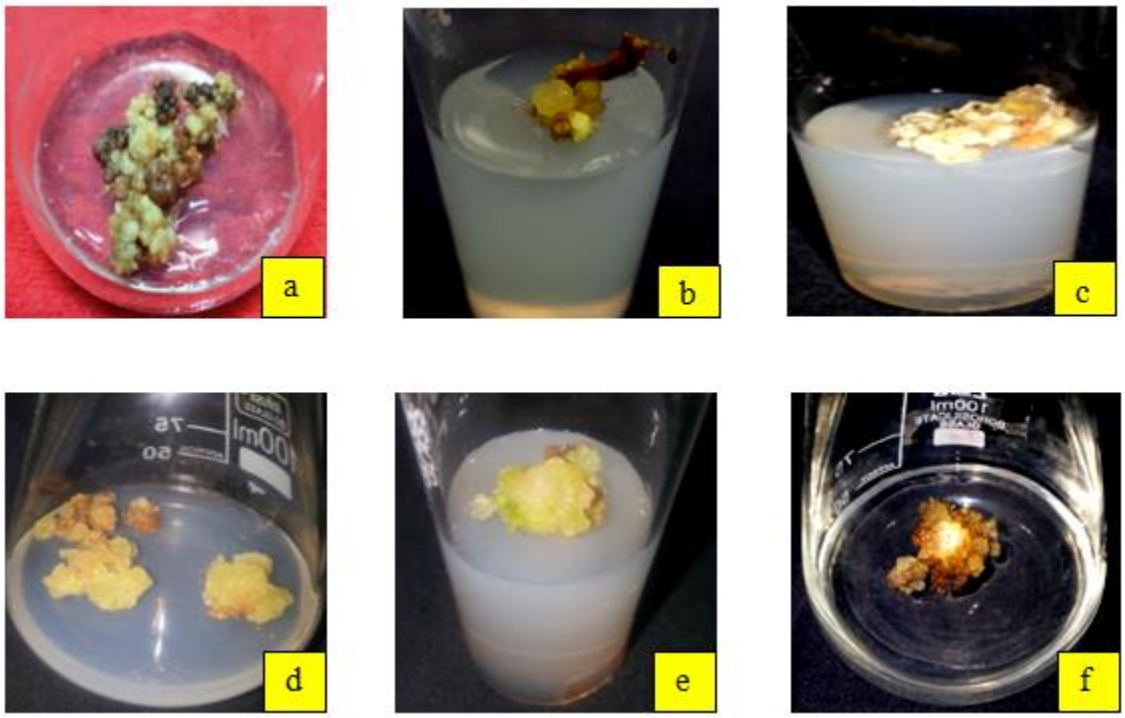

Figure 3

Callus production from Hypocotyl explants on MS medium containing:
BAP (2mg/l) b) Kn (2mg/l) c) 2,4-D (0.5mg/l) d) BAP (3mg/l) + IAA (5mg/l) e) BAP (2mg/l) + NAA (1mg/l) f) Kn (2mg/l) + IAA (1mg/l)

Multiple shoot regeneration

Multiple shoots were produced from hypocotyl callus either on same medium or after subculturing it on MS medium supplemented with different growth regulators (Table 2). Multiple shoot regeneration was obtained without subculturing of callus in 70% cultures with 4.2±0.37 mean number of shoots on MS medium supplemented with BAP (2mg/l) + NAA (1mg/l) within 18 days (Fig. 4a). After subculturing of callus shoot regeneration was achieved on MS medium fortified with BAP alone i:e BAP (2mg/l) as well as in combination with IAA in different concentrations viz; BAP (2mg/l) + IAA (2mg/l) and BAP (5mg/l) + IAA (2mg/l) in 20%; 60% and 40% cultures with 1.8±0.37, 2.6±0.50 and 3.0±0.4 mean number of shoots within 56; 28 and 32 days (Fig. 4b ,4c & 4d) respectively. However, multiple shoots were also obtained when callus was subcultured on MS medium supplemented with IBA (2.5mg/l) in 40% cultures with 4.0±0.54 mean number of shoots within 17 days (Fig. 4e).

