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Induction of photoautotrophy in *Chlorophytum borivilianum* Sant. et Fernand, regenerated *in vitro*

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Abstract

The potential for photoautotrophic growth was studied in *in vitro* cultures of *Chlorophytum borivilianum* Sant. et Fernand. Best *in vitro* shoot differentiation was observed on MS medium supplemented with BAP 5 mg L^{-1}, whereas MS + IBA 2 mg L^{-1} produced maximum root number and root length. The regenerated plantlets were then sequentially transferred to liquid basal medium having a gradual decrease in sucrose concentration [3%, 2%, 1.5%, 1%, 0.5% and 0%] after 48 hours stay in each. The plantlets thus formed were successfully hardened and transferred to sand-soil and farmyard manure mixture [1:1:1]. Approx. 90% of *C. borivilianum* regenerants survived after successful hardening.

Keywords: Micropropagation, photoautotrophy, *Chlorophytum borivilianum*.

INTRODUCTION

For past many decades micropropagation has been extensively used for the rapid multiplication of many plant species [1]. The goal of micropropagation is to obtain a large number of genetically and physiologically identical plantlets with high photosynthetic potential which are able to survive the transfer to *ex vitro* conditions [2]. The transfer of *in vitro* raised plantlets to *ex vitro* conditions is one of the most critical factors in the micropropagation process and cause of higher production costs. High mortality is often observed upon transfer to *ex vitro* conditions as the cultured plants have non-functional stomata, weak root system and poorly developed cuticle [3]. Moreover, the cultivation media used for most plant cell, tissue and organ culture are often supplemented by saccharides as carbon and energy sources. This addition besides inducing heterotrophy or mixoheterotrophy in the cultures also considerably decreases the water potential of the medium and increases the risk of bacterial and fungal contamination. Plantlets developed within the culture vessels grow under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity. These contribute a culture-induced phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field [4].

Photoautotrophy has emerged as an important tool in this light. The concept of photoautotrophic micropropagation was proposed as a means of reducing production cost of the micropropagation process [5]. This strategy assumes that autotrophic cultures have persistent leaves that live longer and would be more photosynthetically productive *ex vitro* [6].

Induction of photoautotrophy could be a very important step in hardening process, where the *in vitro* regenerated plantlets are grown in sugar less medium. These plantlets usually need elevated CO_2 concentration and higher irradiance than conventionally used [7, 8, 9, 10]. Photoautotrophic growth of plantlets on medium without saccharides not only enables the development of fully functional photosynthetic apparatus but also helps to combat the microbial infection arising otherwise and reduced dependence on exogenous growth regulators [11]. This results in a faster acclimatization and high survival rate for plants, when transferred to *ex vitro* conditions [12, 13].

Chlorophytum borivilianum

Chlorophytum borivilianum Santapau & Fernandes (Liliaceae) commonly known as 'Safed Musli' is an important medicinal herb reputed for its tuberous roots. The plant finds wide applications in more than 100 ayurvedic preparations. It is used as an aphrodisiac, immunomodulator, revitalizer, and as a general tonic. The tuberous roots of safed musli contain steroidal saponins, fructans, fructoligosaccharides, acetylated mannans, phenolic compounds and proteins (14, 15, 16, 17). It is used as a remedy for a large number of diseases, as a curative for natal and postnatal problems, as antimicrobial, anti-inflammatory, antitumor agent, and also, to increase the general body immunity (18, 19).

Owing to its therapeutic activity and diversified uses, the demand for *Chlorophytum borivilianum* has increased both in Indian and International markets. The plant primarily propagates through roots due to poor seed setting, low viability (<20%) and long dormancy. The bulk of industry's demand is met through collection from wild forests, due to which the plant has reached an endangered status (20, 21).

The present work was aimed to study the effect of photoautotrophic induction on the *in vitro* raised cultures of *Chlorophytum borivilianum.*

MATERIALS AND METHODS

The material was procured from different institutes/organization viz: JNKVV, Jabalpur (M.P.), S.F.R.I.,

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Jabalpur (M.P.) and from several local private growers. Healthy and vigorous plants were chosen.

