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Original Research Article

In-vitro propagation of *Pluchea lanceolata* (DC) C.B.Clarke a potent antiarthritic medicinal herb through axillary bud

SATENDRA SINGH [1,2]✉*, RITU MISHRA[1,3], AND RAJNEESH K. AGNIHOTRI[2]

[1]Division of Genetics and Plant Breeding, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow -226015 (U.P.), India
[2]Department of Botany, School of Life Science, Khandari Campus, Dr. B. R. Ambedkar University, Agra-282002(U.P.), India
[3]Academy of Scientific and Innovative Research (AcSIR), CSIR-Human Resource Development Centre Campus, Sector-19, Kamla Nehru Nagar, Ghaziabad-201002 (U.P.), India

ABSTRACT

Pluchea lanceolata, commonly known as Rasana, is highly valued for its anti-inflammatory, antiarthritic, and analgesic properties and is extensively employed in treating rheumatoid arthritis, cough, neurological diseases, edema, sciatica, psoriasis, bronchitis, dyspepsia, and piles. Shrinking populations and insufficient variation due to rootstock propagation methods warrant efficient mass multiplication and conservation protocols. In the present study, explant (axillaries bud was cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinins and auxin (PGRs) individually, as well as in combination. The highest mean shoot number (2.06 ± 1.43) and second highest mean shoot length (2.62 ± 1.49 cm) obtained in MS media enriched with 4 mg/L Benzyl amino purine (BAP) + 1 mg/L Naphthalene acetic acid (NAA). Whereas, the best shoots multiplication (2.62 ± 1.41) and shoots elongation (5.88 ± 2.44) responses were displayed when MS medium supplemented with 0.25 mg/L (BAP) +0.50 mg/L Kinetin (KIN) were used.

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1. Introduction

Pluchea lanceolata (DC) C.B. Clarke (Asteraceae) is a small, perennial, xerophytic, widely grown in warm climatic regions of India (Murugan et al., 2008) such as Rajasthan, Gujarat, Punjab, Uttar Pradesh, Haryana, Madhya Pradesh, and Bihar. The herb is an undershrub, commonly a meter tall, rarely attaining a height of 1.5 m., characterized by sessile oblong to oblanceolate leaves and involucre of bracts being oblong, with purple bract-tip. This plant is highly valued for its medicinal importance. It is widely applied in treating arthritis, inflammatory disorders, paralysis, the pain of neurological origin, hemiplegia, bone and muscle pain, constipation, and respiratory diseases (Sharma, 2005; Srivastava and Shanker, 2012). Many classical Ayurvedic texts mention this plant as one of the most crucial Rasayana herbs and are included in formulations of more than 80 medical ailments (Srivastava and Shanker 2012). The plant shows a wide range of therapeutic effects such as anti-inflammatory activity, antigonadotrophic, (Srivastava et al. 1990), immunosuppressive (Bhagwat et al. 2010), analgesic (Arya, 2008), anti-neoplastic, anti-malarial, antioxidant, neuroprotective, and a muscle relaxant (Jahangir et al., 2005; Jahangir and Sultana, 2006; Srivastava and Shanker, 2012; Mohanty et al., 2013; Srivastava et al., 2014; Mundugaru et al., 2018).

The seeds in *Pluchea lanceolata* are non-viable and are propagated only by rootstock resulting in fast declining populations in the wild (Singh, 2004). The word 'Rasana' has found its origin in Sanskrit. The plant is widely used in the indigenous system of medicine, and classical Ayurvedic texts mention utilizing this herb to treat joint pain. The plant contains high amounts of medicinally critical secondary metabolites viz. quercetin, β sitosterol, pluchine, isorhamnetin, daidzein, triterpenes, triterpenoid, etc. Ethnomedicinally, the

