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FACTORS INFLUENCING *IN VITRO* SEED GERMINATION, MORPHOGENETIC POTENTIAL AND CORRELATION OF SECONDARY METABOLISM WITH TISSUE DEVELOPMENT IN *PRUNELLA VULGARIS* L.

HINA FAZAL[1,2], ZABTA KHAN SHINWARI[2], NISAR AHMAD[3] AND BILAL HAIDER ABBASI[2*]

[1]*Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex, Peshawar 25120, Pakistan*
[2]*Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320 Pakistan*
[4]*Center for Biotechnology and Microbiology, University of Swat, Swat 19200 Pakistan*
[*]*Corresponding author's email: bhabbasi@qau.edu.pk*

Abstract

Plant growth regulators (PGRs), polyamines (PAs) and temperature regimes are the key factors that influence morphogenesis and plant architectural development; however, the understanding that how these factors control plant growth and development is still poor and needs further research in *Prunella vulgaris*. In this study, we monitored the effect of these factors on seed germination, morphogenetic potential and secondary metabolism. Different temperature regimes showed that 25°C is the most suitable temperature for seed germination (88.87±1.76%) on Murashige and Skoog (MS) basal medium. The synergistic combinations of kinetin (Kn), 6-benzyladenine (BA) and putrescine (PUT; 2.0 mg l^{-1}) promoted seed germination (90.22±4.51%) after 24 days of inoculation. A combination of Kn and PUT (1.0 mg l^{-1}) encouraged mean shoot length (11.0±1.95 mm) with the optimum amount of chlorophyll content (23.73±1.8 µg cm^{-2}). However, maximum mean root length (13±0.65 mm) was observed on medium containing Kn and spermidine (SPD, 2.0 mg l^{-1}). Maximum calli (71.56±2.63%) were obtained from root explants on ½ MS-medium containing indole butyric acid (IBA) and α-naphthalene acetic acid (NAA; 0.5 mg l^{-1}). Higher number of shoots (78.5±3.75%) was obtained with Kn and PUT (1.0 mg l^{-1}). IBA concentration of 1.0 mg l^{-1} was found effective for root formation (74.71±3.3%). Moreover, PGRs and PAs have a significant effect on accumulation of total phenolics, flavonoids and DPPH activity. This protocol is helpful for consistent plantlets and prunellin production in *P. vulgaris* L.

Key words: *Prunella vulgaris*; Seed germination; Micropropagation; Antioxidants; Phenolics; Flavonoids.

Introduction

P. vulgaris L. is a thermophilic and hygrophilous perennial herb in Lamiaceae family, commonly known as "self-heal" because of its wound healing property (Fazal *et al.,* 2014; Chen *et al.,* 2013; Rasool *et al.,* 2009). The commercial importance of this species is due to the presence of active components such rosmarinic acid and prunellin (Tabba *et al.,* 1989). In Unani formulations it is commonly used for sore throat, cold and headache (Rasool *et al.,* 2009; Shinwari *et al*., 2006). In Korean traditional medicine it is used to treat goiter, nephritis and oedema. In Chinese medicine it is used as detumescene, antifebrile, sedative, mastitis and to treat thyroid gland malfunction (Huang *et al.,* 2013). Modern *In vitro* and *in vivo* clinical studies suggested that *P. vulgaris* has a wide spectrum of biological effects including anti-viral, anti-tumor, anti-inflammatory, stimulation of immune system and enhancement of production of T-lymphocytes and cytokines (Chen *et al.,* 2013; Liu *et al.,* 2009; Chlopcı´kova *et al.,* 2005; Zdarilova *et al.,* 2009).

P. vulgaris is naturally propagated by stolon and seed. The stolon produces large number of plantlets than seed. Because the seed needs more time to germinate while stolon starts plantlets development, wherever it touches the soil. Moreover, seed provides plants of desirable size within 6 months period. If the seed are cold stratified for 1 month, it will shrink the time period of plantlets development to about 8-10 weeks (Clark & Wilson, 2003). Seed germination in common field significantly changes the antiviral properties. The reduction in such activities is because of the 10-fold decrease in bioactive components, especially rosmarinic acid (Qu & Widrlechner, 2011). To date, the literature on the cultivation and improvement of this species is scarce. The demand and collection of the said species for therapeutic remedies are higher than its production. Most of the existing literature reflects phytochemical profile and biological activities rather than cultivation and improvement of the species (Rasool *et al.,* 2009; Huang *et al.,* 2013; Liu *et al.,* 2009; Qu & Widrlechner, 2011). According to the escalating demand, this plant needs improvement especially for producers and end-users.

