

ESTABLISHMENT OF EFFICIENT MICROPROPAGATION SYSTEM IN BISHOP'S WEED (*TRACHYSPERMUM AMMI* L) USING SEED AS EXPLANT

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ABSTRACT

Bishop's weed (*Trachyspermum ammi* L.) is an important medicinal, aromatic and spice plant. The study reports multiple shoot regeneration of bishop's weed using seed explant, cultured on $1.0 \times$ MS medium containing 0.1 - 1.6 mg l^{-1} Kin or TDZ with and without 0.10 mg l^{-1} IBA (10 combinations); and 0.5 - 2.50 mg l^{-1} BA with and without 0.10 mg l^{-1} IBA (10 combinations). Shoot regeneration ranged 1.73 to 2.14 shoots per explant on $1.0 \times$ MS medium containing variants of Kin with and without IBA. Whereas, multiple shoot induction was recorded on $1.0 \times$ MS medium containing variants of TDZ and variants of BAP with and without IBA that ranged 4.05 to 9.73 and 5.74 to 10.05 shoots per explant respectively. The results confirmed that $1.0 \times$ MS medium containing variants of Kin with and without IBA were least suitable for micropropagation purposes. Whereas, $1.0 \times$ MS medium containing variants of BAP with and without IBA induced maximum number of shoots per explant compared to other regeneration media irrespective of the concentration of plant growth regulators. The results further testified successful induction of roots on $1.0 \times$ MS medium containing 1.0 mg l^{-1} IBA supplemented with 75 g l^{-1} sucrose in growth chamber where the plants set seeds under 16 h day length light with 23°C temprature and 90% relative humidity after 8 weeks.

Key wotrds: In vitro, zygotic embryo with two cotyledons, Shoot regeneration, Sucrose, Acclimatization

Abbreviations: BAP – 6-benzylaminopurine; IBA – indole-3-butyric acid; Kin – Kinetin; MS medium - Murashige and Skoog's medium; NAA – -naphthaleneacetic acid; TDZ – Thidiazuron.

INTRODUCTION

Trachyspermum ammi L belongs to Apiaceae or Umbelliferae family, commonly known as bishop's weed (Jeet *et al.* 2012) or ajwain is an important medicinal, aromatic and spice plant that grows widely and is cultured in arid and semi-arid regions of Central, South and West Asia (Joshi 2000, Tubives 2014); on saline soils (Ashraf 2002; Munns 2002). It also grows rarely in Europe and America due to introduction by immigrants. Bishop's weed is 60 - 90 cm tall with branched structure and blooms from July to September each year. The fruit is small and dark khaki colored with bitter pungent taste. Seeds of bishop's weed are medicinally important and contain 38.6% carbohydrate, 11.9% fiber, 18.1% fat, 15.4% protein, 8.9% moisture, and 7.1% minerals (iron, phosphorus, calcium, and nicotinic acid), flavone glycosides, tannins, and saponins (Pruthi 1992). The seeds bear 2 - 4% (v/w) brown coloured oil with 35 - 60% (v/w) thymol as main component (Bairwa *et al.* 2012). These seeds are used as anti-platelet aggregatory (Srivastava 1988), galactagogue, stomach, carminative (Chialva *et al.* 1993), anti pyretic, anti inflammatory (Vadevathy *et al.* 1995), expectorant, antiseptic (Choudhury *et al.* 1998), digestive stimulant (Platel and Srinivasan 2001), Bronchitis, colic pain (Singh *et al.* 2003), antitussive (Boskabady *et al.* 2005), nematicide

(Kwon Park *et al.* 2007), antifilarial (Mathew *et al.* 2008), antilithiasis and diuretic (Ramaswamy *et al.* 2010). Besides that it also used as aflatoxin and detoxificant (Velazhahan *et al.* 2010) in perfumery and preservative in foods (Joshi 2000).

