Plant Archives Vol. 21, Supplement 1, 2021 pp. 168-173 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
doi link : https://doi.org/10.51470/PLANTARCHIVES.2021.v21.S1.031

DOUBLED HAPLOID PRODUCTION IN *CAPSICUM ANNUUM* L. USING ANTHER CULTURE : A REVIEW

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ABSTRACT

Capsicum spp. is a horticulture crop with great potential and is supposed to be the fourth most significant vegetable in the earth. *Capsicum* genus covers several dozens of species which contain vast range of nutritional and medicinal properties. The process of inbreed development for hybrid variety production is fastened by the use of doubled haploid (DH) system. Doubled haploid (DH) system plays an important role in breeding programs and developmental studies. The production of haploid and doubled haploid plants using the anther cultures of the desired genotype is one of opportunities for rapid stabilization of new variants. There is an ongoing research to develop and improve the effectiveness of these methods by looking at the factors involved in different phases like induction and regeneration. Factors predominantly incorporate condition of donor plants, plant genotypes, medium components, cultured gametophytic cells temperature treatments of (cold pre-treatment, heat shock), the developmental period of early on gametophytic cells and use of various segments like Charcoal and plant development regulators. Stress treatment is one of the conditions which are utilized for the incitement of gametophytic cells to modify toward the sporophytic pathway.

Keywords: *Capsicum annuum,* Doubled haploid, Embryoids, Plant regeneration, Genotype.

Introduction

Capsicum genus represents angiosperm of family, *Solanaceae*. Its species are native to America, where they have been cultivated for thousands of years (Lopez-Espana *et al.,* 2016). *Capsicum* spp. is a horticulture crop with great potential and is supposed to be the fourth most significant vegetable in the earth (Sanchez *et al.,* 2020). It is a leading vegetable crop in our country. There is an increase in demand for *Capsicum* varieties due to their good profitability, productivity, and export opportunities (Medina *et al.,* 2006). *Capsicum* genus covers several dozens of species 22 wild and 5 domesticated species which includes, *C. annuum, C. chinese, C. frutescens, C. pubescensand C baccatum* (Boseland, 1996). The terms used to name species of *Capsicum* are confusing because of the high number of expressions used. Words like pepper, chili, chile, aji, paprika and *Capsicum* are used interchangeably in the literature. *Capsicum* when referring to the grossum group is called sweet pepper, due to its less pungency. The more piquant varieties are called chilli peppers. The large and mild forms are called bell pepper or sweet pepper.

In India, *Capsicum* cultivation, exhibits strong domestic and international demand, because of its promised market and taste-wise acceptancy both in rural and urban areas. *Capsicum* cultivation has emerged as bright spot even in bleak areas of cultivation. It can be cultivated both in open fields and green house. These factors have caught the attention of progressive breeders for efficient and promising cultivar development, eyeing its export potentialities. The main traits of breeder's consideration are productivity, agricultural and quality performance, adaptability to marginal lands and regional environments, biotic and abiotic stress tolerance, etc., keeping homozygosity as its major constraint. Breeders have drawn in with hereditary improvement of this cultivar through traditional techniques creating genotypes having qualities identified with creation and protection from biotic and abiotic stresses (Regner, 1996). However, there were natural limitations to this, basically, inequality and equity. However, finding pure or homozygous lines is a time-consuming given that it requires minimum six cycles of self-fertilization (Cravero *et al.,* 2011).

In plant breeding, various tools of biotechnology are used, and one of them is tissue culture, particularly haploid and doubled haploid technology, can effectively help to pick superior plants with novel characteristics. Haploids (H), are plants with gametophytic chromosome number, and doubled haploids (DH), are haploid plants that have under gone chromosomal duplication, either automatically or artificially induced by antimitotic agents (Badu *et al.,* 2017). DH technology represents attractive biotechnological method to accelerate plant breeding, enhancing efficiency as well as helps in addressing the economic and ecological goals. In this case, the assembly of haploid and double *in vitro* plants is an advantageous gizmo because of the pure line obtained in one generation, as a result of which reduces the time and cost of production (Maraschin *et al.,* 2005). As an advantage, DH plants have a magnificent utility in hereditary and cytogenetic investigations (Dwivedi *et al.,* 2015). In addition

to this, haploids can provide valuable material for different types of research such as mapping, genetic analysis, mutations, gametoclonal and somaclonal variation, transformation, somatic hybridization, biochemical and physiological studies, artificial seed production and germplasm storage (Niemirowicz-Szcytt 1997; Lefebvre and Palloix, 1996).