Seed germination under controlled and aseptic conditions reduces the time required for production of clones, bypassing harsh conditions and pathogen attacks (Khan *et al.,* 2013; Nikolic *et al.,* 2006). Temperature and light regimes have a significant effect on plant development and phytochemical profile (Abbasi *et al.,* 2011; Shohael *et al.,* 2006; Fazal *et al.,* 2015; Ahmad *et al.,* 2016). Intact *In vitro* plantlets or cells produce equivalent/higher phenolic, flavonoid and antioxidant components to that of the mother plant (Khan *et al.,* 2014). Furthermore, various advantages of seed explants are also proved from the available literature (Malik & Saxena, 1992; Victor *et al.,* 1999). No information is found in literature regarding the seed germination of *P. vulgaris* on MS-medium to date. However, seed germination in soil is widely reported (Zhang *et al.,* 2008; Zhang *et al.,* 2009; Guo *et al.,* 2009a, 2009b, 2009c). In order to obtain healthy biomass, micropropagation is the only approach that provides consistent and identical clones in a shorter period (Ahmed *et al.,* 2011). Micropropagation has proven advantages over conventional seed germination and stem cutting practices (Ahmad *et al.,* 2013a; Aman *et al.,* 2013). The objective of present study was to investigate the effects of different temperature regimes and synergistic combination of polyamines (PAs) and PGRs on *In vitro* seed germination to investigate the correlation of antioxidant activity, total phenolic and flavonoid content with dry biomass and the development of simple indirect regeneration protocol from root explant.

Materials and Methods

Seed collection and sterilization: Seeds of *P. vulgaris* were collected from wild plants in the Madyan valley of District Swat, Pakistan. These seeds were authenticated through taxonomic markers and the voucher specimen (10459) was prepared following Fazal *et al.* (2010) and deposited in Herbarium, Pakistan Council of Scientific and Industrial Research Laboratories Complex, Peshawar (PES) for future reference. The recent method of Ahmad *et al.* (2014) was used for seed sterilization. Seeds were subjected to 0.2% $HgCl_2$ and 70% ethanol for ~2 min and subsequently washed several times with sterile distilled water. Seeds were dried on filter paper before inoculation.

Selection of temperature regimes for germination on PGRs free medium: Surface sterilized seeds were placed on PGRs free Murashige and Skoog, (1962) [MS] medium containing 30 g l^{-1} sucrose and then solidified with 7-8 g l^{-1} agar. Before autoclaving at 121°C for 25 min, the pH was adjusted to 5.3. Inoculated flasks were placed in growth chambers at different temperature regimes (15°, 20°, 25° and 30°C) in 16 h photoperiod with light intensity of 40-mol $m^{-2}s^{-1}$ for a period of 30 days. Data on percent seed germination was recorded with 3 days interval for a period of 30 days.

Germination on PAs and PGRs augmented medium, and biomass accumulation: In another set of experiments; 16 treatments of PGRs were applied for seed germination. *T*1 (1.0 BA+PUT), *T*2 (2.0 BA+PUT), *T*3 (1.0 Kn+PUT), *T*4 (2.0 Kn+PUT), *T*5 (1.0 BA+SPD), *T*6 (2.0 BA+ SPD), *T*7 (1.0 Kn+SPD), *T*8 (2.0 Kn+SPD), *T*9 (1.0 BA+Kn+PUT), *T*10 (2.0 BA+Kn+PUT), *T*11 (1.0 GA_3+Kn+PUT), *T*12 (2.0 GA_3+Kn+PUT), *T*13 (1.0 BA+Kn+SPD), *T*14 (2.0 BA+Kn+SPD), *T*15 (1.0 GA_3+Kn+SPD), *T*16 (1.0 GA_3+Kn+SPD) and MS0. These cultures were maintained at 25±2°C in 16 h photoperiod with light intensity of 40-mol $m^{-2}s^{-1}$. Data on % seed germination, days to germination, mean shoot length, chlorophyll content and mean root length was recorded after 4-6 weeks of inoculation.

For fresh biomass (FB) determination, plantlets were collected from jars. Solid media particles and surface water were carefully removed by using autoclaved filter paper (Whatman Ltd., England) and finally weighed (Sortorious digital balance; Germany). Similarly, for dry biomass (DB), plantlets were collected and dried in an oven at 60˚C and finally weighed (Thermo Scientific; Germany).

Micropropagation: For micropropagation, root explants were collected from seed derived plantlets of *P. vulgaris*. These root sections (1.5 cm) were placed on ½ strength MS-medium augmented with different combinations of IBA and NAA (0.1-1.0 mg l^{-1}) for callus induction. Data on callus color, morphology and % callus formation was collected after 30 days of inoculation. Fresh callus was shifted to fresh medium for shoot organogenesis containing combination of BA with PUT (1.0-2.0 mg l^{-1}), Kn either with PUT (1.0-2.0 mg l^{-1}) or SPD (1.0-2.0 mg l^{-1}) or Kn and BA with PUT or SPD (1.0-2.0 mg l^{-1}). Data regarding different growth parameters was collected after 4-5 weeks of inoculation. Suitable shoots were carefully transferred to the rooting medium containing NAA (0.5-1.0 mg l^{-1}) or IBA (0.5-1.0 mg l^{-1}) or half and full MS-medium containing a combination of IBA and NAA (0.5-1.0 mg l^{-1}). After 5 weeks, rooted shoots were shifted to plastic pots for acclimatization.

