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IN VITRO CONSERVATION OF *BACOPA MONNIERI* GERMPLASM : A CRITICALLY ENDANGERED MEDICINAL PLANT OF MADHYA PRADESH

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Bacopa monnieri (L) is an endangered important medicinal plant of Madhya Pradesh, India. In the present study, wild accessions of *Bacopa monnieri* (L.) were collected from 14 (BMG1-14) out of 30 districts visited in Madhya Pradesh, between years 2008-2009. The bioactive molecule, Bacoside-A content among the accessions, analyzed by HPLC method, varied from 3.5mg/g (BMG-11) - 13.2mg/g (BMG-3) dry powder. Leaf explants cultured on different levels of BAP (2.22µM-35.5µM) showed indirect organogenesis while on Kinetin (2.33µM- 37.2µM) direct organogenesis. Plant regeneration was achieved on 2.22µM BAP and all the Kn supplemented media. Maximum number of shoots per explant and high percentage of plant regeneration was recorded on 13.95µM Kn. I*n vitro* plant regeneration from leaf explants of 14 accessions (BMG1-14) was observed within 30-40 days on MS +13.95µM/l Kn +2% suc~rose medium. Regeneration response varied between 26 to 94%, with maximum response from BMG-4 and the lowest in BMG-14. Regenerated plants were successfully transferred to field conditions. Thus , the study shows successful collection and *in vitro* conservation of *Bacopa monneiri* germplasm of MP.

Key words- *Bacopa monnieri, Bacoside-A, in vitro* conservation, Madhya Pradesh, Threatened plant.

Bacopa monnieri (L.), commonly known as Brahmi or Jalneem, belonging to family Scrophulariaceae, is a glabrous, somewhat succulent, creeping herb. Brahmi is astringent, bitter, cooling and is reported to improve the intellect. It is used in the indigenous system of medicine for the treatment of asthma, hoarseness, insanity. *Bacopa* has cognitive benefits, specifically its ability to improve memory, learning and concentration (Joshi and Parle 2006, Stough *et al*. 2001). *Bacopa* also possesses antidiabetic, antioxidant, neuroprotective and hepatoprotective activity (Singh and Dhavan, 1997, Tripathi *et al*. 1996, Singh *et al.* 2006, Ghosh *et al.* 2006, 2007, 2007, Kapoor *et al*. 2009). Pharmacological properties of *Bacopa monnieri* are due to the presence of characteristic saponin called "Bacoside".

According to the report of National Medicinal Plant Board- NMPB, (2007), the annual market demand for brahmi during 2000 was around 1,000 tonnes. *B. monnieri* is now placed second in a priority species list of the most important Indian medicinal plants (Rajani 2008) and indicated as one among 32 medicinal plants identified for cultivation and conservation. The plant has been identified as one among the 7 important medicinal plants recommended for immediate attention and included in the list as a highly endangered medicinal plants in India (Ramesh *et al*. 2011).

Bacopa monnieri has also been included in threatened plant category in Madhya Pradesh (FRLHT report 2006). It is because of the conventional propagation of *Bacopa* via seed/vegetative propagation is slow and hindered due to seedling death at two leaved stage and specific habitat requirement (Sharma *et al*. 2007). Over exploitation of *Bacopa* has also been done because of its high medicinal value. Therefore, *in vitro* propagation method may be promising option for multiplication and germplasm conservation of *Bacopa monnieri.*

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In the last few decades various workers reported on micropropagation of *Bacopa* (Thakur *et al*. 1976, Thakur and Ganapathi 1978, Thejavathi, 2001, Tejovathi *et al*. 2011, Chaplot *et al*. 2005, Eascandon *et al*. 2006, Sharma *et al.* 2007). However, collection and conservation of *Bacopa monnieri* germplasm from different geographical regions of India are limited (Darokar *et al*. 2001, Khanuja *et al*. 1997, Karthikeyan *et al*. 2011, Mathur *et al*. 2003). Present study reports *in vitro* conservation of *Bacopa* germplasm of Madhya Pradesh.