Androgenesis

Androgenesisis the basic and most widely used method for obtaining the haploids in *C. annuum*. In androgenesis an immature gametophyte is diverted from its normal development pathway and is induced to sporophytic development. Regardless of the huge number of studies drove with anther culture, the declared reasonability of the system is still low (Irikova *et al*., 2011a). The frequency of embryos is high which are derived from anther but conversion of theses embryos into normal plants are quite low (Segui-Simarro *et al.,* 2011a). In this method anthers are cultured directly in the media. Besides of anther culture shed-microspore culture with a double-layer medium is also used to produce DH plants from a male nucleus *in vitro* (Supena *et al*., 2006). Shed microspore culture lead to less embryo induction (Arı *et al*., 2016), leading to less number of normal looking embryos. Another method to obtain Haploid and DH plants is microspore culture, but at very low rates (Irikova *et al*., 2016). As mentioned there are three methods which lead to production of haploids and DHs. Surprisingly; none of them are applicable to a wide spectrum of genotypes. With these prompts mind, anther culture remains the preeminent famous and favored method for DH production. This might be because of its simplicity and the abundance of experimental outcomes and particular protocols concerning t he massive quantity of studied. The capacity of microspore and shed-microspore culture for microspore-starting embryogenesis has not been recognized now, because of unobtrusive number of attempted genotypes and particular difficulties, for instance, contamination and mechanical separation of microspores (Supena *et al*., 2006; Gemes Juhasz *et al*., 2009; Liu *et al*., 2013). Keeping all factors in mind, a research review has been done in order to summarize androgenesis effecting prospectives in *C. annuum*, using conventional anther culture technique and certain factors influencing it.

Genotype Dependency

The frequency of androgenic plants obtained depends highly on genotype by Dumas de Vaulx *et al.,* 1981; Ltifi and Wenzel, 1994; Mityko *et al.,* 1995; Qin and Rotino, 1993; Sibi *et al.,* 1979. Low rate of haploids limits the utility of anther culture in *C. annuum* by Foroughi-Wehr and Wenzel (1993). As per a few researchers the development of pepper haploids in culture of anthers and microspores are relient on species, variety, hybrid and genotypes of individual plants (Mityko *et al*., 1995; Saccardo and Devreux, 1974; Vagera and Havranek, 1985; Nowaczyket *et al*., 2006). Heterogenic effective androgenic effects were demonstrated by Cheng *et al*. (2013). It was revealed that some genotypes naturally respond better than other genotypes under study, irrespective of further optimal treatments done. The high and low responding genotypes have different essentials for perfect treatment conditions in similar species (Cheng *et al*., 2013).

Donar Growth Conditions

Researchers studied the influence of temperature and photoperiod on course of action of embryoids in anther culture of *C. annuum* among F1 cross breeds of cultivars Jetta, Parma and Trophy (Kristiansen and Andersen, 1993). In the green house plants were developed at temperatures strolling from 16 to 30°C and at a photoperiod of 11-19 h. The anthers were assembled from solitary plants during 5-9 weeks of developing and refined using the procedure of R. Dumas de Vaulx *et al.* (1981). Embryoids were gotten from the source material accumulated from plants created at all temperature frameworks with an improvement perfect at 26°C. Effect of photoperiod on the formation of embryoids is not studied yet whereas the age of donar plant directly impacts the quantity of embryoids. When the anthers are collected from maternal plants matured 12-14 weeks development of embryoids didn't occurred. Similar outcomes had been suggested by J. Mityko *et al*., (1995), who examined 4 breeding lines (*C. annuum* L.), 7 varieties and 4 F1 hybrids using donor plants grown in a greenhouse. These researchers recorded the increased yield of embryoids in anther culture when the blossom buds are aggregated inside about a month from the essential bloom appearance.