Selection of explant is one of the important step for successful in vitro shoot regeneration especially for the recalcitrant plants. Mature or immatre seed as explant is not commonly used for in vitro shoot regeneration. However, there are few reports on use of seed as explant for plant species like spinach (AlKhayri *et al.* 1992), peanut (Li *et al.* 1994; Pacheco *et al.* 2007), chickpea (Polisetty *et al.* 1997), rice (Bano *et al.* 2005), wheat (Malik *et al.* 2004), onion (Khar *et al.* 2005), *Epimedium alpinum* L (Mihaljevi and Vršek 2008), mungbean (Harisaranraj *et al.* 2008), narbon vetch (Kendir *et al.* 2009) and hairy vetch (Aasim *et al.* 2011). Direct organogenesis of plants using seed explants provides faster and a time saving approach (Aasim *et al.* 2011) for obtaining multiple shoots or whole plants without callus interphase to decrease somaclonal variations (Zapata *et al.* 1999).

The study aimed to develop an accelarated and efficient micropropagation system conserving stability among morphological features of newly germinating plants using seed as explant.

MATERIALS AND METHODS

The bishop's weed seeds were obtained from local seeds market of Rawalpindi, Pakistan. The seeds were submerged in water for 10 minutes prior to sterilization to check seed vitality by eliminating the floating seeds for use in sterilization. They were surface sterilized using 2.5 % (v/v) commercial bleach (ACE 5% NaOCl) for 10 min followed by 3 × 5 min rinsing with sterile distilled water. The sterilized seeds were cultured on 1.0 × MS medium (Murashige and Skoog 1962) containing 10 combinations each of 0.10 - 1.60 mg l⁻¹ Kinetin (Kin) with or without 0.10 mg l⁻¹ indole-3-butyric acid (IBA) (Table 1); 0.10 - 1.60 mg l⁻¹ Thidiazuron (TDZ) - with and without 0.10 mg l⁻¹ IBA (Table 2); and 0.5 - 2.5 mg l⁻¹ 6-benzylaminopurine (BAP) with or without 0.25 mg l⁻¹ IBA (Table 3). Each of the shoot multiplication medium was supplemented with 3% (w/v) sucrose and 0.65% (w/v) agar. All culture media were autoclaved for 20 min at 1.4 kg cm⁻². The pH was adjusted to 5.6 - 5.8 with 1M NaOH or 1M HCl. All cultures were grown at 24° ± 1° C with a 16/8-h (light/dark) photoperiod. Light was supplied at intensity of 25 μmol m⁻² s⁻¹ by cool-white fluorescent lamps.

Healthy, elongated and well multiplied shoots were isolated under aseptic conditions for rooting on 1.0 × MS medium containing 0.2, 0.4, 0.60, 0.80 and 1.0 mg l⁻¹ IBA for four weeks. In another experiment, shoots were cultured on 1.0 × MS medium containing 1.0 mg l⁻¹ IBA with 45-90 g l⁻¹ sucrose for four weeks. Rooted plantlets were submerged in water for 6 hours before transfer to pots to reduce transfer shock and harden the plants to external environment. These plantlets were transferred to pots containing peat moss, peat moss + perlite (2:1, 1:1) or perlite. The pots were kept in growth chambers at 24° ± 1° C in 12 h light (35μ Mol photons m⁻² s⁻¹) photoperiod.

Each experimental treatment including the controls used 48 explants, divided into six replicate groups. The data were subjected to one-way ANOVA using F-test in "IBM® SPSS® Statistics Version 20" for Windows selecting Duncan's Multiple Range test to compare means. Care was taken to square root transform all data given in percentage (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

The study evaluated multiple shoot regeneration on NaOCl treated seed explants of bishop's weed cultured on 1.0 × MS medium containing 10 variants each of Kin, TDZ and BAP singly or in combination with IBA. All explants induced shoot regeneration in same framework of shoot induction starting with swelling of explants followed by single shoot initiation ending up

with multiple shoots irrespective of the concentration and combination of plant growth regulator used in the study. The seeds germinated within 7-10 days with initiation of single shoot followed by multiple shoot induction, which sharply changed the regeneration pattern depending on the type of growth regulator, their concentrations and combinations used in the study. It was also noteworthy to see late callus induction after 5 weeks of culture irrespective of growth variants.

