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Induction of Haploids and Doubled Haploids in *Chlorophytum Borivilianum* Santa Pau & Fernandes: An Endangered Medicinal Crop

Chander Prabha[1], Abha Singh[2] and Rolly[1]

[1] Plant Tissue Culture Lab. Dept. of Botany, Patna University, Patna- 800005, Bihar, India.

[2] Microbial Biodiversity Lab. Dept. of Botany, Patna University, Patna- 800005, Bihar, India.

Abstract. Most common *in vitro* method to rapidly generate homozygous breeding lines could be achieved through androgenesis. Present work is designed to develop an efficient regeneration system to produce haploids (Hs) and doubled haploids (DHs) by culturing anthers *in vitro* and their identification in *Chlorophytum borivilianum,* an endangered medicinal crop of India. NN medium was used for callus induction and MS medium for regeneration, multiplication and rooting of cultures. Different growth regulators i.e., 2, 4-D, BAP, IAA and IBA in varying concentrations were used at different stages of cultures while CM was added at all the stages. High conc. of sucrose (6%) was added for callus induction and its low conc. (3%) was used in subsequent stages. 60% anthers developed embryogenic calli (friable and compact), 20% ELS and 16% regular heart shaped embryos. 13% microshoots and 9% rooted plantlets were scored. The putative Hs and DHs were analyzed phenotypically, anatomically and cytologically. 5% of the regenerates were Hs and 4% DHs. The findings are valuable to achieve homozygous lines to be used in future for raising the ploidy levels in the commercial crop.

Keywords: *Chlorophytum borivilianum*, *In vitro*, Androgenesis, Haploids, Doubled haploids, India

1. Introduction

Androgenesis to raise haploids (Hs), doubled haploids (DHs) and colchiploids is a comparatively new area but it provides broad platform for crop improvement as evidenced in several economically important plants such as cereals, vegetables, oil and tree crops [6, 20]. During androgenesis microspores change their gametophytic pathway to embryogenesis and several factors contribute to this process. Successful production of pollen plantlets by culturing anthers of *Datura innoxia* was first reported by two Indian scientists [22]. More often,anthers rather than microspores are cultured, since the extraction and culture methods of pollen grains (PG) are exhaustive. The DH technology is being used on commercial scale in cereals [7, 10, 19] where the ratio of individuals homozygous for genes of interest is higher in DH population than in F_2 sexually derived population. Moreover, homozygosity is achieved in a single generation which accelerates the selection process for breeding to produce new varieties. Unfortunately, in case of medicinal plants this technique is being exploited scarcely. The biochemical profile and therapeutic activities of storage roots of the endangered herb *C. borivilianum* have been extensively investigated [17, 21]. Although, Indian forests are still rich in safed musli, but are unable to meet the growing demands of Indian and foreign biopharmaceutical and neutraceutical companies as its conventional cultivation has not given much dividend due to poor seed germination, low viability and dormancy accompanied by production of small and large tubers in their 1st and 2nd seasons of growth respectively by seed raised plants. So, there always exists scarcity of planting materials [17]. Therefore, several efforts are being made through *in vitro* techniques to balance the demand and supply [2, 14, 17]. The present study aims to device an efficient *in vitro* protocol to produce Hs and DHs by culturing anther and to assess the success of the technique in *C. borivilianum*. As the crop is of economic interest for our nation, the Hs could be further used to raise colchiploids.