Table 2

Effect of different hormones on multiple shoot regeneration from hypocotyls callus

Treatments	Mean number of shoots±SE	Mean number of days taken for shoot regeneration	Percent culture response
MS Basal	No response	-	-
MS + BAP (2mg/l) + NAA (1mg/l)	4.2±0.37	18	70
MS + BAP (2mg/l)	1.8±0.37	56	20
MS + BAP (2mg/l) + IAA (2mg/l)	2.6±0.50	28	60
MS + BAP (5mg/l) + IAA (2mg/l)	3.0±0.44	32	40
MS + IBA (2.5mg/l)	4.0±0.54	17	40

(10 replicates per treatment)

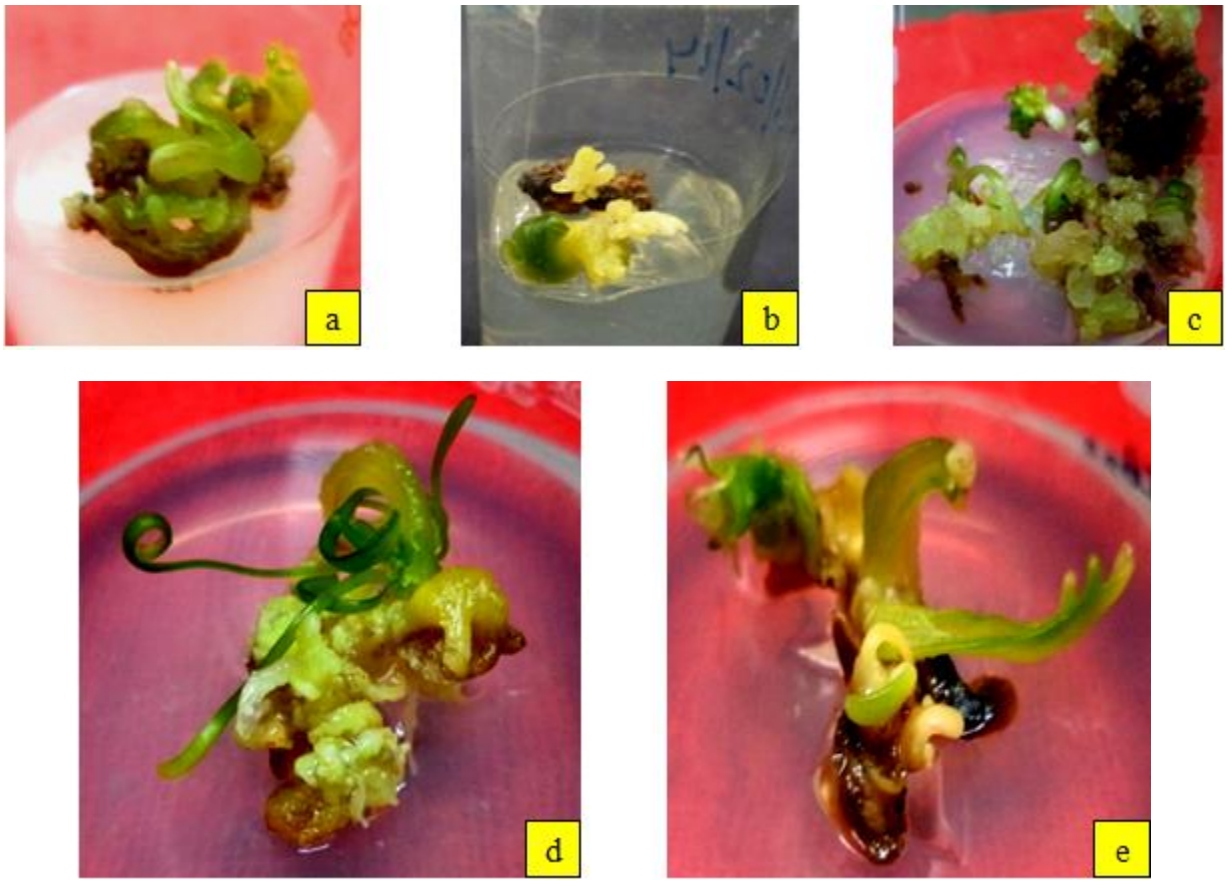

Figure 4

Multiple shoot regeneration from hypocotyl callus obtained on MS medium containing:
BAP (2mg/l) + NAA (1mg/l) b) BAP (2mg/l) c) BAP (2mg/l) + IAA (2mg/l) d) BAP (5mg/l) + IAA (2mg/l) e) IBA (2.5mg/l)