Comparing effects of Kin with and without 0.10 mg l⁻¹ IBA, no callus was induced on explants cultured on 1.0 × MS medium containing variants of Kin used singly (Table 1). Use Kin with IBA in 1.0 × MS medium favored callus induction variably irrespective of the concentration of Kin in the culture medium. Shoot regeneration percentage ranged 72.91 to 97.91% with maximum shoot induction on 1.0 × MS medium containing 0.20 mg l⁻¹ Kin. Variants of Kin used singly were inhibitory and induced lesser number of shoots per explant compared to number of shoots regenerated on 1.0 × MS medium containing variants of Kin with 0.10 mg l⁻¹ IBA (Fig 1a). The result indicated induction of 1.88 - 2.14 and 1.63 - 2.13 shoots per explant on 1.0 × MS medium containing Kin singly or Kin with IBA respectively. Germinated seed induced 1.62 - 5.23 cm long shoots on 1.0 × MS medium containing variants of Kinetin used singly. 1.0 × MS medium containing variants of Kinetin with 0.10 mg l⁻¹ IBA regenerated 1.82 - 4.24 cm long shoots per explant with maximum shoot length gain on 0.1 mg l⁻¹ Kin with 0.10 mg l⁻¹ IBA.

The results testified that variants of TDZ with and without IBA equally induced callusing on seeds in range of 13.38 - 80.55% (Table 2). Maximum callus induction was noted on 0.80 mg l⁻¹ TDZ with 0.10 mg l⁻¹ IBA. Callus induction percentage decreased with increasing concentrations of TDZ used singly. Callus induction percentage increased consistently on variants of TDZ with 0.10 mg l⁻¹ IBA to reach its optimum at 0.80 mg l⁻¹ TDZ with 0.10 mg l⁻¹ IBA.

Shoot regeneration percentage showed no difference among variants of TDZ with and without IBA in 1.0 × MS medium in statistical terms. Shoot regeneration had range of 77.77 to 94.44%. Maximum shoot regeneration percentage was noted on 1.0 × MS medium containing 0.10 mg l⁻¹ TDZ. Shoot regeneration was also noted on cotyledonary leaves on some explants cultured on TDZ plus IBA containing 1.0 × MS medium (Figure 1d). Multiple shoot induction was recorded on variants of TDZ plus IBA (Figure 1b) after two weeks of culture on 1.0 × MS medium. Number of shoots per explant showed significant differences among variants of TDZ with and without IBA. They had range of 6.81 - 9.73 on 1.0 × MS medium containing variants of TDZ without IBA. Number of shoots per seed explant changed from 4.05 - 5.81. The results suggested that TDZ with IBA was inhibiting in terms of number of shoots per

explant. Shoot length had range of 0.47 to 1.28 cm with maximum gain in shoot length on $1.0 \times$ MS medium containing 0.10 mg l^{-1} TDZ with 0.10 mg l^{-1} IBA.

Callus induction percentage varied on MS medium containing variants of BAP with 0.25 mg l^{-1} IBA ranged 33.33 to 75.00% with maximum callusing on $1.0 \times$ MS medium containing 0.50 mg l^{-1} BAP – 0.25 mg l^{-1} IBA (Table 3). Multiple shoot induction was registered on variants of BAP with and without IBA (Figure 1c) after three weeks of culture. Shoot regeneration percentage had range of 83.33 – 100%. Again maximum shoot induction was noted on seeds germinated on $1.0 \times$ MS medium containing 0.50 mg l^{-1} BAP plus 0.25 mg l^{-1} IBA. Shoot regeneration was also registered on cotyledonary leaves on some explants cultured on BAP plus IBA (Figure 1e) containing $1.0 \times$ MS medium. Number of shoots per explant changed from 5.74 to 10.05 per explant. Maximum number of 10.05 and 9.99 shoots per explant were recorded on $1.0 \times$ MS medium containing 0.50 mg l^{-1} BAP with and with out 0.25 mg l^{-1} IBA. Shoot length increased from 0.79 to 1.07 cm on $1.0 \times$ MS medium containing variants of BAP with and with out 0.25 mg l^{-1} IBA. The longest shoots were noted on $1.0 \times$ MS medium containing 1.50 mg l^{-1} BAP with 0.25 mg l^{-1} IBA followed very closely by $1.0 \times$ MS medium containing 2.00 and 2.50 mg l^{-1} BAP with 0.25 mg l^{-1} IBA and 0.50 mg l^{-1} BAP used singly.

