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Chilli Anther culture for haploid and doubled haploid production

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Abstract:

Plants with a gametophytic chromosome number are called haploids, while those with two chromosomes duplicated are called doubled haploids. When compared to the conventional breeding method that uses several generations of egging, the production of haploids and doll bled haploids (DHs) through gametic embryo gene allows for the single-step development of complete homozygous lines from heterozygous parent plants, significantly reducing the time required to produce homozygous plants. Agriculture systems have been greatly impacted by the development of haploidy technology and protocols to generate homozygous plants, as well as the generation of haploids and DHs, which is a particularly attractive biotechnological tool. These biotechnologies now play a crucial role in the breeding programs of numerous crops that are significant to agronomy. Local chilli cultivars in Vietnam possess a unique scent and pungency. There haven't been many reports of pure line development from pollen culture in the local hot chilli, though. The purpose of this study was to determine how flower bud size related to the developmental phases of microspores and how changes in culture media concentration affected the impact of plant growth regulators on in vitro androgenesis. Based on the size of the petals and sepals, flower buds were gathered at random and visually sorted into three stages. The anthers were subsequently cultivated on MS basal medium with varying concentrations of BA (0.5 - 1.5 mg/L), kinetin (1.0 - 3.0 mg/L), and NAA (0.1 - 0.7 mg/L). The findings demonstrated that the 2.5 mm long, light violet anthers were made up of 80% haploid and 20% Dihaploid anthers. With in vitro anther culture, twelve different pepper genotypes (Capsicum annuum L.) were studied to ascertain the impact of genotype and medium on haploid plant production. After being cultivated in O1 and O2, the buds' cultured conditions were found to have an impact on the commencement of plantlets, embryonic development, and anther development. The cv. Flinta F1 genotype produced the greatest rate of 12.79% when the anther developmental status was assessed, whereas the SU-31 genotype produced the lowest result, 2.47%. With an astounding 25.0% growth rate in the O2 medium, the cv. Dolphin variety

yielded the highest growth rates. Out of the eight embryos and five plants, the cultivar Dolphin had the best genotype.

Keywords: Introduction, Material and method, Description of Experimental Area Results, Discussion, Conclusion

Introduction:

Chilli pepper (*Capsicum annuum*), belong to the genus Capsicum, also known as pepper, sweet pepper, or bell pepper, is a member of the Solanaceae family and is one of the most significant vegetable crops in the world (Hegde *et.al*.,2017). This fact is due to the high biological value of the fruits (high content of dry substance, vitamin C and B-complex, minerals, essential oils, carotenoids, etc.) and their various kinds of utilization in the culinary and food industry of different countries (Irikova *et.al*.,2011).

Chilli popularly called “capsicum”, known for its green fruits and pungency. The word capsicum has been derived from the Greek word Kapsimo meaning “to bite” or “to swallow”. *Capsicum annuum* contain capsaicin, this is a chemical that produces a burning sensation in the mouth when eaten. Capsaicin (C18H27NO3) is responsible for the pungency in chilli, it is a condensation product of 3-hydroxy-4 ethoxybenzylamine and decylenic acid. When ingested or applied topically, capsaicin and related compounds referred to as capsaicinoids, these substances gives chilli peppers their intensity. The red color of chilli is due to the presence of Capsanthin, which is actually a mixture of estersof capsanthin, capsorubin, zeaxanthin, cryptoxanthin and other carotenoids. The amount of capsaicin in peppers varies and is dependent on genetics. Capsaicin extracted from chillies is used in manufacturing pepper spray and tear gas as chemical irritants, forms of less-lethal weapons for control of unruly individuals or crowds. There is an increase in demand for Capsicum varieties due to their good profitability, productivity, and export opportunities (Medina *et.al.*, 2006).