Different explants viz. stem discs (0.5-1.0 cm) bearing young shoot buds and excised leaf parts. were chosen as explants for the *in vitro* multiplication of the plant. The explants were first washed in running tap water for 2-3 times thoroughly so as to wash off the adhering soil particles. They were then thoroughly washed with 0.01% Labolene [10 min] followed by repeated tap water washing for 2-3 times. The explants were then transferred to laminar air flow hood where they were surface sterilized in 70% ethanol for 5 minutes after which they were washed with distilled water for 2-3 times and then with 0.1% Mercuric chloride [5 min]. Explants were finally washed with sterilized distilled water four times and aseptically transferred onto the solidified Murashige and Skoog [MS] medium. The MS media was prepared by using MS stocks and adding 3% sucrose to it. The pH of the media was adjusted to 5.6 –5.8 before adding agar [8 gm 1^{-1}]. Media was sterilized at 15 lbs pressure for 15 min by autoclaving. Cultures were incubated at 25 ± 2^0C at photoperiodic cycle of 16 hr light [approx 1500 lux] and 8 hr dark.

Induction of shooting

MS media supplemented with different concentrations of cytokinins [BAP/KIN] were used for determining the role of best possible cytokinin hormone concentration for initiation of shooting and subsequent multiple shoot formation.

Induction of rooting

The microshoots thus obtained were subcultured onto varying concentrations of auxins [IAA, IBA and NAA] for root induction. All cultures were sub-cultured on the fresh medium at every 21 days interval. The plantlets with healthy roots thus obtained were further subjected to photoautotrophic and hardening treatments. *In vitro* response from each explant was recorded every week. Fifteen explants were taken for each treatment and the procedure was repeated at least thrice.

Induction of photoautotrophy

The regenerated plantlets were sequentially transferred to liquid basal medium [MS media without any agar and phytohormones] having a gradual decrease in concentration of sucrose [3%, 2%, 1.5%, 1%, 0.5% & 0%] after 48 hours stay in each.

Hardening and acclimatization

The plantlets were transferred to pots containing sand-soil and farmyard mixture [1:1:1], covered with polythenes bearing 6-8 holes (fig.6). Plantlets were irrigated with sterilized distilled water thrice a week. After 10-12 days the polythenes were removed for an hour each. The exposure time was gradually increased in the following weeks and the polythene covers were completely removed after the 6th week (fig.7).

RESULTS AND DISCUSSION

Effect of cytokinins on shooting paramaters

Out of the different explants used, only the stem discs bearing shoot buds produced maximum morphogenetic response. Green shoot like structures from the apex of the stem-disc explants were observed with subsequent thickening of base. Shoot initiation started after 6-7 days of inoculation (Fig.1). Of the two cytokinins attempted BAP to be at its higher concentration [5mg L^{-1}] was observed to be better than KIN which brought about organogenesis [through shoot differentiation]. The shoots sub-cultured on higher concentrations of BAP [5 mg L^{-1}] in different subculture passages produced highest number of shoots (Fig.2 & Graph 1), whereas, BAP at 1 mg L^{-1} concentration supported optimum shoot length (Graph 2). This is in corroboration to the earlier reports of many workers who found BAP more suitable than KN [22, 23, 24, 25] (Table 1).

Table 1.Effect of cytokinins on shooting paramaters.

S. No.	Cytokinin	Conc. [mg L^{-1}]	MSN	MSL
1.	BAP	0.1	1.90 ± 0.14	0.78 ± 0.09
		0.5	7.0 ± 0.30	1.72 ± 0.17
		1.0	9.2 ± 1.02	2.89 ± 0.25
		5.0	23.2 ± 1.22	2.30 ± 0.18
2.	KIN	0.1	2.19 ± 0.06	1.0 ± 0.08
		0.5	2.70 ± 0.15	5.56± 0.14
		1.0	5.19 ± 0.18	2.46 ± 0.09
		5.0	5.42 ± 0.19	1.86 ± 0.12

[Results are shown as mean ± SE, n=15]
BAP- Benzyl Amino Purine, KIN- Kinetin, MSN- Mean Shoot Number, MSL- Mean Shoot Length