✉Corresponding author: Satendra Singh
Tel: +05222718544; Fax: +05222718544
E-mail address: satendras.s30@gmail.com, **doi: 10.30495/tpr.2021.680483**

plant's decoction has been used for relieving dysentery, lumbago, leucorrhoea, dysuria, hemorrhoids, gangrenous ulcer, and disorders causing cachexia (Ayurvedic Pharmacopoeia, 1989; Khare, 2007). Since the seeds being nonviable and the rootstock being only the method of propagation limits this herb's availability in the wild. Additively, the ruthless exploitation and extraction of this herb from the wild may pose a risk of extinction for this herb. Therefore, suitable alternatives methods need to be established for the rapid multiplication of this herb. The only solution to this problem can be offered by applying appropriate tissue culture techniques that provide alternative methods for the rapid regeneration and proliferation of desirable clones. Therefore, in the present study, suitable culture protocols have been established to prioritize this rare herb conservation.

2. Experimental

2.1. Collection of explants

The disease-free, healthy, young axillary bud of *P. lanceolata* (CSIR-CIMAP-herbarium no Boucher No 13063) was used as an explant in the present study. Axillary buds collected from plants growing in the grassland of the Department of Botany, School of Life Sciences, Khandari Campus, Dr. B.R. Ambedkar University, Agra, India. Geographically, the University registered at 26º 44′ N to 27º 25′ N and 77º 26′ E to 78º 32′ E, is located in the extreme southwest of Uttar Pradesh. The plants can be easily identified in the months of August to April when the plants are either in a glowering or fruiting state. However, in the present study, the explants were collected during the month of late April and early May (Fig. 1).

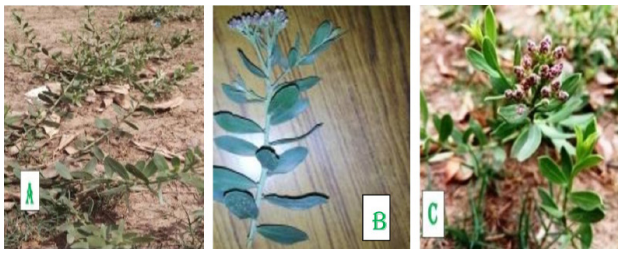

Fig. 1. Field view and morphological condition in Pluchea lanceolata.

2.2. Source of chemicals

For the preparation of the stock solution of culture media (Murashige and Skoog, 1962), all the chemicals used in this study (experiment) were of analytical grade and obtained from Hi Media Laboratory Pvt. Ltd, Merck (India) Ltd. or Sigma Chemicals Co., USA.

2.3. Sterilization of glassware and instruments

All the glassware was boiled in 10% washing soda solution (w/v) for 40 mins and were thoroughly washed under running tap water and left overnight to dry at room temperature. The next day, the glassware was kept in an oven at 80-100˚C for one hour and then placed inside the laminar airflow for UV treatment for about 20-30 min to check the virus and bacteria contamination. All the tools (such as knives, scalpel, forceps) used in the experiment were sterilized with 70% alcohol and then wrapped in fresh aluminum foil. Aluminum foil wrapped tools and media were finally autoclaved at 12-15 psi presser and 121 ˚C for 20 min.

2.4. Sterilization of explants

About 1-2 cm long explants of *P. lanceolata* were excised carefully and washed under running tap water for 20 minutes to wash off external dust particles and other contaminants. Explants were kept in distilled water for 24 hours to facilitate the leaching of alkaloids. Explants were soaked in tween-20 (neutral detergent) solution (2 mg/L) for 20 minutes with continuous stirring and rinsed thoroughly with distilled water 2-3 times. Then, the explants were soaked in a 1% aqueous Bavistin solution (w/v) containing (BASF, India Limited) for 5 minutes and were washed with distilled water 3-4 times to remove any other contaminants. Explants were sterilized with streptomycin (0.1% v/v) for 10 minutes, rinsed with distilled water 2-3 times, and were finally taken to a laminar flow hood under sterile conditions. Under laminar flow, the explants were sterilized with mercuric chloride ($HgCl_2$, 0.1% v/v) aqueous solution for 1 minute and washed thoroughly 3-4 times with sterile double distilled water. These washed and sterilized explants were used for inoculation.