Analytical methods: The DB of plantlets was grinded in mortar and pestle for extract preparation. Total phenolic content in each treated sample was determined by using the method of Ahmad *et al.* (2014). Briefly, 0.1 ml (2N) Folin-Ciocalteus reagent was mixed with 0.03 ml extract and 2.55 ml sterile distilled water. Before incubation for 6 min, the mixture was centrifuged (10,000 rpm; 14 min) and then filtered through 45 µm membrane in UV visible spectrophotometer (Shimadzu-1650; Japan) cuvette. The absorbance of resulted mixture was measured at 760 nm. Gallic acid (Sigma; 1.0-10 mg/ml; $R^2 = 0.9878$) was used for plotting standard calibration curve. Results as Gallic acid equivalent (GAE) mg/g of DB were obtained from % TPC by using the following equation.

$$\% \text{ total phenolic content} = 100 \times (A_S\text{-}A_B)/ (C_F \times D_F)$$

where A_S is the absorbance of the sample and A_B is absorbance of blank. C_F is the conversion factor from standard curve and D_F is the dilution factor.

The TFC in plantlets DB was determined following Ahmad *et al.* (2014). Methanolic extract (0.25 ml) of the treated samples was mixed with sterile distilled water (1.25 ml) and 0.075 ml 5% (w/v) $AlCl_3$. Before incubation (5 min) and centrifugation (10,000 rpm; 14 min), the solution was mixed with 0.5 ml of NaOH (1 M). The absorbance was checked at 510 nm with a UV-visible spectrophotometer (Shimadzu-1650PC, Japan). Rutin (Sigma; 1.0-10 mg/ml; $R^2 = 0.9866$) was used for plotting standard calibration curve. The total flavonoid content was expressed as rutin equivalent (RE) mg/g-DB of extracts

DPPH radical scavenging (DRSA) in germinated plantlets was determined according to the method of Ahmad *et al.* (2013b) with little modifications. Briefly, methanolic extract (1.0 ml; 5 mg/20 ml) of each sample was mixed with 2.0 ml of DPPH free radical solution (0.3 mg/20 ml ×4). The mixture was incubated in the dark for approximately 30 min. The absorbance of resulted mixture was measured at 517 nm at room temperature by using UV-visible spectrophotometer model (Shimadzu-1650PC, Japan). Finally the radical scavenging activity was calculated as percentage of DPPH discoloration using the following equation;

$$\text{DRSA (\%)} = 100 \times (1 - A_P/A_D)$$

where A_P represents absorbance of plantlets extract at 517 nm and A_D is the absorbance of the DPPH solution without tissue extract

Statistical analysis: Analysis of replicated mean values, standard errors (±), and least significant difference (LSD) were carried out by using Statistix software (8.1 versions) and Origin Lab (8.5) software was used for graphical presentation.

Results and Discussion

Seed response to temperature regimes: In the present study, different temperature regimes (15°, 20°, 25° and 30ºC) were applied for *In vitro* seed germination on PGRs free MS-medium. The cultures of *P. vulgaris* were maintained under constant photoperiod (16L/8D h). Data on % germination was recorded with 3 days interval for 30 days period. The germination started on 3rd day and % germination increased with increasing number of days. Among different temperature regimes, 25ºC was found optimum (88.87±1.76%) for *In vitro* seed germination (Fig. 1). The current results are consistent with the observations of Khan and Gulzar (2003) that optimum temperature for perennial plants lays between 20°-30°C. Highest (30ºC) and lowest (15ºC) temperature minimized % germination (38.10±2.5% and 66.67±3.33%). Qu *et al.* (2008) observed that temperature increment after 30°C significantly reduced germination response in *Halocnemum strobilaceum***.** However, 20 ºC induced 70.83±2.16% germination on PGRs free medium. Contrary to our data, Yogeesha *et al.* (2007) observed that 20°C is the suitable temperature for *Carica papaya* seed germination. According to these results, 25°C is the optimum temperature for consistent plantlets production. Therefore, it is suggested that *P. vulgaris* seeds should be favorably sown in the field during the month of April in such areas, where the forecasted temperature is below 30°C.