Rooting of regenerated shoots

For root induction, both full strength and half salt strength MS media supplemented with auxins like IAA, IBA, NAA and 2,4-D were used both individually at various concentrations as well as in combination with cytokinins viz: BAP and Kn. However, roots were regenerated from shoots sub-cultured on half salt strength MS medium augmented with IBA (1.5mg/l) and IAA (2.5mg/l) within 45 days (Fig. 5a) and 53 days (Fig. 5b) respectively.

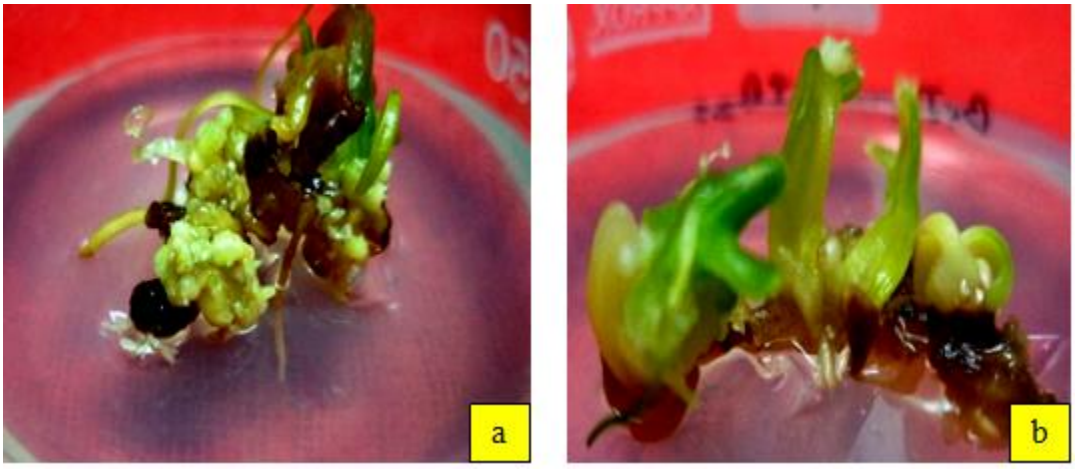

Figure 5

Root regeneration from *in vitro* raised shoots:
IBA (1.5mg/l), b) IAA (2.5mg/l)

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Hardening of *in vitro* raised shoots

For acclimatization *in vitro* raised plantlets were taken out of culture vials. The medium adhering to the basal portion of plantlets was washed with double distilled water. After washing they were transferred to jiffy pots containing autoclaved soil combined with the bark of *Betula utilis* and maintained under controlled conditions in culture room (Fig. 6).

Figure 6
Hardening of *in vitro* raised plants

5. DISCUSSION

During the present study different plant growth regulators both auxins and cytokinins, used either individually or in combination produced callus, multiple shoots and roots from hypocotyl explants of *Bunium persicum.* Maximum callus differentiation was achieved on MS medium fortified with BAP (2mg/l) in 80% cultures within 24 days. Maximum shoot regeneration (70%) with 4.2±0.37 mean number of shoots was achieved without subculturing of callus on MS medium fortified with BAP (2mg/l) + NAA (1mg/l) within 18 days. The results are in accordance with the study of Sharifi and Pouresmael, (1995) who also achieved shoot regeneration in *B. persicum* on MS medium augmented with Kn (2mg/l) + NAA (0.1mg/l). Best root regeneration was achieved on half salt strength MS medium fortified with IBA (1.5mg/l) within 45 days. Our results are in contrast with the study of Valizadeh and Tabar, (2009) who obtained rooting in case of *B. persicum* on MS basal medium.

6. CONCLUSION

A rapid *in vitro* propagation protocol was developed for callus induction, multiple shoot regeneration and rooting of regenerated shoots from hypocotyl explants of *Bunium persicum.* Different plant growth regulators both auxins and cytokinins were used. Among all the plant growth regulators BAP proved to be most effective for both callus induction as well as shoot regeneration. However best root regeneration was achieved on half salt strength MS medium augmented with IBA.

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