2.5. Inoculation of explant

These surface-sterilized explants of appropriate sizes (1-2 cm long) were inoculated into the MS medium (Murashige and Skoog, 1962). The essential salts of culture media were supplemented with sucrose (3 w/v%) and agar-agar powder (0.8 w/v%) with plant growth regulators (PGRs). Before heating, the culture medium's pH was adjusted to 5.8 with NaOH (1 N) and HCl (1 N). The autoclaved culture medium was transferred to culture tubes, and then culture tubes were allowed to solidify at room temperature. The explant was inoculated in the culture tube containing solidified culture media. The tubes containing explants were placed in Culture Room maintained at 25 ± 1°C under 16 h photoperiod B2000-3000 lux intensity and 8 h dark period with 55 ± 5% humidity.

3. Results and Discussion

3.1. Culture establishment

After the sterilization, 60% or more of the explants were free of contamination (Table 1). Bacterial and fungal contamination is a common problem encountered during the culture establishment and subculturing of the explant. However, all possible sterilization steps

Table 1
Survival rate of explant of *Pluchea lanceolata*.

S. No.	Medium	Total explant inoculation	Explant survival		Explant dead	
			No.	%	No.	%
1	Water agar	20	13	65	7	35
2	MS medium	20	15	75	5	25
3	BAP	16	12	75	4	25
4	NAA	16	13	81.25	3	18.75
5	KIN	16	12	75	4	25
6	BAP+NAA	16	11	68.75	5	31.25
7	BAP+KIN	16	14	87.5	2	12.5

were followed to minimize the chance of contamination. The chances of contamination were minimized by selecting meristems as the explant for *in-vitro* culture because of their high totipotency and least vulnerability to diseases. Hence, the nodes present near the shoot apical meristem were preferred for culturing to reduce bacterial and fungal contamination. After the primary inoculation, the explants were transferred after 15-18 days into the new fresh MS medium. The different concentrations of PGRs exerted a significant effect on the percentage of shoot induction, elongation, and multiplication. Phytagel showed superiority over the agar medium for both the factors (Agnihotri et al., 2009). The 70% response shoot induction was found in MS medium supplemented with Plant hormones, Benzyl amino purine (BAP), Kinetic (KIN), and further exhibited shoot proliferation and elongation in the same medium (Arya and Patni, 2013). Likewise, in the current set of experiments, the highest survival response (81.25%) and minimum (65%) was recorded; as responding explant for shoot induction, while in multiplication medium (BAP+KIN), the response was observed 87.50% (Table1).

3.2. *In vitro* shoot induction

Based on plant growth regulators and their concentration, the axillary bud break varied. The sprouting of bud generally occurred within 7 to 8 days (Fig. 2-B) but well sprouting was achieved within 2 to 3 weeks after inoculation (Fig. 2-C and D). However, the results show that different combinations of PGRs used in the present study had different effects on cultures. Out of all concentration strength of PGRs, used in the present study, NAA at 2 mg/L, BAP at 1.5 mg/L, and KIN at 1.5 mg/L gave maximum response for shoot induction (Table 2). Whereas, the maximum average number of shoot response was achieved with a combination of BAP (4 mg/L) and NAA (1 mg/L). These findings were congruent with the findings of Kanugo and Sahoo (2011) on their studies involving *Withania somnifera* and the combination of BAP and NAA, however of two concentrations (Fig. 7). However, higher concentrations of BAP, NAA, and KIN has inhibitory effects on shoot induction and vice versa. Individual application of NAA, BAP, and KIN in the culture medium shows that