MATERIAL AND METHODS

Germplasm Collection and Maintenance

Bacopa monnieri is one of the threatened medicinal plants of Madhya Pradesh (FRLHT, 2006, IUCN 2010), grows in dam marshy areas, near boundaries of water bodies. Between 2008- 2009, we systematically visited various districts of MP looking for the *Bacopa* germplasm. Branches with roots from each site were collected along with soil and carefully brought to the college in polythene bags. Each accession was numbered as- BMG 1, 2, 3 etc. in the order of collection. Part of the plant material was maintained in the green house and the remaining was used for HPLC analysis.

Analysis of Bacoside A content by HPLC and HPTLC

The Baciside A content in all the 14 accessions was analysed on Shimadzu HPLC system, at CIMAP, Lucknow, and HPTLC at MPCST, Bhopal as per the procedure standardized by Gupta *et al*. (1998).

Tissue culture studies

Conventional propagation of *Bacopa monnieri* is very slow. *In vitro* multiplication therefore, is the only way for large scale propagation and conservation of *Bacopa monnieri* germplasm.

Leaves of accession BMG-1 were surface sterilized and cultured according to the procedure given by Tejovathi *et al*. (2011). About 0.5-1.0 cm size leaf explants were inoculated on Murashige and Skoog's (MS) (1962) medium supplemented with 2% sucrose. Various concentrations of 6-benzylamino purine (BAP) (2.22µM-35.5µM) and Kinetin (Kn) (2.33 µM/l- 37.20 µM/l) were tested for their influence on organogenesis. Agar-Agar Type-I (HiMedia, India) was used (0.8%) as gelling agent in all experiments.

In vitro conservation of *Bacopa monnieri germplasm*

The leaves of all the accessions collected from various regions of Madhya Pradesh were inoculated on MS medium supplemented with 13.95µM /l Kn. Regenerated plantlets of the accessions were initially transferred to the filter paper (Whatman's filter paper No. 1) wick culture for better root development. After 1-2 weeks, plants were transferred to the thermo-coal cups or root trainers containing mixture of autoclaved soil and manure in the ratio of 1:1. Plants were then covered with the polythene bags and kept in the culture room at 25±1ºC under cool white fluorescent light of 2000 Lux. Regenerated plants were watered daily with tap water and polythene covers were gradually removed. Finally the established *in vitro* regenerated plants were transfer to the green house for hardening.

All the experiments were repeated thrice with 30 replicates per experiment. The data was recorded as- percentage response, no. of plantlets/ explant and the influence of genotype on plant regeneration from 4 week old cultures.

RESULTS AND DISCUSSION

In the present study, about 30 districts of MP were covered between the years 2008-09 and wild accessions were available only at 14 regions (Figure-1). All the germplasm accessions collected from various regions were brought and maintained in the net house at Boston College Gwalior. Each germplasm

accession was assigned with a code (BMG 1-14), according to the sequence of collection (Figure-1).

Bacopa is one among the 7 important medicinal plants recommended for immediate attention and included in the list of "highly endangered medicinal plant in India" by NMPB (National Medicinal Plant Board) and Technology Information Forecasting and Assessment Council (TIFAC), Department of Science and Technology, Government of India (http://www.nmpb.nic.in/prioritisemedicinalplants.htm) and also by the Madhya Pradesh state. In India, collection and conservation of *Bacopa* germplasm was initiated since past few decades. Limited reports are available in literature on the conservation of germplasm and its diversity studies. Mathur *et al*. (2003) collected 27 accessions of *Bacopa monnieri* from various geographically distinct locations in India and estimated Bacoside-A content among the accessions, which ranged from 0.98% to 1.93%. Similarly, Darokar *et al.* (2001) evaluated 23 indigenous wild germplasm collections from different geographic regions of India and one exotic collection from Malaysia and evaluated Bacoside-A content among them. However, the central state of India (MP) was almost untouched. Probably ours is the first attempt for collection, conservation and analysis of the genetic diversity of *Bacopa monnieri* from different regions of Madhya Pradesh. Among 14 accessions collected from MP the content of Bacoside -A varied between 3.5 mg/g -13.2 mg/g dry power (Figure 2). The accession with highest Bacoside- A content was BMG-3 (Katni) having 13.2 mg per gram dry powder followed by BMG-2 (Chitrakoot). While the accession with lowest Bacoside-A content was BMG-11 (Bhopal sp) with 3.5 mg per gram dry powder (Figure-2).