Effects of synergistic combination of PAs and PGRs on germination parameters: Highest germination (90.2±5.58%) was observed in *T*10 after 24 days of inoculation (Fig. 2). It showed that higher concentrations of Kn, BA and PUT (2.0 mg l^{-1}) significantly enhanced the germination percentage. However, *T*3 showed 86.8±7.08% germination in reduced time period (15 days) than *T*10 and control (30.5±5.58%; 25 days). There is a paucity of information about the synergistic role of PAs and PGRs on *P. vulgaris* seed germination. Afzal *et al.* (2009) concluded from their experiments that synergistic combination of PAs, improved seed germination in tomato. Farooq *et al.* (2008) observed that treatment of *Oryza sativa* seeds with 10 ppm PUT significantly enhanced germination parameters. Similarly, Sorkheb *et al.* (2011) reported that PAs promoted pollen germination and pollen tube elongation.

In the present study, maximum mean shoot length of 11.0±1.95 mm and 11.0±0.81mm was observed in *T*3 and *T*12 seedlings as compared to MS0 (30.5±2.77 mm; Fig. 3). However, the chlorophyll content was higher in *T*3 (23.73±1.8 µg cm^{-2}) than *T*12 (19.49±1.38 µg cm^{-2}) and control (10.93±0.63 µg cm^{-2}). From the current results, it is proved that PUT augmented media not only encouraged shoot length but also enhanced chlorophyll content (Fig 3). The cross talk of PAs with PGRs has never been analyzed in *P. vulgaris* for mean shoot and root length. However, the effect of a similar set of PGRs on root and shoot development is widely reported in other plant species (Ahmad *et al.,* 2013a; Khan *et al.,* 2013). As compared to shoot length, maximum mean root length (13±0.65 mm) was observed in *T*8 seedlings in response to the synergistic combination of Kn and SPD (2.0 mg l^{-1}; Fig. 3). Wu *et al.* (2010) observed that the addition of SPD alone to the medium did not show improvement in root growth of *Poncirus trifoliate.* A comparative response was also reported by Hausman *et al.* (1995). The addition of exogenous SPD suppressed the endogenous PUT level and increased the production of SPD and SPM (Jia *et al.,* 2010). In the current study combination of Kn and SPD were used to enhance root growth.

Development of an efficient micropropagation system: In the present investigation, an efficient indirect regeneration system *via* callus culture from root explants in *P. vulgaris* was developed (Fig. 4). The reason for this system development is because of the fact that this species is inching rapidly towards extinction due to overexploitation for domestic and marketable drug preparations.

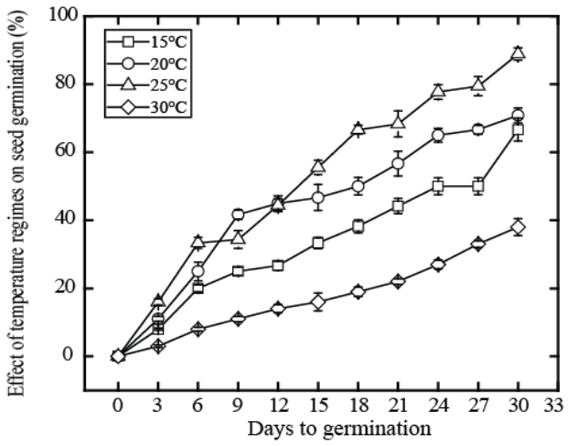

Fig. 1. Effects of temperature regimes (15°, 20°, 25° and 30ºC) on *In vitro* seed germination on PGRs free medium in *P. vulgaris* L. Data was collected after 3 days intervals from three independent experiments. Mean values (n = 3; ± S.E) are significantly different at $p<0.05$.

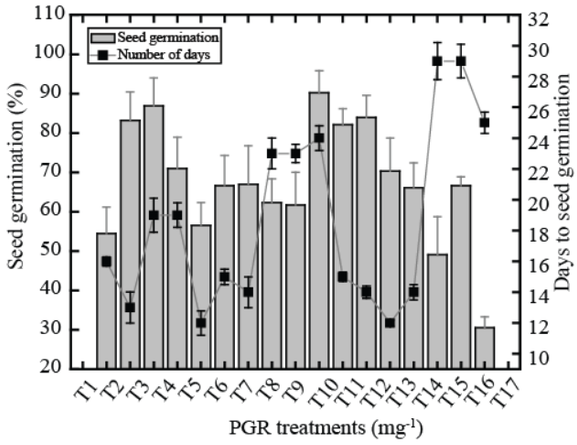

Fig. 2. Effects of synergistic combinations of putrescine and spermidine with PGRs on *In vitro* seed germination in *P. vulgaris* L. Data was collected from three independent experiments. Mean values (n = 3; ± S.E) are significantly different at $p<0.05$.