Capsicum is native to the tropical and subtropical Americas, and the majority of the genetic diversity is concentrated in Bolivia,Peru, Brazil, and Mexico (Víctor García-Gaytán *et.al*.,2017). Christopher Columbus brought chillies to Europe in 1493. In India, it was introduced by the Portuguese in the seventeenth century. Major chilli growing states in India are Andhra Pradesh, Karnataka, Maharashtra, Orissa, Tamil Nadu, Madhya Pradesh, West Bengal and Rajasthan which accounts for more than 80% of the total area and production of the country. The North-Eastern states are bio-diversity hotspots for myriad variants of this crop. Chillies from India are exported mainly to Sri Lanka, USA, UK, Canada, Saudi Arabia, Malaysia, Singapore and Germany (Pandit *et.al*.,2020). Conventional breeding in chilli pepper is a long-term and labor-intensive process due to uncontrolled pollination and the requirement for isolation to stop the genetic degeneration of breeding material. This might be overcome through in vitro methods for haploid plant production. Hence, alternative biotechnological approaches, the more efficient and sustainable than conventional methods, are being used to produce uniform doubled haploid (DH) Plants through in vitro anther culture. Anther culture is an important tool for crop improvement and plant breeding because it allows the generation of homozygous lines in shorter time by doubling the number of haploid chromosomes, as opposed to the classic traditional methods of self-fertilization and backcrossing, which are used to fix or stabilise the desired traits in hybrid plants resulting from parental crosses.

Additionally, plant recessive mutants that would typically be extremely challenging or nearly impossible to obtain using conventional techniques are also made achievable by this in vitro system.

The chilli pepper is a highly significant horticultural plant to which anther culture techniques have been applied in order to produce haploid or doubled haploid plants for crop improvement and breeding programmes as well as for breeders to shorten the breeding process required to produce homogenous plants that can be used directly as superior pure line varieties or in the production of hybrids, which would of greater benefit to the farmers. Also, haploids can be utilized to facilitate the detection of mutations and recovery of the unique recombinants.

Keeping the above views in mind, the present investigation was undertaken with the following objectives

1. To develop double haploid Chilli (*capsicum annuum*) plantlet through anther culture.

Material And Method :

Material

Scientific Equiptments:

- Laminar Air Flow
- Autoclave
- PH Meter
- Weighing Machine
- Magnetic stirrer

Methods

Collection of Plant Material

Flower buds of chilli at the stage, the microspores were at the uninucleate stage and the anther was about 1mm long collected from Ankur Seeds Pvt Ltd Breeding Support Center, Nagpur.

Preparation, sterilization and storage of culture media

Murashige and Skoog (MS) Media- The stock solutions for micronutrients, macronutrients, vitamins, iron and growth regulators were prepared with sterile distilled water. To make up one litre of medium, the required amount of each stock solution was added into a glass beaker. A known quantity of glass distilled water was added into the beaker and the required quantity of macronutrients and sucrose were weighed, added as solids and dissolved fully and then the volume was made unto one litre with distilled water. All the components of the medium were mixed and final volume was made by adding double distilled water and the pH was adjusted to 5.8±0.05 by using 1N NaOH or 1N HCl followed by addition of agar. The anther culture medium were sterilized in an autoclave at 121°C, 15psi for 20 minutes. The sterilized

medium was poured into Petri-plates in laminar air flow cabinet. The autoclaved medium was stored at 25±2 °C and used within 4-5 days of its preparation.

Preparation and storage of stock solutions of growth regulators

Stock solutions of cytokinins (Kinetin, BAP, Zeatin), auxins(NAA, IBA, 2,4- D) and thidiazuron (TDZ) stock solution was prepared. These prepared stock solutions were stored at 4ºC in refrigerator and used whenever needed. All stock solutions were used within one month of their preparation.

Table 1. Composition of plant growth regulators

Sr. no	Growth regulator	Concentration
1.	Auxins 2,4-dichioro Phenoxy Acetic Acid L Naphthalene Acetic Acid	1 mg l^{-1}
2.	Cytokinins 6 Benzyl Amino Purine Kineti	1 mg l^{-1}

Table 2. Composition of basic MS media. (Murashige and Skoog, 1962)