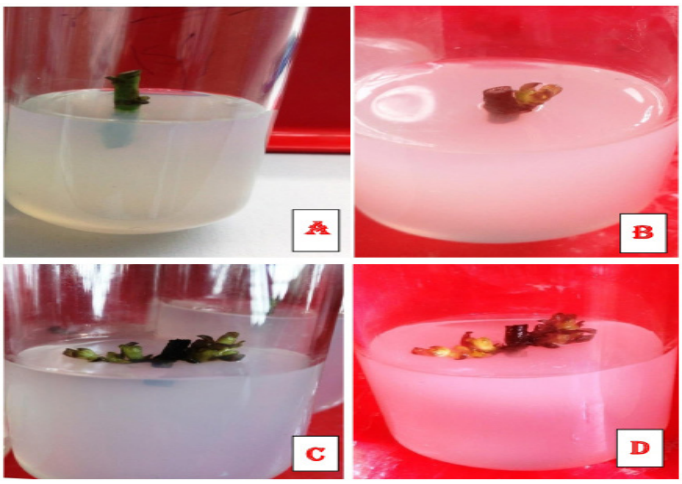

Fig. 2. *In-vitro* propagation in *Pluchea lanceolata* having four different conditions in MS medium.
A. Establishment of axillaries bud explant on MS medium.
B. Shoot induction form explant on shoot induction medium after 15 days of culture.
C. After 21 day of culture.
D. After 28 day of culture.

Table 2
Effect of difference concentration single or combination of PGRs on shoot induction and number of shoot or shoot length per culture.

NAA (mg/L)	BAP (mg/L)	KIN (mg/L)	Average no of shoot Mean ± SD	Shoot length Mean ± SD	No of shoot per culture
0.5	-	-	0.68 ± 0.60	0.79 ± 0.64	1-1
1	-	-	0.87 ± 0.61	0.99 ± 0.60	1-1
1.5	-	-	1.06 ± 0.68	1.20 ± 0.65	1-2
2	-	-	1.68 ± 1.07	1.32 ± 0.73	1-2
2.5	-	-	1.06 ± 0.68	1.16 ± 0.48	1-1
3	-	-	0.96 ± 0.57	0.54 ± 0.55	1-1
4	-	-	0.81 ± 0.54	0.93 ± 0.55	1-1
-	0.25	-	0.75 ± 0.77	0.90 ± 0.85	0-1
-	0.5	-	1.06 ± 0.99	1.41 ± 1.16	1-1
-	1	-	1.87 ± 1.58	2.18 ± 1.90	1-2
-	1.5	-	1.93 ± 1.38	2.30 ± 1.53	1-4
-	2	-	1.75 ± 1.18	2.62 ± 1.49	1-3
-	2.5	-	1.62 ± 1.02	2.37 ± 1.28	1-3
-	3	-	1.56 ± 0.81	2.28 ± 1.02	1-2
-	3.5	-	1.31 ± 0.70	1.72 ± 0.90	1-2
-	4	-	1.25 ± 0.68	1.42 ± 0.66	1-1
-	-	0.25	0.93 ± 0.99	0.68 ± 0.63	1-1
-	-	0.5	1.06 ± 0.85	1.00 ± 0.71	1-1
-	-	1	1.12 ± 0.95	1.20 ± 0.93	1-1
-	-	1.5	1.87 ± 1.50	2.12 ± 1.38	1-3
-	-	2	1.68 ± 1.01	1.56 ± 0.84	1-2
-	-	2.5	1.50 ± 0.96	1.53 ± 0.83	1-2
-	-	3	1.12 ± 0.80	1.21 ± 0.78	1-1
-	-	3.5	1.06 ± 0.68	1.17 ± 0.63	1-1
-	-	4	1.00 ± 0.51	0.81 ± 0.471	1-1
1	2		0.68 ± 0.60	0.43 ± 0.38	0-1
1	3		1.12 ± 1.02	0.46 ± 0.40	1-1
1	4		2.06 ± 1.43	0.53 ± 0.45	1-2
2	3		1.06 ± 0.85	0.46 ± 0.43	1-1
3	3		0.87 ± 0.67	0.76 ± 0.58	0-1