Microspore Developmental Stage

The developmental stage of microspore is one of the mostimportant factors that influence the formation of embryoids. George and Narayanaswamy (1973) stated that microspores of later stages give best result. Flower bud whose corolla is slightly larger than sepals contain microspores having partial anthocynin colouration are said to be of later stages (Mityko *et al*., 1995; Novak, 1974; Morrison *et al*., 1986a). Dolcet-Sanjuan *et al*. (1997) confirmed the development stage of microspores (10% binucleate) using the method by Pace *et al.* (1987), by staining with mithramycin A. DAPI staining was also done for best stage determination (Coleman and Goff, 1985). Lantos *et al*. (2009) has experimented with collected buds of late uninucleate and early binucleate stages of microspore development for effective embryogenesis to occur in *C. annuum*. The presence of microspores in the optimal phase directly was difficult to ascertain, as it was time consuming so indirect indicators were used. According to this buds having length of corolla petals equal to length of calyx sepals include microspores at late uninucleate stage, whereas the buds with longer corolla petals include microspores entering the first mitotic division or in the binucleate phase (Ozkum Ciner and Tipirdamaz, 2002). Nowaczyk and Kisiala (2006) studied that in the anther culture of *C. annuum*, embryos originating from buds with longer corolla petals than calyx sepals did not reveal any ability of direct embryogenesis, producing only callus. Kim *et al*. (2004) believed that the early binucleate phase of microspores is the most optimal one as far as the induction of embryos in anther culture.

Pretreatment of Anther

Pre-cultivation flower buds are often used to increase the yield of haploids (Dumas de Vaulx, 1981; Saccardo and Devreux, 1974; Sibi *et al.,* 1979). This can be done by using both low (4°C) (Saccardo and Devreux, 1974; Sibi *et al.,* 1979; Oskum *et al*., 2001; Supena *et al*., 2006) and high (35° C) temperature (Dumas de Vaulx, 1981) treatment. According to Gonzalez-Melendi *et al*. (1996) the treatment of flower buds with 4°C for 2–4 days gives better result. One-

day pretreatment of buds with 4°C was ideal and it emphatically influenced embryogenic process (Ari *et al*., 2016a; Supena and Custers, 2011). A study by Popova *et al*. (2016) stated that when flower bud is pretreated with low temperature in *C. annuum* genotypes a reduction in embryogenic response is seen. Whereas when a cold pretreatment of 48 h is given slight increase in direct embryo formation was reported. 72h and 96 h cold pretreatment decreased the embryo formation. A ratio of direct embryos formation with respect to callus was the highest at 24 h cold pretreatment (19.05%) (Papova *et al*., 2016).