2. Material and Methods

Planting Materials (discs with fingers) were procured from authentic source, grown in polyhouse (28 - 34+2$_0$C temp., 16h photoperiod and 65% humidity) on raised beds. Anthers were cultured by the technique [25], cultures were initiated on NN medium [12] and the subsequent stages in MS medium [27] (Table 1). Spikes with young flower buds were collected, surface sterilized with 70% alcohol and chilled at about 5-7$_0$C for 7 days in a refrigeration unit. The buds were separated from floral axis, surface sterilized with 70% alcohol for 3 min.followed by 2.5 % sodium hypochlorite for 20 min. and then rinsed with dd H_2O. 90 anthers (without filament) were placed horizontally on CPM with 2, 4-D (2mg/l), Sucrose (6%), CM (10%) and Agar (0.8%) for callus induction. Cultures were initiated in petriplates (12 anthers/plate) and culture tubes (1 anther/tube). The developmental stage of microspores were determined simultaneously (uninucleate to early binucleate). The cultures were incubated in dark at 25 + 2$_0$C for 15 days and then shifted to light (300 Lux). Callus initiation started after two weeks.The calli (2-3 mm dia) were transferred in fresh CPM. The developed ELS were sub cultured in RM. Plates/tubes were sealed with parafilm and placed under moderate intensity florescent light,27+2$_0$C temp. and 16h light/8h dark photoperiod. After 2- 3 weeks, embryoids (0.2-0.3 cm long) were separated from callus and transferred to MM. Individual microshoots (1.0-1.2 cm long) were separated from the clumps, transferred in rM. Roots appeared after 4-5 weeks and the rooted plantlets (5 cm long) were transplanted into water saturated soil on cloudy day. The regenerates were screened for Hs and DHs by morphological, anatomical and cytological (root tips mitosis) parameters.

3. Results and Discussion

In order to avoid unnecessary colchicine treatments, the utility of Hs in plant breeding is required to produce DHs through doubling of genetic materials which occurs spontaneously. Pure lines and DHs could be generated through androgenesis [13]. Several workers [4, 10, 19, 24,] have shown that anther culture depends on diverse factors: developmental stage of PGs, cold pre-treatment of anthers, growing conditions of donor plants, culture medium, orientation of plated anthers and plant genotype. In the present study all the factors associated with androgenesis were carefully optimized. The findings are presented in Table 2. Anthers were procured from lower buds as they appear in basipetal succession on floral axis (Fig. 1) and cultured in CPM. In several plants it has been reported that frequency of androgenesis is higher when anthers are harvested at the onset of flowering and it declines with maturity [10]. 60% of anthers produced embryogenic calli (friable and compact) in NN medium with 6.0%sucrose, 10%CM and 2mg/l 2,4-D (Fig. 2). The high percentage of anthers showing callusing is indicative of the positive role played by sucrose, 2,4-D and CM which is complex mixture of nucleic acids, sugars, growth hormones and some vitamins. The effect of cold treatment on anthers, casein hydrolysate and high sucrose concentration on callusing were thoroughly investigated in pea anther culture where 60% calli were obtained [16]. Artificially induced stress (cold, heat, chemical factors, etc.) influences the natural developmental and differentiation process of male and female gametes [1, 19]. High sucrose conc. (30 – 50%, 10%, 13%, 130 g/l and 40 g/l) were used in apple, *Brassica carinata*, Czech winter rape, *Gentiana triflora* and wheat respectively [10, 18,19]. The carbohydrate source promotes pollen callusing and inhibits proliferation of somatic tissues [12]. In this study 6% sucrose was added and the results were similar to the previous findings. CM (5, 10 and 15%) has been invariably used for androgenesis [7] and in the present case, 10% has been used for induction and 5% regeneration, multiplication and rooting. In wheat 2,4- D (100mg/l) and in rice (10^{-7}- 5x 10^{-4} M) induced haploid plants from anthers [9, 19], where as 2,4-D (0.5 mg/l), NAA (1.0 mg/l) and BAP (0.5 mg/l) were used for callus induction in rice in another report [15]. Presently, BAP, 2,4-D and IAA were found to be most effective and essential for regeneration and multiplication of embryoids and IBA to induce rooting in microshoots (Table 1). Embryogenic calli were transferred in CPM and after 15 days of dark incubation 20% ELS appeared (Fig. 3).The media used for callus induction, regeneration, multiplication and rooting contained Iron in chelated form as it is more effective for androgensis [3, 12].Through several works, now it is well established that anther wall provides nursing effect to induce pollens into embryoids. The division stage of pollen is very important because the young pollens are induced to undergo sporophytic mode of development. During androgenesis, the vegetative cell which was programmed for gametophytic development de-differentiates