Comparing ability of Kin, TDZ and BAP with and without IBA; Kinetin used singly did not induce callusing at all. However, varying percentage of callus induction was noted on TDZ and BAP with and without IBA. Whereas, high shoot induction percentage (>72.91%) was noted on Kin, TDZ and BAP with and without IBA. Comparing number of shoots per explant, it was noted that maximum number of 2.51, 9.73 and 10.05

shoots per explant were noted on Kin with IBA, TDZ without IBA and BAP with IBA respectively. Comparing shoot length it was registered that relatively, longer shoots were obtained from Kin-IBA (Table 1) cultured medium compared to TDZ plus IBA (Table 1) and BAP plus IBA (Table 1) containing media.

The experimental results about the effects of $1.0 \times$ MS containing 0.2, 0.4, 0.6, 0.8 and 1.0 mg l^{-1} IBA supplemented with 30 g l^{-1} sucrose showed variable behaviors after 4 weeks of rooting. All shoots induced roots and also multiple shoots at the cut ends of rooted shoots (Figure 1f). These shoots were transferred to pots containing either peat moss, peat moss + perlite (2:1, 1:1) or perlite, covered with plastic bags and shifted to growth room at $24^\circ \pm 1^\circ \text{ C}$ and 16 h light photoperiod for acclimatisation. Once the covering plastic bags were removed, they wilted and died within one week irrespective of substrate due to loss of moisture .

It was noted that multiple shoot induction was lowest at highest IBA concentration. Therefore, sregenerated shoots were rooted on $1.0 \times$ MS containing 1.0 mg l^{-1} IBA with higher sucrose concentration (45-90 g/l) which also induced 100 % rooting. However, multiple shoot induction from the base end decreased with sucrose concentration. The plantlets rooted on 1.0 mg l^{-1} IBA supplemented with 75 g l^{-1} sucrose (Figure 1g) were hard and sturdy and were easy to acclimatise when transferred to pots containing peat moss, moss+perlite (2:1, 1:1) or perlite (Figure 1h,i) and kept in the growth chamber. Plants flourished profusely once the covering plastic bags were removed from the plantlets after initial period of acclimatisation (one week). The acclimatised plants showed 100% survival with flowering (Figure 1j) and seed set after 8 weeks.

Table 1. Shoot regeneration from mature seed explant of Bishop's Weed on different KIN-IBA concentrations

KIN (mg l^{-1})	IBA (mg l^{-1})	Callus Induction (%)	Shoot Regeneration (%)	Shoots per explant	Shoot length (cm)
0.10	-	0.00 ^c	95.83 ^{ab}	1.88 ^{ab}	5.23 ^a
0.20	-	0.00 ^c	97.91 ^a	1.93 ^{ab}	3.54 ^{ab}
0.40	-	0.00 ^c	95.83 ^{ab}	1.73 ^b	2.31 ^{de}
0.80	-	0.00 ^c	85.41 ^{abcd}	1.81 ^b	1.74 ^e
1.60	-	0.00 ^c	95.83 ^{ab}	2.14 ^{ab}	1.62 ^e
0.10	0.10	8.30 ^c	83.33 ^{bcd}	2.13 ^{ab}	4.24 ^{ab}
0.20	0.10	29.16 ^b	87.49 ^{abc}	1.85 ^b	3.04 ^{bcd}
0.40	0.10	39.58 ^{ab}	72.91 ^d	2.00 ^{ab}	2.82 ^{cde}
0.80	0.10	49.99 ^a	91.66 ^{ab}	1.63 ^b	3.93 ^{bc}
1.60	0.10	27.08 ^b	74.99 ^{cd}	2.51 ^a	1.82 ^e

Values in a column followed by different letters are significantly different ($p < 0.05$) according to Duncans Multiple range test

Table 2. Shoot regeneration from mature seed explant of Bishop's Weed on different TDZ-IBA concentrations.