Medium composition and growth regulators

In *C. annuum* the protocol by, Dumas de Vaulx *et al*. (1981) is still the basis *in vitro* anther culture. Gudeva *et al*. (2007) utilized five distinctive medias for anther culture, N (Nitch, 1969) enhanced with Kinetin (Kn) (1.0 mg l^{-1}), indole-3-acetic acid (IAA) (0.001 mg l^{-1}); MS (Murashige and Skoog, 1962) enhanced with kinetin (Kn) (1.0 mg l^{-1}), 2.4-dichlorophenoxyacetic acid (2.4-D) (0.01 mg l^{-1}) and indole-3-acetic acid (IAA) (0.001 mg l^{-1}); LS (Linsmaer and Skoog, 1965) enhanced with Kinetin (Kn) (3.0 mg l^{-1}) and indole-3-acetic acid (IAA) (1.0 mg l^{-1}); NN (Nitch and Nitch, 1969) enhanced with Kinetin (Kn) (0.01 mg l^{-1}) and 2.4-dichlorophenoxyacetic acid (2.4-D) (0.001 mg l^{-1}) and CP (Dumas de Valux *et al*., 1981) enhanced with Kinetin (Kn) (0.01 mg l^{-1}) and 2.4-dichlorophenoxyacetic acid (2.4-D) (0.01 mg l^{-1}). Only in the CP medium the formation of haploid embryos was seen when exposed to heat thermal stress (+35°C), which has similarities with the findings of De Valux *et al*. (1981). Callus formation was observed at minimum rates using CP medium. However, there are no embryos formation for an equivalent medium and cultivation conditions (Irikova and Rodeva, 2004). Nervo *et al*. (1995) revealed that addition of silver nitrate to (5mg l^{-1}) CP medium could significantly improve effective embryogenesis. Nowaczy and Kisiala, (2006) employed silver nitrate (5mg l^{-1}) to CP media for embryogenesis with positive results depending on the genotype. Sibi *et al*. (1979) and Dumas de Vaulx *et al*. (1981) increased the rate by incubating anthers in two successive media at different incubation condition to induce embryogenesis. The process involved incubating explants in a semisolid CP medium containing 2,4-D at 0.01 mg l^{-1}, 0.05 mMFe EDTA, 3%w/v sucrose, 0.8% agar and after incubation of anther on CP medium for 12 to 14 days the anthers were moved to the R1 regeneration medium with an addition of 0.1or 0.2 mg 1^{-1} of kinetin but without 2,4-D, resulting embryoids in one month. The yield of haploid plants is improved by adding vitamin B12 to a medium that enhance the development of embryoids (Sibi *et al*., 1979). Coconut milk is also used by some researchers for increase in embryoid development (Wang *et al*. 1981), yeast extract, casein (George and Narayanaswamy, 1973), carrot extract and activated charcoal (Vagera and Havranek, 1983). Various modified versions of this protocol have been tested since then (Mityko *et al*., 1995; Morrison *et al*., 1986a, 1986b; Munyon *et al*., 1989). Two phase culture system by Dolcet-Sanjuan *et al*. (1997) further enhanced the efficacy, embryo yield as the limiting factor.

Popova *et al*. (2016) stated that embryo induction is more suitable when the combination of kinetin and low concentration of 2.4-D (0.004 to 0.1 mg L^{-1}) is used (Luitel and Kang, 2013; Olszewska *et al*., 2014). The use of different mixtures of plant growth regulators had been additionally stated with unique success (Taskin *et al*., 2011; Irikova *et al*., 2011). It was found by Qin and Rotino (1993), that for promoting embryogenesis in some recalcitrant pepper genotypes the combination of BA(0.6 mg L^{-1}) with 2.4-D is used, while Ozkum and Tipirdamaz (2011) stated that NAA(4.0 mg l^{-1}) combined with BA(1.0 mg l^{-1}) initiate microspore embryogenesis. To sum up, androgenesis is initiated in growth regulator-free medium (Kim *et al*., 2008) just as in media upgraded with 5 µM indole-3-acetic acid (IAA) and 2.5 µM zeatin (Supena *et al*., 2006, 2011), or in the media of 0.5 mg·l^{-1} kinetin (Kin) and 0.5 mg·l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) stated by Lantos *et al.* (2009).

Incubation treatment

As parIrikova and Rodeva, (2004) and Gudeva *et al*., (2007) the impact of various media and distinct incubation treatments showed the induction of embryogenesis. In anther culture the heat treatment of anther at 35°C on CP medium induced embryogenesis (Dumax de Valux *et al*., 1981). To correlate with such finding Gurdeva *et al.* (2009) used basic media by, Dumas de Vaulx *et al*. (1981). CP medium with heat temperature stress (+35°C) gave good embryo induction with incubation of 8 days dark followed by 4 day incubation in growth chamber (25°C, photoperiod of 12h light/12 dark). Embryo induction rates were in concord with the findings of Dumas de Valux *et al*. (1981).Gudeva *et al*. (2007) stated the response of anthers cultured on different media in different thermal incubation treatment. When microspore division of pepper is stimulated at (35°C)heat thermal stress has more noteworthy impact than cold one (7° C) (Barany *et al*., 2005; Kim *et al*., 2004). Despite the fact that the instrument of temperature stun in instigating higher androgenesis isn't known, one of the hypotheses recommended is that the decrease of ABA levels inside the cultured anthers (Dolcet-Sanjuan *et al*., 1997).