Tissue culture studies

Development of shoots from callus was observed within 20 days after explant culture. About 18-79% explants showed adventitious shoots on 2.22-22.22µM BAP supplemented MS medium, while at higher concentrations no shoots were developed (Table.1). Among the various concentrations tested, frequency of shoot inducing response was highest on the lowest concentration (2.22µM) of BAP (Figure-3a). Numerous shoot buds were developed on BAP supplemented media with size varying from 0.5-4.0 cm long (Figure 3b). All shoot buds however could not grow and elongate properly. Rooting and well developed of plantlets were recorded only on 2.22µM BAP medium. None of the other concentration tested showed root initiation (Table.1). Plantlets regenerated on BAP supplemented medium were weak and stunted, with size varying from 0.5- 4.0cm. Although high frequency of shoot initiation was observed on BAP medium only 13 plantlets/ explant were recovered within one month. Sub culturing on to basal medium was required for the shoots of BAP supplemented medium for rooting and plantlet regeneration.

Direct organogenesis i.e. adventitious shoots induction was the common phenomenon observed on all kinetin supplemented media within 12-15 days. This was followed by rhizogenesis at the base of the shoot bud within 15-18 days of inoculation (Figure 3c). No callus initiation was observed on any of the Kn supplemented media . The highest percentage of shoot inducing (76.38%) and plant regeneration (65.5%) response was recorded on 13.95µM Kn followed by 11.62 µM Kn (72.38% and 55.5% respectively) while least observed on 34.88 µM Kn (Table.1). The shoots and the plants developed on Kn supplemented medium were more vigorous. The size of the shoots in 4 weeks old cultures measured 3.0-4.0cm with internodes size varying from 0.5-1.0cm. Some of the shoots developed anthocynin pigment both in root and shoots.

6- Benzylaminopurine and Kinetin are widely

Figure:1 Map of Madhya Pradesh showing the districts of germplasm collection and their accession numbers

used either alone or in combinations with auxin for the shoot initiation and plantlet regeneration for different medicinal plants (Vincent *et a.,* 1992, Bhuyan 1997, Jayakumari and Jayabalan 2002, Nagori and Purohit 2004, Martin and Madassery 2005*,* Rai *et al*. 2009). Differential response of *Bacopa* to different levels of BAP has been reported earlier. Tiwari *et al*. (1998, 2001), observed shoot, root and callus development from 2.22-6.67µM BAP supplemented media, while Sharma *et al* (2007) reported shoot and root induction on 0.2-2.0mg/l BAP. Shrivastava and Rajani (1999), Tiwari *et al* (2001) and Mohpatra and Rath (2005) obtained shoots alone on 1.0-6.0µM, 0.44-22.2µM and, 1.0-5.0 mg/l BAP respectively. In the present study we obtained callus, shoots and roots on 2.22µM BAP, callus and shoots on 4.4 - 22.22µM and only callus on 24.44 - 35.5 µM BAP. Our results show similarity with earlier studies (Shrivastava and Rajani 1999, Tiwari *et al.* 2001 and Mohpatra and Rath 2005) but differed with of Tiwari *et al*. (1998) and Sharma *et al.* (2007), this may probably due to the variations in other parameters such as genotype, explants size, physiological age etc.

Direct organogenesis and plantlet regeneration was observed on Kinetin supplemented medium (Figure 3c.). Our results are in accordance with the work done by Shrivastava and Rajni (1999), who reported shoots development directly from explant without intervening callus, however, are in contrast with Tiwari *et al*. (1998).

The plant regeneration on Kn supplemented medium was achieved in single step i.e shoot initiation, elongation and subsequently root initiation on the same medium. These plantlets were healthy and varied in size from. 3 to 4 cm in length. Leaf and internode size were also found to be more as compared to plantlets on BAP supplemented medium. About 9-10 plants regenerated /explant /month. Further maintenance of shoots on the Kn medium increased the number of plants.

In vitro conservation

Conservation of *Bacopa monnieri* is mandatory to keep it off from extinction.