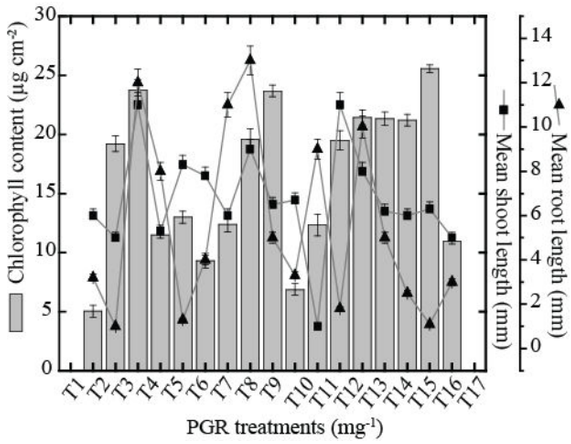

Fig. 3. Correlation of mean shoot length with chlorophyll content and mean root length on medium containing combination of PAs and PGRs. Values are mean of three independent replicates (n = 3; ± S.E). Mean values are significantly different at p<0.05

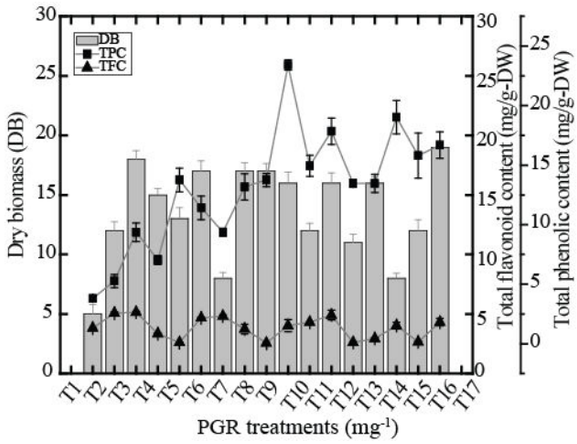

Fig. 5. Correlation of total phenolic (GAE mg/g-DB) and flavonoid content (RE mg/g-DB) with dry biomass in regenerated plantlets of *P. vulgaris* in response to synergistic combination of PAs and other PGRs. Data was collected from 3 independent experiments. Mean values with standard errors (n = 3; ±) are significantly different at $p<0.05$.