to change its programme from gametophyte to sporophyte. Most of the works on *in vitro* anther and pollen cultures, have recommended uninucleate to early binuleate stages for efficient and effective androgenesis and in this study most of PGs were at uninucleate to early binucleate stages of division when they were examined under microscope. In *C. borivilianum* the low seed setting, viability and germination indicate the lesser amount of embryogenic pollens which accounts for a very low frequency of Hs formation. In order to increase the number of embryogenic pollens, chilling treatment to the floral buds is essential [25]. The SDHs are formed directly due to endo-duplication, fusion between pollen nuclei, cytomixis, C- mitosis and formation of abnormal microspores [1]. Efforts are being made to increase their frequency, so that these can be multiplied and directly field tested. Cytomixis had been reported in PMCs of *C. comosum* where migration of chromatin materials resulted into erratic meiosis and drastically reduced the fertility [23]. The recovery of 20% ELS in CPM may be due to abnormal meiosis affecting the number of normal viable pollens which could only be ascertained by meiotic analysis. A trait of anther-donor clones associated with the generation of monohaploid plants is the low production of 2n pollens [26]. Approximately, 16% embryoids produced regular heart shaped embryos (Fig. 4). In MM, 13% putative haploids and DHs (Figs. 5,6) were recorded and 9% of them produced healthy roots (Fig.7) in rM supplemented with low levels of BAP and IBA after 4-5 weeks (Table 2). Spontaneous chromosome duplication to generate DHs depends upon genotype of the donor but their frequency could beincreased by using improved plant regeneration rates [19]. In this study, the recovery of 9% rooted plantlets (5%Hs and 4% DHs) may be attributed to the genotype which could be improved by cultural conditions e.g. media,GRs, etc. In winter and spring cultivars of German wheat 15-44% and 25-68% SHs were reported respectively [4, 5]. The most effective method to detect and identify Hs and DHs is the use of genetic markers.Morphological and anatomical characterization should be followed by cytological confirmation [11]. For initial screening, stomata size, pollen and pollen abortion are considered. Phenotypic characterization started at the regeneration stage where some cultures had thin and light green leaves (Fig. 5), while in others healthy, broad (almost double in width) and dark green leaves appeared (Fig.6). Stomata size was almost reduced to half in Hs (3.44 x 2.21 μm) than DHs (6.69 x 3.1 μm) confirming the phenotypic status of the regenerates. Further, the genotypic status was confirmed cytologically by mitosis when roots appeared. In the mitotic preparations of putative Hs and DHs the n= 8 and 2n=16 were observed (Figs. 8, 9). The chromosome number (2n=16) has been reported for this herb [8]. From the morphological, anatomical and cytological analysis, it is evident that 5% of the rooted plantlets were Hs and 4% DHs. Last but not the least, this study lays ground work for the potential use of anther culture in *C.borivilianum* to raise Hs and DHs which could be further utilized to obtain colchiploids for meeting the medicinal and neutraceutical market demands at global level.

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5. References

[1] A.G. Juhasz, and M. Jakse. Haploids in the improvement of miscellaneous crop species (Cucurbitaceae, Liliaceae, Asparagaceae, Chenopodiaceae, Araceae and Umbelliferae). In: C. E. Palmer et al (eds.). *Haploids in Crop Improvement II*. 2005, **56**: 259-276.

[2] A. Kumar, D. Aggarwal, P. Gupta, M. S. Reddy. Factor affecting in-vitro propagation and field establishment of *Chlorophytum borivilianum. Biologia Plantarum*. 2010, **54** (4): 601- 606.