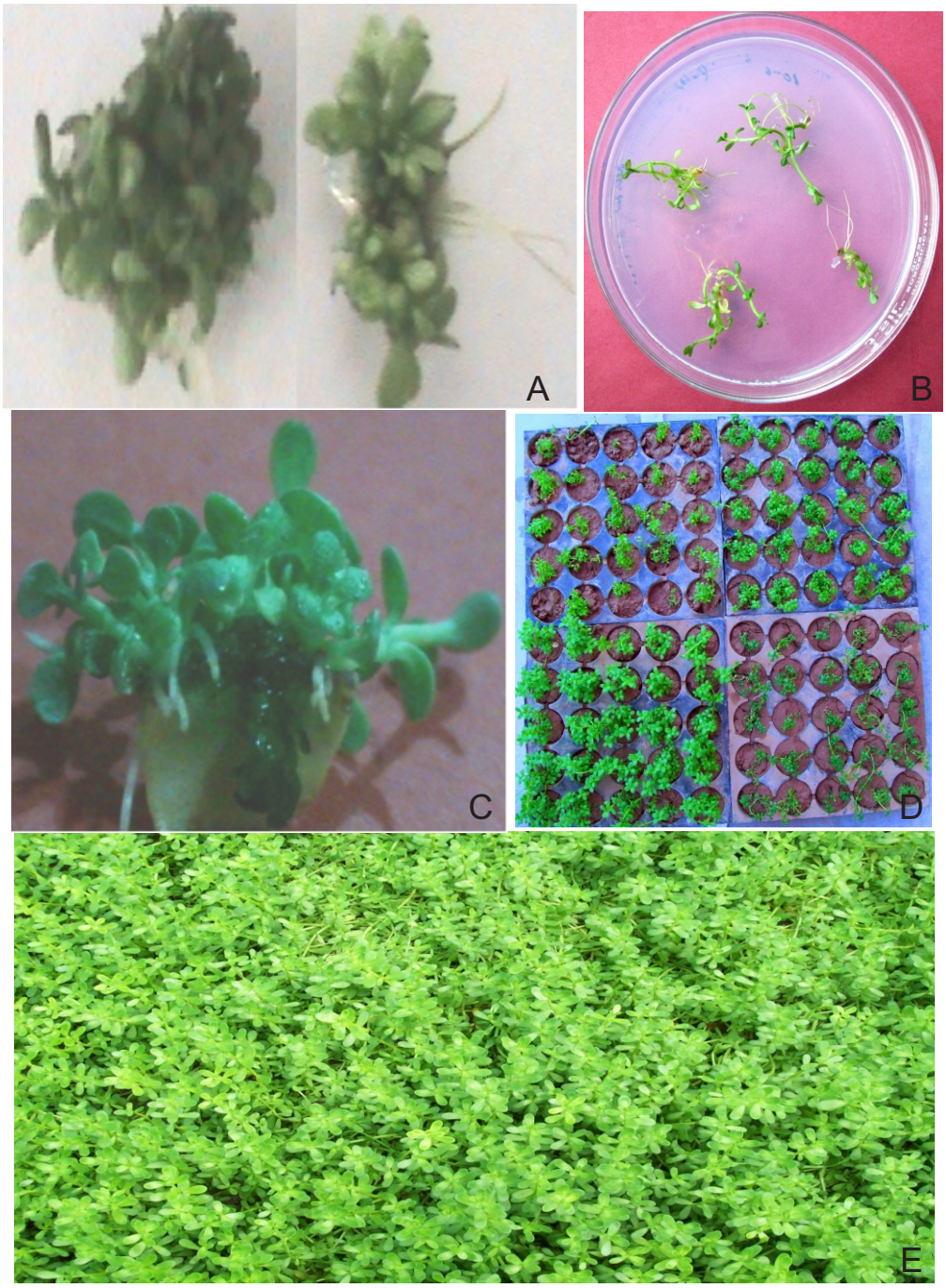

Figure 3. *In vitro* multiplication of *Bacopa monnieri* L. 3a. Multiple Shoot inductions and rooting on BAP (2.22µm) medium 3b. Weak and different length shoots rooting on MS based medium 3c. Multiple shoot induction and rooting on Kn (13.95µm) medium 3d. *In vitro* regenerated plantlets transferred to root trainers. 3e. Reqenerated plants successfully growing in the field conditions.