Effect of auxins on rooting paramaters

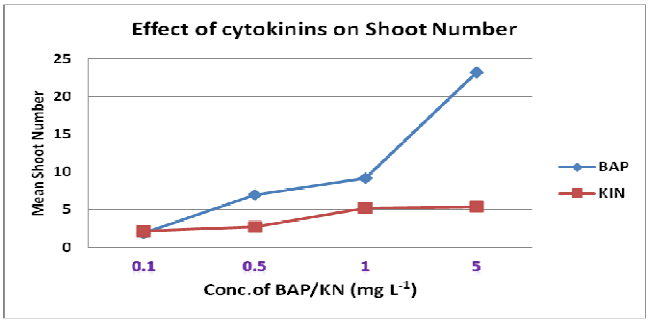
Thin slender, hairy tuberous roots were obtained from the multiple shoots when cultured on liquid MS medium containing filter bridges for support and supplemented with different concentrations of IBA, IAA and NAA. Among all the auxins IBA at 2mg L^{-1} (Fig.3) produced maximum root number and root length (Table 2, Graph 3 &4). Suri *et al.* [26] and Ramawat *et al.* [27] had also reported about the *de-novo* tuberous root formation in *C. borivilianum* from the stem disc region in a medium containing IBA, glucose and sucrose.

Table 2.Effect of auxins on rooting paramaters.

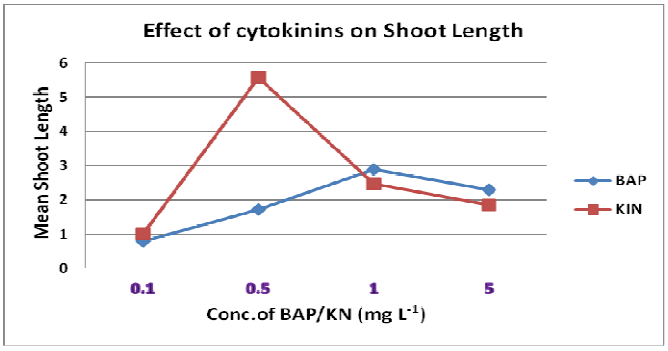
S. No.	Auxin	Conc. [mg L^{-1}]	MRN	MRL
1.	Control		4.75±0.34	2.5±0.17
2.	IBA	0.1	5.67±0.81	2.1±0.23
		0.5	5.9±0.66	1.98±0.33
		1.0	6.2±1.03	1.8±0.26
		2.0	10.46 ± 0.48	4.05 ± 0.56
3.	NAA	0.1	7.5±0.83	1.94±0.28
		0.5	6.9±0.98	1.82±0.20
		1.0	6.8±1.13	1.74±0.29
		2.0	4.2±.026	1.08±0.17
4.	IAA	0.1	5.2±0.74	1.71±0.19
		0.5	4.8±0.8	1.5±0.21
		1.0	5.2±0.58	1.23±0.2
		2.0	1.86±0.16	0.88±0.09

[Results are shown as mean ± SE, n=15]
IBA- Indole Butyric Acid, NAA- Napthalene Acetic Acid, IAA- Indole Acetic Acid, MRN- Mean Root Number, MRL- Mean Root Length