different concentrations were required for optimum shoot induction. A concentration of 2.0 mg/LL NAA and KIN was found optimum which produced 1.68 ± 1.07 and 1.68 ± 1.01 (Fig. 4 and 6) respectively, shoot buds per explant (Table 1) and 1.5 mg/L BAP 1.93 ± 1.38 shoots (Fig. 5). MS medium supplemented with different concentrations of BAP (0.5-3.0 mg/L) had a positive effect on the induction of shoot buds. Any deviation from these optimum concentrations resulted in a decrease in the average shoot number per explant. For the multiple shoot's inductions, the presence of BAP in the medium played a very significant role in direct organogenesis. At higher concentrations, BAP reverses its action on multiplication and reduces the number ofshoots formed ultimately resulting in stunted growth. For several medicinal plant species, the effect of BAP on multiple shoot formation has been reported (Tiwari et al., 1998; Wang et al., 2004; Espinosa et al., 2006). Similarly, 1.5 mg/L BAP and 1.5 mg/L KIN, the maximum number of shoot induction was obtained. For shoot bud proliferation, the application of auxins viz., NAA (0.25-2.0 mg/L) was combined with the optimal concentrations of BAP (0.5 mg/L). Any deviation from the recommended auxin concentrations had inhibitory actions, as in *Pluchea lanceolata* (Arya et al., 2008).

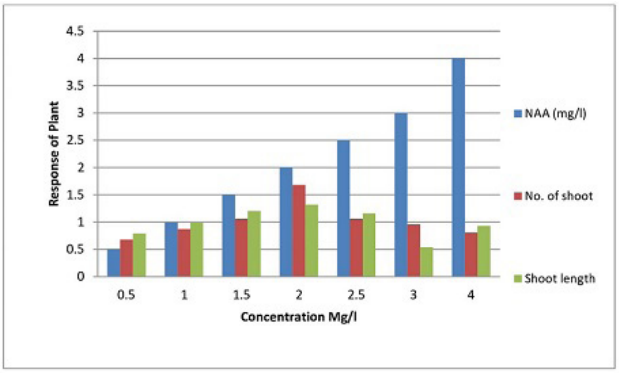

Fig. 4. Number of shoot/explant and shoot length (cm) at different concentration of NAA (mg/L).

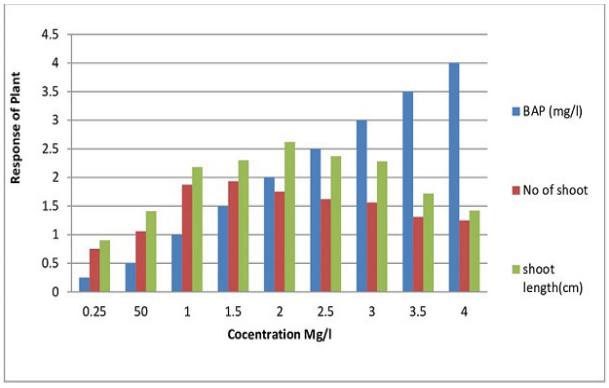

Fig. 5. Number of shoot/explant and shoot length (cm) at different concentration of BAP (mg/L).

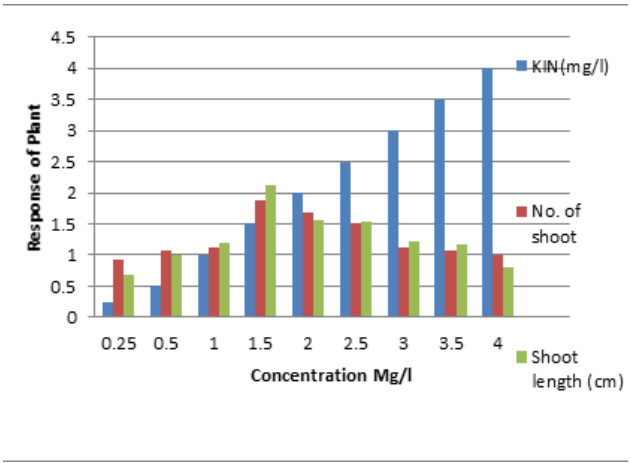

Fig. 6. Number of shoot/explant and shoot length (cm) at different concentration of Kin (mg/L).