Table-1: Percentage response of *Bacopa monnieri* leaf explants (BMG-1) on MS medium with various concentration of BAP (µM)

S. No.	Conc. of growth Regulator µM	Callus response (%)	Shoot response (%)	% Plant regeneration	No. of micro shoot/ explant Mean±S.E.
1	2.22	55.88	79.41	0.05	13±2.44
2	4.44	73.68	40.9	-	3.33±1.10
3	6.67	91.66	70.58	-	2.33±0.77
4	8.88	82.6	47.42	-	00±00
5	11.11	74.07	48.14	-	2.5±0.83
6	13.33	84.61	34.61	-	2.33±0.7
7	15.55	52.63	18.42	-	2.5±0.83
8	17.78	60.46	30.23	-	2.0±0.66
9	20.0	70	25	-	1.66±0.54
10	22.22	82.14	45.45	-	2.33±0.77
11	24.44	82.75	-	-	-
12	26.67	67.74	-	-	-
13	28.89	60.46	-	-	-
m14	31.11	46.42	-	-	-
15	33.33	72.72	-	-	-
16	35.5	50	-	-	-

Table-2: Percentage response of *Bacopa monnieri* leaf explants (BMG-1) on MS medium with various concentration of Kn (µM)

S.R. No.	Conc. of G.R.	Shoot % response	Root % response	No. of micro shoots\ explant Mean±S.E.
1	2.33	68.93	27.27	5.10 ± 0.79
2	4.65	52.68	47.36	6.05 ± 0.72
3	6.98	62.5	50	6.75 ± 2.25
4	9.30	57.14	50	6.83 ± 2.83
5	11.62	72.38	55.55	4.67 ± 0.47
6	13.95	76.38	65.51	7.16 ± 0.58
7	16.27	63.88	33.33	5.22 ± 0.39
8	18.60	58.06	48.38	5.94 ± 0.78
9	30.0	60.86	39.13	5.38 ± 0.62
10	23.24	60	46.66	4.94 ± 1.74
11	25.58	53.57	32.14	5.33 ± 1.74
12	27.90	61.9	47.61	5.16 ± 2.17
13	30.23	54.54	9.09	4.88 ± 1.15
14	32.56	62.96	37.03	5.44 ± 1.21
15	34.88	61.11	27.77	3.0 ±1.15
16	37.20	61.53	53.94	3.88 ±1.15

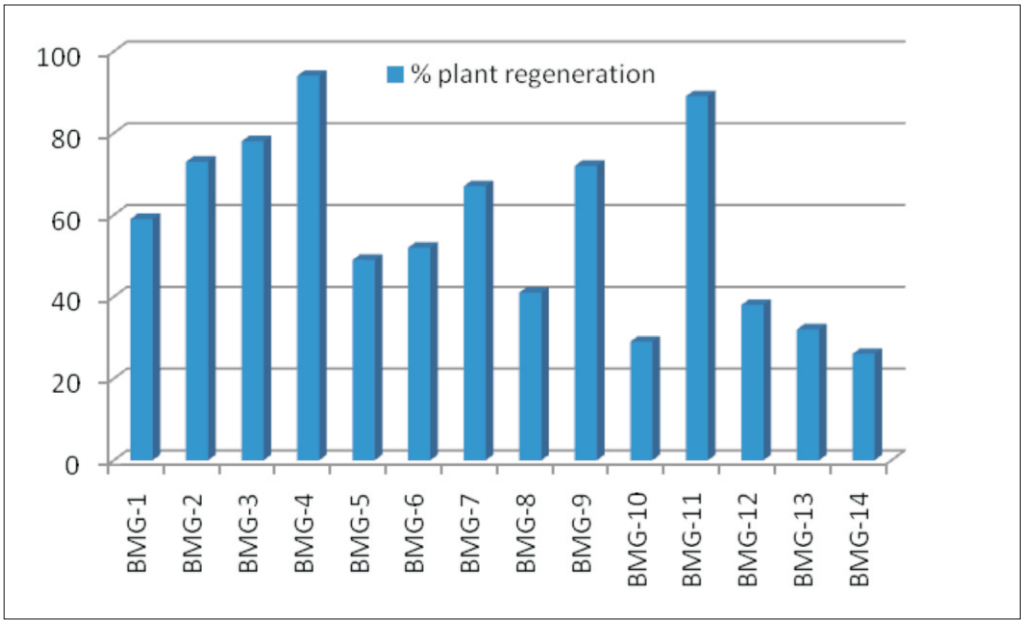

Figure-4 *In vitro* Micropropogation response (%) of 14 accessions (BMG 1-14) *Bacopa monnieri* L.