Effect of cytokinins on shooting paramaters

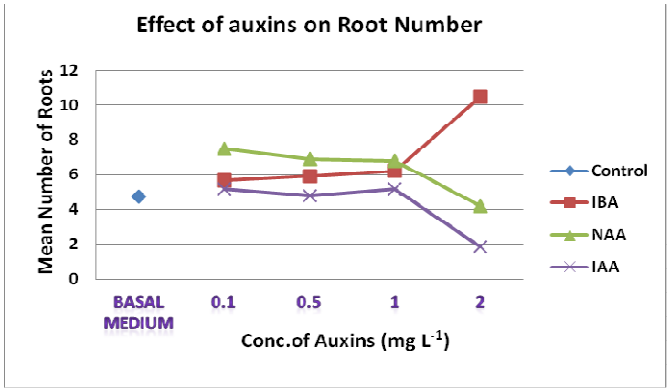


Graph 1. Effect of cytokinins on Shoot Number

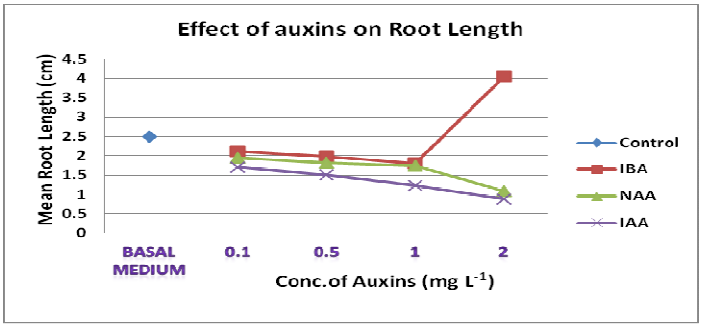


Graph 2. Effect of cytokinins on Shoot Length

Effect of auxins on rooting paramaters



Graph 3.Effect of auxins on Root Number



Graph 4. Effect of auxins on Root Length

Photo plate: In vitro regeneration of Chlorophytum borivilianum and induction of photoautotrophy.

Fig 1. Shoot initiation from stem disc on MS + BAP 5 mg L^{-1}.
Fig 2. Multiple shoot induction on MS +BAP 5 mg L^{-1}.
Fig 3. Rooting on MS + IBA 2 mg L^{-1} supported on filter bridges.
Fig 4. Plantlets on MS+2% sucrose medium.
Fig 5. Plantlets growing on sucrose free medium.
Fig 6. Hardening of plantlets on sand-soil-farmyard manure (1:1:1) and covered with polythene bag.
Fig 7. Plantlets after removal of covers (3-4 weeks).
Fig 8. Successfully hardened plantlets after 6 weeks.

Effect of photoautotrophic induction

Transfer of plantlets to liquid medium containing varying concentrations of initially showed yellowing of the leaf tip (Fig: 5), followed by the withering of leaves. Approx. 40% plantlets exhibited tuber formation along with the initiation of new roots and shoots when brought down to 1.5% sucrose concentration. This was due to the decreased concentration of sucrose in the medium which was a nutritional shock from a sucrose based heterotrophic to the induced autotrophic state leading to the initiation of photosynthetic machinery. Similar results were also reported by Srivastava and Aggarwal in *Sesbania aculeata* [28].

Photoautotrophy has also been stimulated in *chrysanthemum* meristems by growing them on sucrose free medium [29]. Plantlets cultured under this regime exhibited comparable rates of photosynthesis to those found in seedlings. Dang and Donnelly [30] also reported that sucrose in the medium promoted plantlet growth but depressed photosynthesis and reduced *in vitro* hardening. Rooting and acclimatization *in vitro* or *ex vitro* or both, can be achieved more easily in photoautotropic–micropropagation. Plant regeneration was achieved from chlorophyllous root segments derived from *in vitro* rooted plants of *Holostemma annulare* and showed 80% survival after a hardening period of four weeks by adjusting the humidity conditions inside the mist chamber by removing the polythene covering for 1 h during the first week and increasing the exposure time in subsequent weeks [31]. In curry-leaf micropropagation [32], simultaneous *ex vitro* rooting and acclimatization could be achieved using soilrite and soilrite with farmyard manure as a carrier. Better rooting [80–91%] and very high *ex vitro* survival [90–97%] was also achieved in Citrus using these carriers by Parthasarathy *et al.* [33] while Singh *et al.* [34] recorded 60% survival in citrus.

Tissue culture-based propagation techniques in *C. borivilianum* using various explants in solid and liquid media (35, 36, 37) reported so far have met with limited success because of high mortality at transplantation stage due low establishment in soil, cytological instability and early loss of regeneration potential of the *in vitro* cultures.

Approx. 90% of *C.borivilianum* regenerants survived after the successful hardening (fig.8).

CONCLUSIONS

Induction of photoaututrophy in regenerated plantlets could be an important step in hardening and acclimatization process. Also, the enhanced rooting and early tuberisation as seen in this case shows an added advantage, which thus reduces the time required for tuberisation. Besides, the protocol is cost effective and eliminates the chances of contamination, thereby reducing the transplantation shock in the regenerants.

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