Chemical constituents of the culture media used	MS medium (1962)
a.Major constituents	1900
Potassium nitrate	370
Magnesium sulphate (anhydrous)	1650
Ammonium nitrate	
Potassium dihydrogen orthophosphate	170
Calcium chloride	440
b. Minor constituents	Mg/l
Boric acid	6.20
Manganese sulphate (mono/hydrate)	22.30
Sodium molybodate	0.25
Potassium iodide	0.83
Zinc sulphate (heptahydrate)	8.60
Copper sulphate (pentahydrate)	0.025
Cobalt chloride (hexahydrate)	0.025
c. Chelating agents	Mg/l
Na: EDTA (disodium salt dehydrate)	37.25
Ferrous sulphate (heptahydrate)	27.80
d.Vitamins	Mg/l
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Nicotinic acid	0.5
Others	Mg/l
Glycine (Free base)	2.0
myo-Inositol	100

L-Glutamine	
Total(g/l)	4.41

Identification of anther stage :

For the identification of proper stage of anther, flower buds were collected at three different stages of microspore development . The size and morphology of flower buds can be used as an indirect indication for determining the stage of microspore development. Anthers from flower buds of different stages were subjected to cytological examination by staining with 2 per cent acetocarmine dye after squashing and observed under microscope at 100magnification.

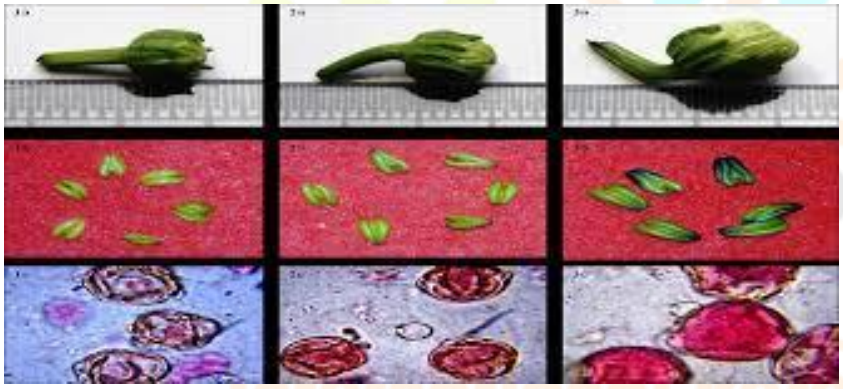

Fig 1. Flower buds, anther morphology and developmental stages of pepper micros pores.

Based on anther morphology and microspore stage of development the flower buds collected were divided into three different groups (1a, 2a & 3a).

- Colour of the anthers is viewed as good indicators of stage of microspore development (1b, 2b & 3b).
- Stages of microspore development in anthers were determined using a electron microscope, (1c, 2c & 3c).
- Group-1 flower buds consisted of anthers with uni-nucleate microspores (1a–c); Group-2 flower buds consisted of anthers with 80% uni-nucleate and 20%bi-nucleate microspores (2a–c); (3a–c). The purple colour tipped anthers were selected; invariably it contains the uni-nucleate stage of microspores (Group-1).

Cold Treatment :

The collected buds were given cold treatment (30C-100C) for 24hrs before excising the anther. Cold treatment plays a significant role in androgenesis as its increases the percentage of development of embryoids.

Explant Surface Sterilization :

For the explant sterilization, the buds were firstly treated with 0.1mg/ml Mercuric Chloride(HgCl2) for 7-8 minutes with vigorous shaking. After washing with HgCl2, buds were washed with sterile distilled water for 5 mintues.

Anther Inoculation :

Sterilized explant were transferred aseptically to a sterilized glass petri plate under the laminar flow. The explant were dried out with the help of tissue paper and kept in the autoclaved petri plates. Then sepals and petals of the buds are removed carefully with the help of needles and 2324 forceps without damaging the anthers and then filaments were detached. Each anther was aseptically placed on the petri plate containing medium with the help of forcep, 12 to 15 anthers were plated in 60 × 15 mm Petri plate. The forceps were rinsed with 70% of Ethanol to avoid any chances of contamination. The petri plates were labeled with the date and media code and lastly wrapped with cling wrap. The same procedure was undertaken for all the buds. This culture plates were kept in the dark room with temperature 25±20C for 25-30 days. The cultures were observed with the interval of 7 days for contamination and *in vitro* response.

Embryo Inoculation :

The Embryos started forming at 1 to 1.5 months of anther culturing. There was a formation of 0.3 to 0.4 cm length embryos. This formed embryos were picked up asceptically with the help of forcep and cultured in petri plates containing regeneration medium and incubated in the light room for 8-10 days .