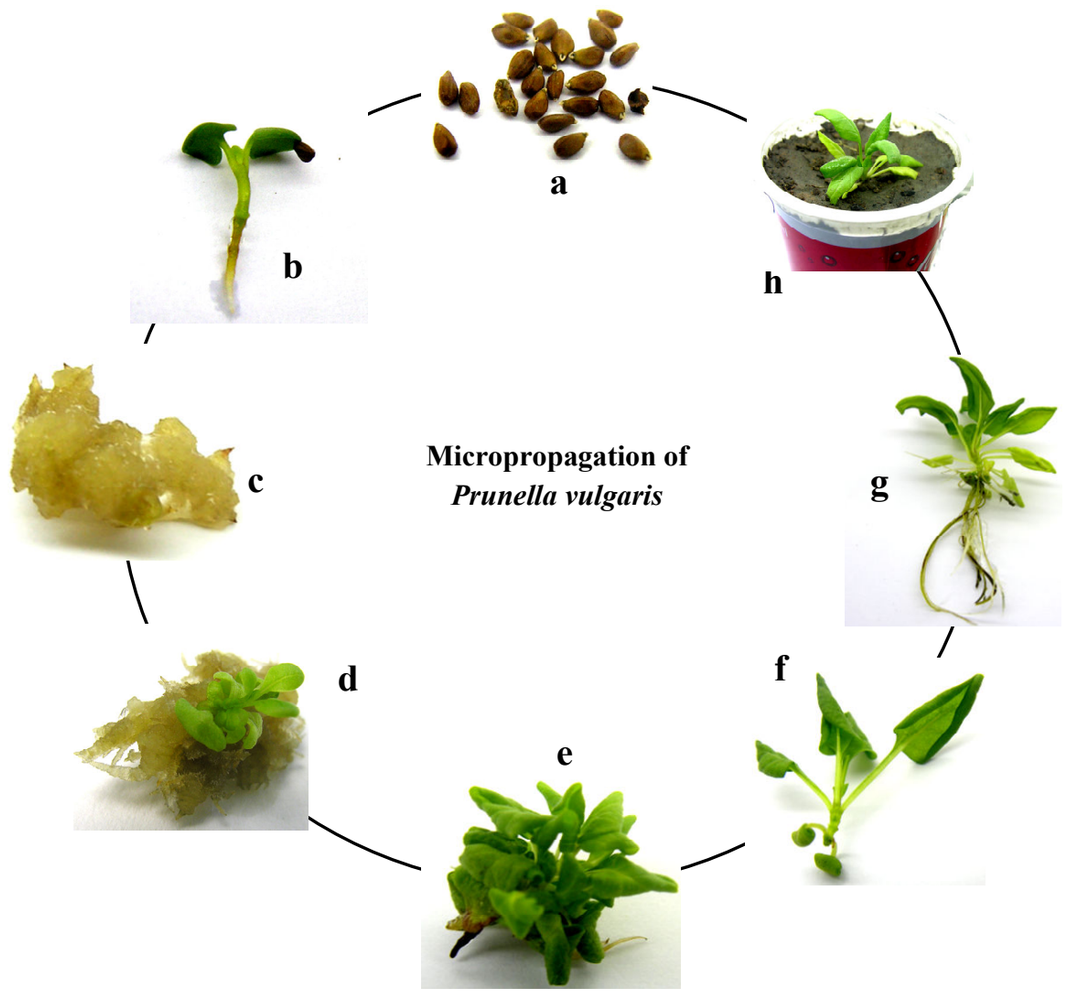

Fig. 4. Micropropagation of *Prunella vulgaris* (a) Seeds of *P. vulgaris* collected from wild place of Madyan, Swat (b) germinated seedling (c) callus formation from root explants (d) shoot regeneration from callus cultures (e) shoot multiplication (f) shoot elongation (g) rooted shoot and (h) acclimatization of *In vitro* regenerated plantlet in cup containing soil; sand and manure.

During present experiment, best callus response (71.56± 2.63%) was observed on ½ strength MS-media containing IBA and NAA using root explant (*T*3; 0.5 mg l^{-1}). Lower (*T*1 and *T*2) (*T*4 and *T*5) and higher concentrations of IBA + NAA produced lower amount of callus (Table 1). No callus induction was observed on MS0. However, callus morphology and callus color were found same for all combinations. Transparent friable callus was obtained in all treatments, which is helpful in the establishment of cell suspension culture. To date, reports on callus production in *P. vulgaris* are scarce. Recently, Kour *et al.* (2014) followed the indirect regeneration protocol for *P. vulgaris* and developed indirect regeneration system through callus culture. Zamir *et al.* (2012) reported that combination of IBA + NAA (0.5 mg l^{-1}) with other auxin induced more than 70% callus in *Saccharum officinarum* L. More recently, Sen *et al.* (2014) concluded that NAA with other auxin significantly influenced callus response in *Achyranthes aspera*. Bucchini & Ricci (2013) recorded 72% callus in *Inula crithmoides* on NAA and 2, 4-D augmented medium. Furthermore, Cheruvathur & Thomas (2013) recorded higher callus induction in *Rhinacanthus nasutus* root explants on Kn plus IBA augmented medium.

Friable and transparent callus was inoculated on fresh medium containing different treatments for shoot organogenesis. Here, we observed maximum shoot formation (78.5±3.75%) in *T*3 and *T*4 (75.0±2.88%) containing combinations of Kn and PUT (Table 2). Addition of Kn and SPD to medium also showed better response than other combinations (Kn+BA+PUT or SPD) and control. Moreover, these treatments also exhibited maximum mean shoot length of 5.30±0.41 (*T*3) and 4.70±0.1 (*T*6). Furthermore, highest shoot number (18.0±1.15; 17.0±1.15) was recorded in *T*7 and *T*8. However, other treatments showed the average number of shoot production (Table 2). We noticed that, *T*3 and *T*4 promoted shoot regeneration but for multiplication, BA is essential as observed in *T*7 and *T*8. Turker *et al.* (2009) also observed that the addition of BA to the medium enhanced shoot multiplication in *P. vulgaris*. Rasool *et al.* (2009) obtained 15±0.4 shoots on medium containing 15 µM BA. They followed the direct regeneration system rather than through callus cultures. In this study maximum rooting (74.71±3.30%) with 3.70±3.81cm mean length and 7.90±1.67 roots per plantlet was observed in *T*4 (Table 3). The current results are consistent with those reported by Turker *et al.* (2009). Successful acclimation was observed after 21 days in pots containing a combination of soil, sand and manure (2:1:1).

Table 1. Effects of different concentrations (0.1-1.0 mg l^{-1}) of IBA and NAA in half MS medium on % callus induction, callus morphology and callus color in *P. vulgaris*. Data on each parameter was collected from 3 independent experiments (*n* = 3).

Callogenesis				
Treatments	**MS+PGRs (mg l^{-1})**	**Callus formation (%)**	**Callus morphology**	**Callus color**
*T*1	½ MS + IBA + NAA (0.1)	26.30 ± 0.75d*	Friable, transparent	Half-white
*T*2	½ MS + IBA + NAA (0.3)	56.08 ± 3.51c	Friable, transparent	Half- white
*T*3	½ MS + IBA + NAA (0.5)	71.56 ± 2.63a	Friable, transparent	Half- white
*T*4	½ MS + IBA + NAA (0.7)	63.80 ± 2.19b	Friable, transparent	Half- white
*T*5	½ MS + IBA + NAA (1.0)	53.79 ± 2.19c	Friable, transparent	Half- white
*T*6	½ MS0	0.00e	NA	NA

* Mean values (± S.E) with common superscripts are significantly different at $p<0.05$

Table 2. Effects of polyamines (PUT and SPD) and PGRs (BA and Kn) on % shoot formation, mean shoot length and number of shoots per explant in *P. vulgaris*. Data on each parameter was collected from 3 independent experiments (*n* = 3).