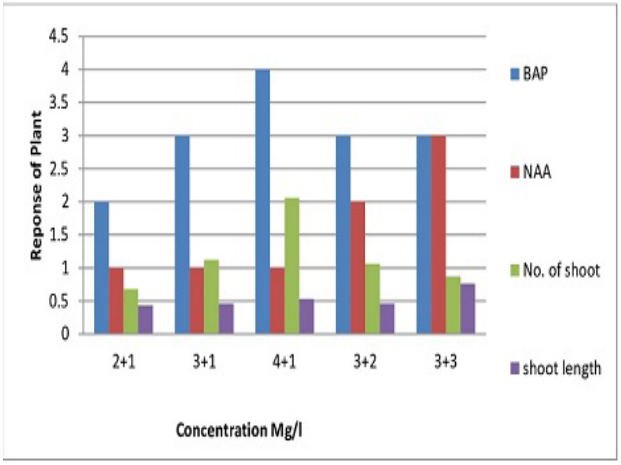

Fig. 7. Number of shoot/explants and shoot length (cm) at different concentration of BAP and NAA (mg/L).

3.3. Shoot multiplication and elongation

The well sprouted axillary bud explants of *Pluchea lanceolata* were transferred to multiplication medium with different concentrations of PGRs after 3 ± 1 weeks of primary culture and observed the various changes in such as height and number of the shoot and its duration of time (Fig. 3-A). The process of multiplication was repeated by sub-culturing after 18 ± 2 days (Fig. 3-B, C, and D). The maximum shoot multiplication and elongation was obtained at the lower concentration of PGRs. The different concentrations of cytokinins BAP (0.25 -1.0) mg/L and KIN (0.25-1.5) mg/L were used in combination but the most favorable combination (0.25 + 0.5) mg/L of BAP and KIN was found optimum for multiplication (Table 3, Fig. 8). The maximum average number of shoots and the highest shoot length were 2.62 ± 1.14 and 5.88 ± 2.44, respectively. The rates of shoot multiplication increased to the fourth subculture with 87.50% survival rate (Table 1). When explants failed to show further shoot growth after the appearance of shoot buds, then a different medium is required for promoting the shoot growth. Therefore, a different medium for the proliferation of shoot buds was prepared, i.e., proliferation medium. Maximum elongation of shoot bud into shoots was achieved on MS medium supplemented with a lower concentration of BAP (0.25 mg/L) and KIN (0.5 mg/L) (Table 3, Fig. 8) termed here as 'shoot elongation medium'. This led to the conclusion that media with lower concentrations of BAP (0.25 mg/L) and KIN (0.5 mg/L) favored the elongation of the shoot. Similar effects of lower concentration of cytokinins on shoot elongation have also been established by previous workers (Gayathri et al., 2009; Uranbey et al., 2010 and Meena et al., 2012). However, Arya and Patni (2013) suggested that higher concentrations promoted shoot multiplication, and lower concentrations promoted shoot elongation (Table 3, Fig. 8).

3.4. Direct shoot induction through axillary bud explant (organogenesis)

The shoot initiation happened after 10 ± 2 days of inoculation of axillary bud on MS medium fortified with different concentrations of PGRs. The best sprouting of axillary bud was obtained on MS medium supplemented

Table 3
Effect of different concentration and combination of BAP and KIN on shoot multiplication and elongation.

BAP (mg/L)	KIN (mg/L)	Average no of shoot Mean ± SD	Shoot length Mean ± SD	No of shoot per culture
0.25	0.25	1.87 ± 1.02	2.8 ± 1.52	1-2
0.25	0.5	2.62 ± 1.14	5.88 ± 2.44	1-3
0.5	0.5	2.43 ± 1.09	3.58 ± 1.85	1-2
1	1.5	2.25 ± 1.06	3.03 ± 1.42	1-2