TDZ (mg l ⁻¹)	IBA (mg l ⁻¹)	Callus Induction (%)	Shoot Regeneration (%)	Shoots per explant	Shoot length (cm)
0.10	-	58.33 ^{ab}	94.44 ^{ns}	9.73 ^a	1.03 ^{ab}
0.20	-	36.10 ^{bcd}	88.88	7.17 ^{ab}	0.74 ^{bc}
0.40	-	24.99 ^{cd}	77.77	7.46 ^{ab}	0.77 ^{bc}
0.80	-	16.66 ^d	88.88	6.81 ^{ab}	0.51 ^c
1.60	-	13.88 ^d	86.10	7.25 ^{ab}	0.47 ^c
0.10	0.10	44.44 ^{bc}	86.11	5.72 ^b	1.28 ^a
0.20	0.10	69.44 ^a	83.33	5.81 ^b	0.84 ^{bc}
0.40	0.10	69.44 ^a	91.66	4.44 ^b	0.72 ^{bc}
0.80	0.10	80.55 ^a	91.66	5.20 ^a	1.01 ^{ab}
1.60	0.10	44.44 ^{bc}	77.77	4.05 ^a	0.90 ^b

Values in a column followed by different letters are significantly different ($p < 0.05$) according to Duncans Multiple range test

Table 3. Shoot regeneration from mature seed explant of Bishop's Weed on different BAP-IBA concentrations

BAP (mg l ⁻¹)	IBA (mg l ⁻¹)	Callus Induction (%)	Shoot Regeneration (%)	Shoots per explant	Shoot length (cm)
0.50	-	36.10 ^{ab}	94.44 ^{ab}	9.99 ^a	1.06 ^a
1.00	-	66.66 ^{ab}	97.22 ^{ab}	9.50 ^a	0.78 ^b
1.50	-	58.33 ^{ab}	91.66 ^{ab}	6.40 ^{bc}	0.79 ^b
2.00	-	58.33 ^{ab}	83.33 ^b	6.20 ^{bc}	0.79 ^b
2.50	-	33.33 ^b	91.66 ^{ab}	6.40 ^{bc}	0.93 ^{ab}
0.50	0.10	75.00 ^a	100.00 ^a	10.05 ^a	0.92 ^{ab}
1.00	0.10	72.22 ^{ab}	88.88 ^{ab}	8.03 ^{ab}	0.99 ^a
1.50	0.10	59.91 ^{ab}	94.44 ^{ab}	6.74 ^{bc}	1.07 ^a
2.00	0.10	47.21 ^{ab}	88.88 ^{ab}	5.90 ^{bc}	1.06 ^a
2.50	0.10	41.66 ^{ab}	86.11 ^{ab}	5.74 ^c	1.02 ^a

Values in a column followed by different letters are significantly different ($p < 0.05$) according to Duncans Multiple range test



Figure 1 *In vitro* shoot regeneration, rooting and acclimatisation of Bishop's Weed using mature seed explant (a) single shoot regeneration from Kin-IBA (b) multiple shoot regeneration from TDZ-IBA and (c) BAP-IBA. (d) shoot initiation from cotyledary leaf on TDZ-IBA and (e) BAP-IBA. (f) multiple shoot induction with rooting in IBA rooting medium (g) rooting and hardening at higher concentration of sucrose (h,i) acclimatised plants in pots with different substrate and (j) plant with flowering.