Shoot organogenesis				
Treatments	**MS+PGRs (mg l^{-1})**	**Shoot formation (%)**	**Average shoot length (cm)**	**Number of shoots per explant**
*T*1	BA (1) + PUT (1)	28.0 ± 4.04e*	1.08 ± 0.53cd	10.03 ± 0.27cd
*T*2	BA (2) + PUT (2)	34.0 ± 3.46e	1.20 ± 0.46cd	11.30 ± 0.12cd
*T*3	Kn (1) + PUT (1)	78.5 ± 3.75a	5.30 ± 0.41a	12.30 ± 0.46c
*T*4	Kn (2) + PUT (2)	75.0 ± 2.88a	4.20 ± 0.46b	11.80 ± 0.17cd
*T*5	Kn (1) + SPD (1)	65.0 ± 1.73bc	3.80 ± 0.12b	10.90 ± 0.23cd
*T*6	Kn (2) + SPD (2)	73.0 ± 8.66ab	4.70 ± 0.17ab	10.30 ± 0.17cd
*T*7	Kn (1) + BA (1) + PUT (1)	58.0 ± 2.31cd	1.80 ± 0.14cd	17.00 ± 1.15ab
*T*8	Kn (2) + BA (2) + PUT (2)	61.0 ± 2.31cd	2.10 ± 0.64c	18.00 ± 1.15a
*T*9	Kn (1) + BA (1) + SPD (1)	53.0 ± 4.04d	1.30 ± 0.17cd	15.00 ± 0.57c
*T*10	Kn (2) + BA (2) + SPD (2)	57.0 ± 3.46cd	1.17 ± 0.48cd	15.50 ± 0.29bc
*T*11	MS0	10.0 ± 1.15f	1.00 ± 0.46d	5.20 ± 0.46e

* Mean values (± S.E) with common superscripts are significantly different at $p<0.05$

Table 3. Effect of NAA alone (0.5-1.0 mg l^{-1}) or IBA alone (0.5-1.0 mg l^{-1}) or combination of NAA and IBA in half or full MS-medium on root induction (%), mean root length and number of roots per plantlet in *P. vulgaris*. Data on each parameter was collected from 3 independent experiments (n = 3).

	Root organogenesis			
Treatments	**MS+PGRs (mg l^{-1})**	**Roots formation (%)**	**Mean root length (cm)**	**Number of roots/ Plantlet**
*T*1	NAA (0.5)	32.30 ± 2.14f*	2.10 ± 1.04c	3.40 ± 0.23e
*T*2	NAA (1.0)	45.04 ± 2.91e	2.60 ± 1.91abc	3.70 ± 0.40e
*T*3	IBA (0.5)	63.29 ± 4.21b	3.50 ± 3.46ab	6.10 ± 0.64b
*T*4	IBA (1.0)	74.71 ± 3.30a	3.70 ± 3.81a	7.90 ± 1.67a
*T*5	Full MS + IBA + NAA (0.5)	55.34 ± 3.08cd	2.70 ± 2.08abc	5.20 ± 0.12bcd
*T*6	Full MS + IBA + NAA (1.0)	61.75 ± 2.74bc	2.80 ± 2.25abc	5.60 ± 0.35bc
*T*7	½ MS + IBA + NAA (0.5)	48.37 ± 5.41de	2.20 ± 1.21c	4.30 ± 0.75cde
*T*8	½ MS + IBA + NAA (1.0)	58.06 ± 4.65bc	2.50 ± 1.73bc	3.90 ± 0.52de
*T*9	MS0	0.00g	0.00d	0.00f

* Mean values (± S.E) with common superscripts are significantly different at $p<0.05$

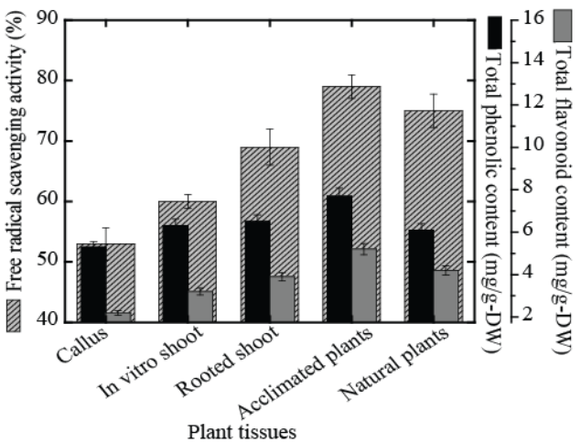

Fig. 6. Correlation of total phenolic (GAE mg/g-DB) and flavonoid content (RE mg/g-DB) with free radical scavenging activity in regenerated tissues in *P. vulgaris*. Data was collected from 3 independent experiments. Mean values with standard errors (n = 3; ±) are significantly different at $p<0.05$.