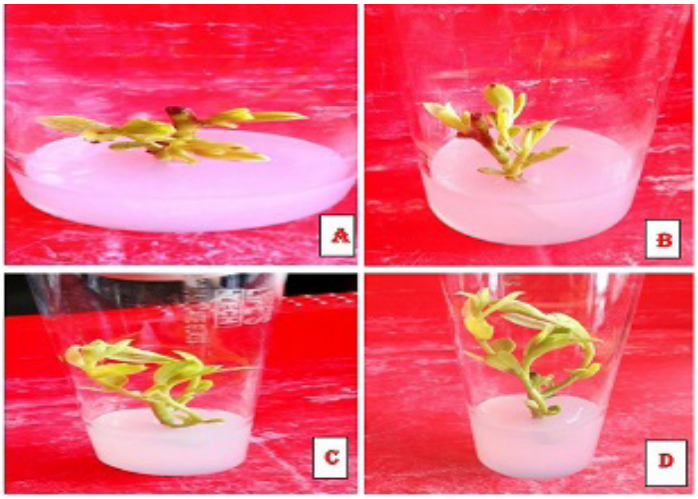

Fig.3. Shoot multiplication in *Pluchea lanceolata*.
A. Multiplication of shoots on multiplication medium.
B. Isolated shoot on elongation medium.
C. Elongated shoot after primary culture.
D. Elongated shoot after secondary culture.

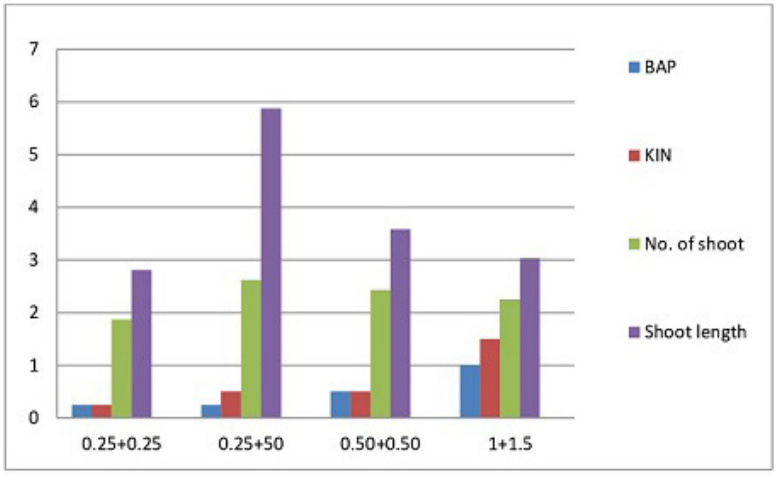

Fig. 8. Number of shoot/explants and shoot length (cm) at different concentration of BAP and Kin (mg/L).

with BAP + NAA (4 mg/L + 1 mg/L) after 2 to 3 weeks. One to four shoots per explant were obtained in MS medium supplemented with 1.5 mg/L BAP (Table 3, Fig. 5). Ullah and Bakht (2016) reported the same hormonal concentrations for *in vitro* micropropagation of *Periploca hydaspidis* where shoots were induced from nodal Shoot explants. Shoot sprouting in their study started within 7-8 days after inoculation. The combination of BAP 4 mg/L and NAA 1 mg/L was found best for the induction of maximum shoot length (6.25 cm) while minimum induction was noted in the combination of BAP 2 mg/L and NAA 0.1 mg/L (1.75 cm).

4. Concluding remarks

The wild populations of *Pluchea lanceolata* (Rasana) are declining at a fast pace and their inability to reproduction through seeds. Therefore, the present study offers a promising approach for *in vitro* multiplication of the species that can be applied for the conservation of this medicinally important species. The present investigation establishes the efficient methods for rapid and successful in vitro propagation that can be employed for maximum shoot induction (1.68 ± 1.07) and shoot length (1.32 ± 0.73) through MS medium supplemented with NAA alone at 2 mg/L and BAP at (1.5 mg/L) concentration showed the optimum shoot induction (1.93 ± 1.38) and shoot length (2.30 ± 1.53) with 1-4 number of shoot per explant, whereas in Kin medium, shoot induction was 1.87 ± 1.50 and shoot length 2.12 ± 1.38 cm. Out of all the separate concentrations of PGRs, BAP concentration is more effective for shoot induction with 75% survival rate. The highest percentage of survival rates (81.25%) was observed in MS medium supplemented with 2 mg/L NAA. The studies also confirmed that application of individual PGR, such as BAP, NAA, and KIN, exerted less effect on shoot induction as compared to the application of a combination of two phytohormones, such as BAP+NAA or BAP+KIN.

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