[3] A. Rashid, and H. E. Street. Segmentation in microspores of *Nicotiana sylvestris* and *Nicotiana tobacum* which lead embryoid formation in the anther culture. *Protoplasma.* 1974, **80**: 323.

[4] A. Stober, and D. Hess. Spike pre-treatment, anther culture conditions, and anther culture response of 17 German varieties of spring wheat (*Triticum aestivum* L.). *Plant Breed*. 1997, **116**: 443-447.

[5] B. Barnabas. Protocol for producing doubled haploid plants from anther culture of wheat (*Triticum aestivum* L). In: M. Malupszynski, et al (eds.). *Doubled Haploid production in crop plants*. *A manual*. FAO/IAEA Division, Wicn. 2003, pp. 65-70.

[6] B. S. Purwoko, I. S. Dewi, N. Khumaida. Rice anther culture to obtain Doubled-Haploids with multiple tolerances. *As. Pac. J. Mol. Biotech.* 2010, **18** (1): 55-57.

[7] B. Roy, and A. B. Mandal. Anther culture response in indica rice and variations in major agronomic characters among the androclones of a scented cultivar, Karnal local. *Afr. J. Biotech*. 2005, **4** (3): 235-240.

[8] C. D. Darlington, and A.P. Wylie, 1955. In: *Chromosome Atlas of Flowering Plants*. George Allen & Wnwin Ltd. (London). 1955, pp. 365. [9] H. Niizeki, and K. Oono. Introduction of haploid rice plant from anther culture. *Proc. Japan Acad*. 1968, **44**: 554-557.

[9] I. Smykalova, M. Vetrovcova, M. Klima, I. Machackova, M. Griga. Efficiency of microspore culture for Doubled Haploid production in the breeding project "Czech Winter Rape". *Czech. J. Genet. Plant Breed*. 2006, **42** (2): 58-71.

[10] J. M. Dunwell, M. J. Wilkinson, S. Nelson, S. Weninf, A. C. Sitorus, D. Mienanti, Y. Alfiko, A. E. Croxford, C. S. Ford B. P. Foster, P. D. Caligari. Production of haploids and doubled haploids in oil palm. *BMC Plant Biology* 2010, **10**: 218.

[11] J. P. Nitsch, and C. Nitsch. Haploid plants from pollen grains. *Science*. 1969, **163**: 85-87.

[12] J. M. Segui-Simarro, P. Corral-Martinez, V. Parra-Vega, B. Gonzalez-Garcia. Androgenesis in recalcitrant solanaceous crops. *Plant Cell Rep*. 2011, **30**(5): 765-788.

[13] N. Kemat, M. A. Kadir, N. A. P. Abdullah, F. Ashraf. Rapid multiplication of Safed Musli (*Chlorophytum borivilianum*) through shoot proliferation. *Am. J. Biot*. 2010, **9** (29): 4595-4600.

[14] O. T. Thuan, V. D. Tuan, B. B. Bong. Study of anther culture of F_1 plants from crosses between aromatic and improved rice cultivars. *Omonrice*, 2001, **9**: 41-45.

[15] P. Sidhu, and P. Davies. Pea anther culture: callus initiation and production of haploid plants. In: I. I. Bennett, et al (eds.). *Proceeding of the Australian Branch of the IAPTC2B*. Perth, Western Australia. 2005, pp. 180- 186.

[16] R. Haque, S. Saha, A. Roy, T. Bera, T. Micropropation of a medicinal plant *Chlorophytum borivilianum* through shoot crown bud and characterization and analysis of active ingredients of roots by TLC. *Int. J. Ph.sci*. 2009, **1** (2): 250- 260.

[17] R. Pathirana, T. Frew, D. Hedderley, G. T. Vaughan, E. Morgan. Haploid and doubled haploid plants from developing male and female gametes of *Gentiana triflora*. *Plant Cell Rep*. 2011, **30**: 1055-1065.

[18] S. K. Basu, M. Datta, M. M. Sharma, A. Kumar. Haploid production technology in wheat and some selected higher plants. *Aus. J. of Crop Sci*. 2011, **5** (9): 1087-1093.