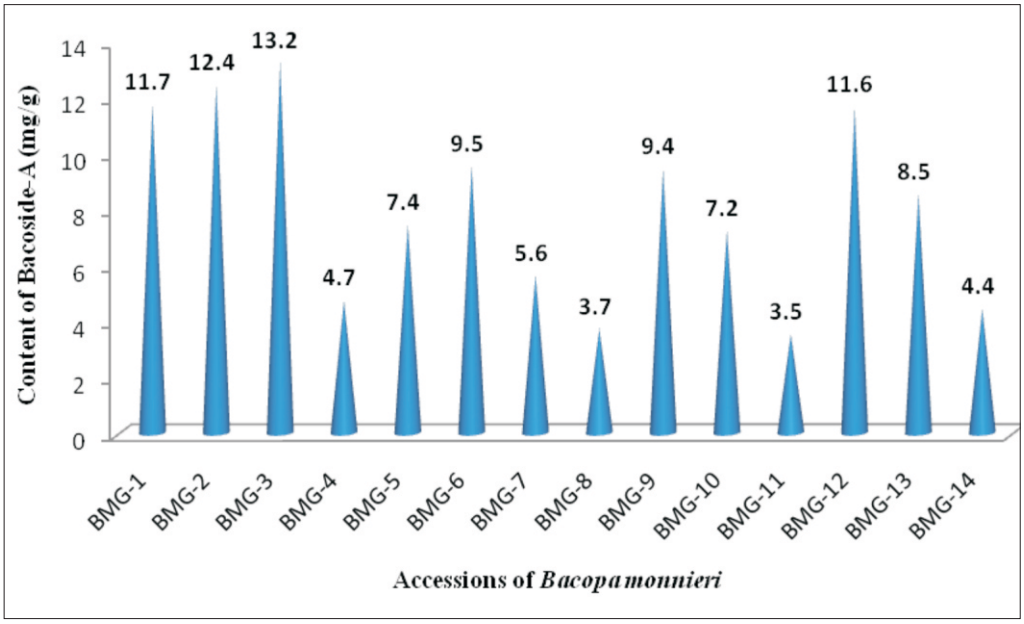

Figure : Bacoside A content present in 14 accessions of Bacopa monnieri (mg/g dry weight)

Impact of low multiplication can be minimized by rapid *in vitro* multiplication, therefore, the propagation can be at an increased rate as compared to the vegetative propagation irrespective of the season, temperature and altitude. *In vitro* micropropagation can reintroduce these plants into the natural habitat and conserve it from extinction.

The earlier published work on tissue culture of *Bacopa* mainly concern with regeneration from different explants through adventitious shoot formation, somatic embryogenesis and/or callus regeneration. To the best of our knowledge, there is no published literature regarding any attempt to conserve the *Bacopa monnieri* germplasm, especially of Madhya Pradesh. In the present study, *r*esults obtained on shoot initiation and plantlet regeneration of 14 accessions (BMG1- 14) has been given in Figure. Results clearly show the *in vitro* multiplication of all the accessions collected. Out of the 14 accessions tested, maximum percentage response of shoot initiation was shown by BMG-4 (94 %) followed by BMG11 (80%), BMG- 3 (78%) and BMG-2 (73%) (Figure- 4). Least response was shown by BMG-14(Figure 4). In the present work we could see variation with respect to Bacoside-A content and the regenerating ability of accessions, although there was no clear indication of relation between the both parameters (Figure 2, 4). Differential regeneration response of accessions may be due to various reasons, genotype variation being one of the important factors.

Finally, *in vitro* regenerated plants were transferred to the root trainers and to green house for hardening and 100% plants were successfully 100% establishment in the field conditions (Figure 3d,e). The Bacoside-A content in the regenerated plants (3 Month old analyzed by HPTLC) found to vary from 14.25 to 22.36mg/g dry plants, indicating the increase in the Bacoside-A content in regenerated plants as compared to the natural accessions (data under communication). Thus, we have successfully regenerated plantlets from all the accessions collected from Madhya Pradesh and transferred to field.

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