DISCUSSION

The study presents multiple organogenesis, rooting and adaptation of bishop's weed, using seed as explant. Mature or immature seeds provide possibility to direct organogenesis (axillary shoot regeneration), gene transfer or somaclonal variations using biotechnology tools (Barik *et al.* 2005, Vaz Patto *et al.* 2011, Ochatt *et al.* 2013). Seed as explant has been previously reported in different plants for inducing callogenesis followed by shoot or bud initiation. However, Gagliardi *et al.* (2000) failed to induce shoot regeneration in peanut using seed explant cultured on $1.0 \times$ MS medium or $1.0 \times$ MS medium containing $10 \mu\text{M}$ TDZ. In this study, results clearly showed the efficacy of seed explant and plant growth regulators affected the callus and shoot regeneration frequency, number of shoots per explant and mean shoot length.

In present study, different cytokinins (Kin, TDZ and BAP) with or without IBA were tested for multiple shoot induction on mature seed explants. All explant showed almost same pattern of shoot induction by the swelling of explants with single shoot initiation ending up with multiple shoot induction in line with the findings of Malik *et al.* (2004) and Aasim *et al.* (2011). The results further illustrated that presence of IBA in the culture medium affected frequency of callus induction variably in line with the findings of Karatas *et al.* (2013). The results on shoot regeneration frequency clearly verified non significant effects of TDZ plus IBA and significant effects of Kin plus IBA and BAP plus IBA on shoot regeneration in agreement with Aasim *et al.* (2011), who reported positive effects of the presence of IBA with TDZ on shoot regeneration frequency of hairy vetch using mature seed explants.

Results on number of shoots per explants revealed the importance of growth variants for multiple shoots as Kin plus IBA induced relatively less number of shoots per explant compared to other growth regulators used in the study in agreement with Karatas, *et al.* (2013) using Kin singly compared to TDZ in dwarf hygro. Results further illustrated that explants showed variable response to concentrations of growth regulators as number of shoots per explant decreased with increase in the concentration of TDZ and BAP, and increased with increase in Kin concentration. Sahin-Demirbag *et al.* (2008) obtained maximum shoot regeneration on $1.0 \times$ MS medium containing 0.45 mg l^{-1} TDZ and their results suggested that a increase in TDZ concentration inhibited shoot regeneration in the Hungarian vetch. The trend of decreasing number of shoots with increasing concentrations of BAP was also reported by Park *et al.* (2011). However, they reported that, TDZ showed a different behavior from BAP and Kin and with increasing concentration on the number of regenerating shoots per explants.

Results on mean shoot length was clearly influenced by the type and concentration of growth regulators. Although, Kin with or without IBA was less efficient in regeneration of number of shoots per explant, yet the shoot were significantly longer compared to those induced on TDZ or BAP with and with out IBA. The results are in agreement with Park *et al.* (2011); who obtained longer and reduced number of shoots per explant on *Liriope platyphylla* using Kin compared to BAP or TDZ. Karatas *et al.* (2013) also reported suppressive effects of TDZ used singly on shoot length compared to Kin used singly in dwarf hygro. Results further revealed the suppressive effects of increased cytokinin concentrations irrespective of type on shoot length in line with Kendir *et al.* (2008, 2009) in Narbon vetch.

Results on rooting showed that regenerated shoots responded well to all concentrations of IBA used in the experiments. Multiple shoot regeneration with callus induction is an unknown phenomenon and has been reported in different legumes like lentil (Aasim *et al.* 2012) and chickpea (Aasim *et al.* 2013). Rooted plantlets with multiple shoots transferred to pots for adaptation died immediately after removal of plastic bags due to water deficiency as root induction from callus failed to transfer minerals and water to the shoots. On the other hand, increased sucrose concentration (7.5 %) was helpful to gain mass and easy hardening of plants. The results are in partial agreement with Aasim *et al.* (2013), who used 60 g l^{-1} sucrose for hardening of rooted chick pea plants. Rooted plantlets were successfully established in all substrates in growth chamber where flowering and seed set was obtained after two months.

The research meets objectives of the study and reports accelerated and efficient shoot regeneration of bishop's weed under in vitro conditions. The results of study can be helpful in future production of true to the type clonally multiplied plants for uniform production of pharmacologically active compounds.

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