[19] S. S. Bhojwani, and M. K. Razdan. Haploid Production. In: *Plant Tissue Culture: Theory and practice*, Publ: Elsevier, New Delhi, India. 2006, pp. 167-213.

[20] S. L. Deore, and S. S. Khadabadi. Development and evaluation of Safed Musli formulation. *Archives Appl. Sci. Res*. 2010, **2** (1): 324-328.

[21] S. Guha, and S. C. Maheshwari. Development of embryoids from pollen grains of Datura *in-vitro*. *Phytomorphology*. 1967, **17**:454 - 461.

[22] S. K. Lattoo, S. Khan, S. Bamotra, A. K. Dhar, A. K. Cytomixis impairs meiosis and influences reproductive success in *Chlorophytum borivilianum* (Thunb) Jacq. – An additional strategy and possible implications. *J. Biosci*. 2006, **31** (5): 629-637.

[23] S. T. Mercy, and F.J. Zapata. Position of anthers at planting and its influence on anther callusing in rice. *Plant Cell Rep.* 1987, **6**: 319-328.

[24] T. P. Croughan. Anther culture for double Haploid production, In: O. L. Gamborg, and G. C. Phillips (eds.). *Plant Cell, Tissue and Organ Culture*: *Fundamental Methods*. Narosa Publishing House. 1996, pp. 143 – 154.

[25] T. Gueye, and K. N Ndir. *In vitro* production of doubled haploid plants from two rice species (*Oryza sativa* L. and *Oryza glaberrima* Steudt.) for rapid development of new breeding material. *Scientific Research and Essays*. 2010, **5** (7): 709-713.

[26] T. Murashige, and F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 1962, 15: 473- 497.

Table 1: Abbreviated names for different Media combinations

Sl. No.	*In vitro* stages	Media Composition	GRs (mg/l)***	Abbreviations
1.	Callus Induction	NN*+ Sucrose (6.0%) + CM (10%) + Agar (0.8%)	2,4-D (2.0)	Callus Producing Medium (CPM)
2.	Regeneration	MS**+ Sucrose (3.0%) + CM (10.0%)+Agar(0.8%)	BAP(4.0)+ 2,4-D (2.0)	Regeneration Medium (RM)
3.	Multiplication	MS**+ Sucrose (3.0%) + CM (10.0%)+Agar(0.8%)	BAP(2.0)+ IAA(0.5)	Multiplication Medium (MM)
4.	Rooting	MS**+ Sucrose (3.0%) + CM (5.0%)	BAP(2.0)+ IBA(3.0)	Rooting Medium (rM)

*Nitsch & Nitsch Basal Medium; **Murashige & Skoog Basal Medium; ***Growth Regulators

Table 2: Showing % of Calli, ELS, Embryos, Rooted Plantlets, Haploids and Doubled Haploids

No. of Anthers inoculated	Calli	Embryo Like Structure (ELS)	Heart Shaped Embryos	Putative Haploids and Dihaploids	Rooted Plantlets	Haploids	Dihaploids
90.0	**60.0**	**20.0**	**16.0**	**13.0**	**9.0**	**5.0**	**4.0**

Fig. 1: Inflorescence of *C.borivilianum*.

Fig. 2: Developing calli from anthers in CPM (3 weeks old).

Fig. 3: Callus with ELSs in RM (4 weeks old).

Fig. 4: Germinating Embryos: Putative Haploids(a) and Doubled Haploids(b) in RM (3 weeks old)

Fig. 5: Putative Haploid showing thin, light green leaves in MM (5 weeks old).

Fig. 6: Putative Doubled Haploid showing healthy, green leaves in MM (5 weeks old).

Fig. 7: Vigorous rooting rM (6 weeks old).

Fig. 8: Chromosome count in Haploid showing n=8

Fig. 9: Chromosome count in Doubled Haploid showing 2n=16