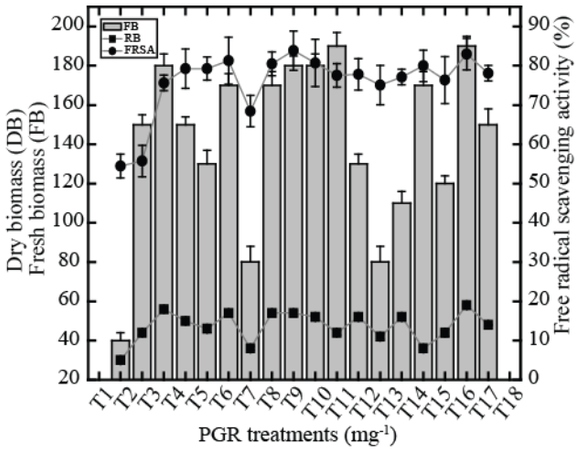

Fig. 7. Correlation of antioxidant activity (DRSA) with fresh and dry biomass in response to synergistic combination of PAs and other PGRs. Data was collected from 3 independent experiments. Mean values with standard errors (n = 3; ±) are significantly different at $p<0.05$.

Production of secondary metabolites: Highest TPC was observed in *T*10 (23.415 GAE mg/g-DB) with DB of 16.01g (Fig. 5). However, *T*3 produced maximum DB (18.0g) but the TPC (13.75 GAE mg/g-DB) was lower than *T*10. It is concluded from these results that the phenolic content in *P. vulgaris* culture varies with the application of PGRs rather than biomass. A similar production of TPC is widely reported in different *In vitro* cultures of *Artemisia absinthium*, *Thymus lotocephalu*s, *Piper nigrum* and 3 endemic species of *Ephedra* (Ali *et al.,* 2013; Ahmad *et al.,* 2014; Costa *et al.,* 2012; Parsaeimehr *et al.,* 2010). In contrast, maximum TFC (2.634 RE mg/g-DB) and DB (18.0g) were observed in *T*3 as compared to other treatments (Fig. 5). However, other treatments did not show linear correlation with biomass accumulation. TPC and TFC were not found to be strictly growth dependent. The current observations are consistent with the results of Ali *et al.* (2013) that production of phenolics and flavonoids in cell culture of *A. absinthium* are not restricted to biomass accumulation. In contrast, a strong correlation of TPC and TFC production with biomass accumulation existed in micropropagated tissues as compared to control. These secondary metabolites increased from calli to acclimated plantlets (Fig. 6). Similar increments in secondary metabolites were also reported by Ahmad *et al.,* (2013b, 2014) in *Piper* regenerated tissues.

Correlation of antioxidant activity with biomass accumulation: In the present experiment, DRSA did not show positive correlation with biomass accumulation. Like TPC and TFC, DRSA was found to be strictly PGRs dependent. Here, maximum DRSA (83.86%) was observed in *T*9 (Fig. 7). Ali *et al.* (2013) also reported that DPPH free radical scavenging activity is independent of biomass accumulation in *Artemisia absinthium*. Dependency of DRSA on PGRs is widely available in previous reports (Khan *et al.,* 2013; Ahmad *et al.,* 2013a,

2014). Contrary to the results of Ali *et al.* (2013) who reported linear correlation of DRSA with TPC and TFC, no linear correlation was found in this study. In contrast to seed germination, a strong correlation of TPC and TFC with DRSA existed in various regenerated tissues of *P. vulgaris* (Fig. 6). DRSA is not restricted to specific antioxidant component, solid or liquid samples but used for the overall antioxidant capacity of the samples. In some cases, *In vitro* cultures showed higher DRSA than synthetic butylated hydroxytoluene antioxidant agent (Güllüce *et al.,* 2003). Therefore, intact plant or cell growth under *In vitro* conditions are widely exploited for such bioactive components production that are either difficult to synthesize or are produced in limited quantities in wild plants (Ali *et al.,* 2013).

Conclusion

In conclusion, through these studies *In vitro* seed germination and micropropagation systems were optimized for *P. vulgaris*. According to current results, it is essential to germinate seed in the field condition at 25 ºC. The synergistic combination of PUT and SPD with Kn is very useful for *In vitro* seed germination. The micropropagation system established here not only helps in consistent plantlets production but also in transformation for genetic improvement and production of promising secondary metabolites including anti-HIV prunellin. High level of TPC, TFC and natural antioxidants are useful for commercial production in bioreactor culture.

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