Sub-culturing :

After the incubation of 8-10 days there was a formation of leafs. After attaining two leaf stages, the plantlets were transferred and cultured in test tubecontaining MS medium supplemented with different concentrations of auxins for root

Results and Discussion

The results on the studies of production of double haploid through anther culture conducted at plant tissue culture laboratory of Ankur Seeds Pvt Ltd Breeding Support Center, Nagpur are presented below.

The anther culture of capsicum annuum genotype ARDC 23/05 was carried out on MS medium modified with addition of growth regulators and growth additives (Fig.1). The results showed that the embryo structure were established 7-8 weeks after culturing the anther (Fig.2). The effect of induction media was significant for the embryo frequency. The result obtained confirm that the induction media supports the production of embryo. In the present experiment, the frequency obtained for genotype ARCD 23/05 was 2.9% (Table 1).

Percent of embryo formation frequency

Per cent response of anthers to embryo formation at the end of 60 days of culture was assessed by calculating number of anthers responded for embryo formation and expressed in percentage.

$$\text{Per cent of embryo formation frequency} = \frac{\text{Number of anthers with embryo formation}}{\text{Total number of anther cultured}} \times 100$$

Table 1

No. of anther inoculated	No. of embryo formation	Frequency (%)
23,240	361	1.55%

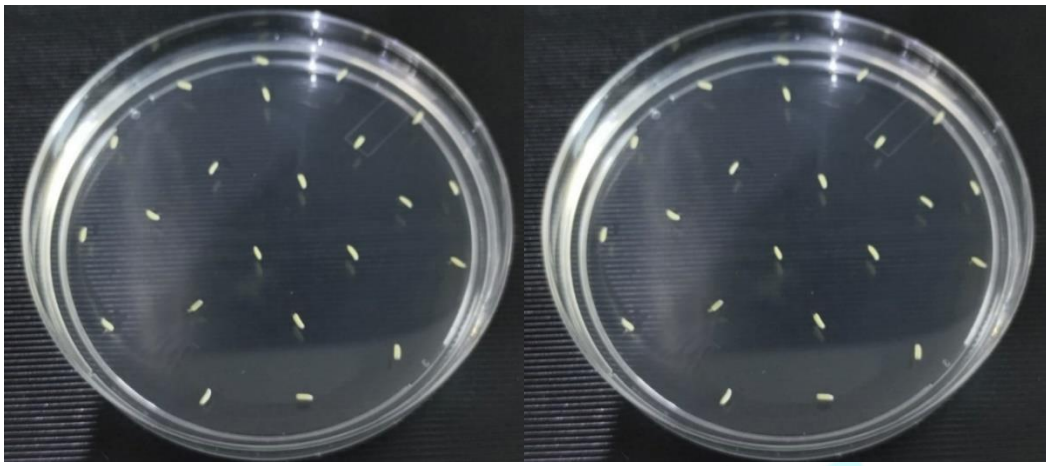

Fig 2. Anther Inoculation of *capsicum annuum* Genotype ARCD 23/05

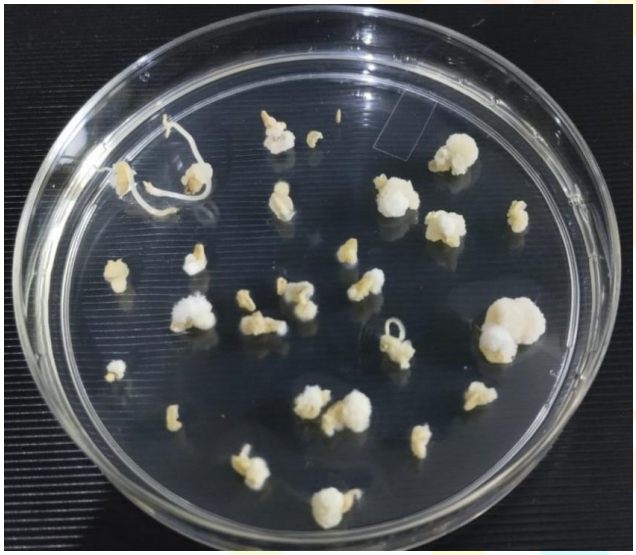

Fig 3. Direct development of embryo from cultured anther

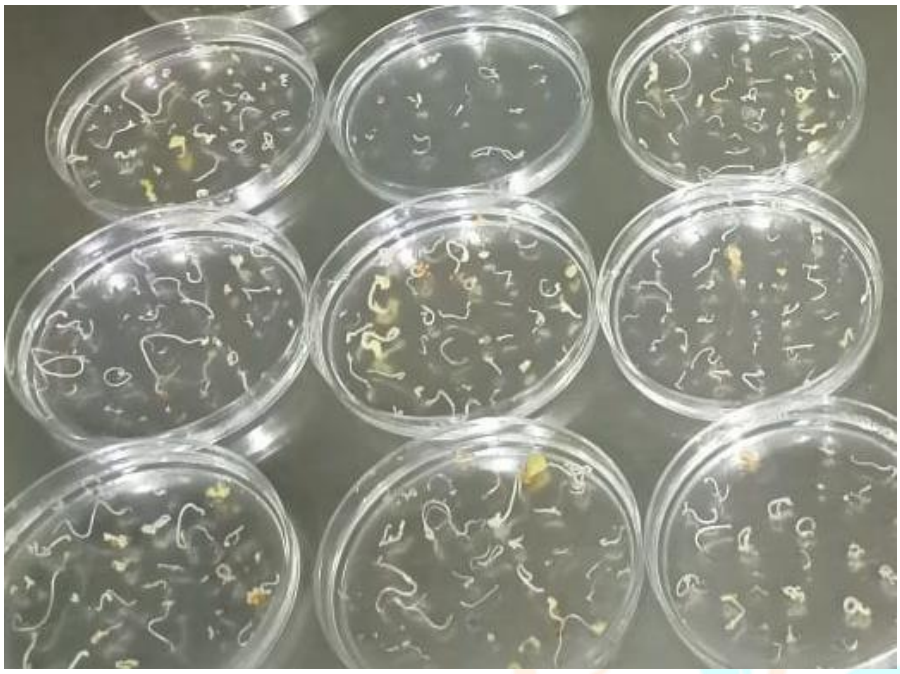

Fig 4. Embryo Inoculation on ½ MS media

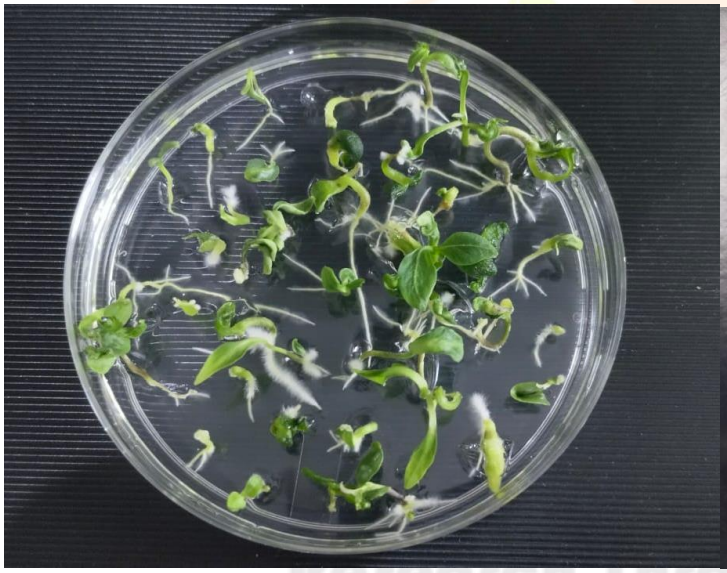

Fig 5. Root induction on rooting medium

Fig 6. Sub-Culturing

CONCLUSION :

From this experiment, the study in chillies has indicated the importance of cold pre treatment of flower buds, microspore developmental stages, and media and growth regulator combination for double haploid plant production and its growth. The anther culture experiment demonstrated that genotype under study showed successful production of double haploid plants through embryogenesis. The genotypes ARCD 23/05 under study showed 2.9% embryo formation frequency.

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