Activated Charcoal

The clarification for the advancement of embryogenesis by AC isn't yet very much explained (Chang *et al*., 2013). When AC is added to tissue culture media, it is regularly thought to evacuate development inhibitory substances emitted by tissues or present in the ingredient of the medium because of the solid adsorptive property of AC (Supena *et al*., 2006) depicted significant increase in the *in vitro* androgenesis using AC. The effect of activated charcoal in anther culture medium has been described previously (Johansson, 1983; Anagnostagis, 1974; Typyrdamaz & Ellialtyoulu, 1998). The promontory materials also can be absorbed and made inaccessible to the plant (Johansson *et al*., 1990, 1983; Vagera, 1990; Vagera and Havranek, 1985; Weatherhead *et al*., 1979) resulting in small embryos in pepper. Supena *et al*. (2006) stated that parallel results cannot be obtained between the number of total embryo with increasing concentrations of charcoal and if the concentration of charcoal is 2% then it slow down the embryonic development in pepper.

Carbon Source

The type and concentration of sugar employed have significant effect on androgenesis. Finnie *et al*. (1989) and Karsai *et al*. (1994) demonstrated the positive effect of maltose in inducing haploid formation in barley and inwheat respectively. The stimulatory effects of maltose were first

studied by Dolcet-Sanjuan *et al*. (1997). Earlier it was suggested that androgenic response was much sensitive to glucose to fructose(Finnie *et al*., 1989). Maltose is converted to glucose more slowly avoiding the inhibitory effects of other sugar. 3% w/v sucrose with 0.8% agar is reported efficient carbon source in embryo induction media by in pepper by Dumas de Vaulx *et al*. (1981).

Regeneration Media

The percent of developing embryoids multiplied when the embryoids were removed from anthers and cultured separately in the presence of FeEDTA in the culture medium (Vagera and Havranek,1985). The normal cotyledonary embryos from the anthers when transferred to solid B5 basal medium with 2% (w/v) sucrose, and held under light, turned green and initiated active root growth within 5 days, and subsequently developed into plantlets (Kim *et al*., 2007). The embryoids when transferred to MS medium (Murashige and Skoog, 1962) containing 3%(w/v) sucrose, 0.8% (w/v) agar, 4 mg l^{-1} NAA, 1mg l^{-1} BA or 1 mg l^{-1} NAA and 4 mg l^{-1} BA with or without 0.25% activated charcoal improved efficiency(Ciner and Tipirdamaz, 2000). The cultures were incubated at 29°C in continuous light conditions (2000 lux). However, one of the most important factors is growth regulators in androgenic culture of pepper and for better understanding of this factor need further study. The embryoids developed when transferred to MS media (without growth regulators) for further regeneration at 25° C with 16 h photoperiod gave best proliferation (Bhattacharaya *et al*., 2014).

Diplodization

The characteristic feature of the Caspicum is that the presence of haploid and diploid plants among the androgenic regenerants. Plants in which spontaneous diploidization did not occur it is necessary to use the factor provoking the duplication of the number of chromosomes and restoring fertility. Available literature offers studies concerning the colchicine treatment of haploid regenerants obtained by androgenesis of *Capsicum annuum* genotypes (Gemesne *et al.,* 2001; Lantos *et al.,* 2009).

Conclusion

In conventional breeding the production of haploid and doubled haploid plants is applicable as it shortens the time required to acquire new varieties. DH plants contain genetically homozygous lines. By using DH lines researchers can fix new gene combinations rapidly resulting in individuals with resistance to diseases, pests and greater production. Despite the fact that anther culture is considered as forthcoming strategy for the creation of DH plants, still effective convention for genotype free framework is yet to be created. Crucial factors like growth conditions, collection of explants and use at optimal stage, composition of medium, culture conditions need to explore further for efficient promising protocol for androgenesis in *Capsicum annuum*. Along with androgenesis regeneration is again a key factor to be considered. After regeneration how much percentage of diplodized plants got achieved again is a big issue to develop protocol of DH for any crop.

Acknowledgments

The authors acknowledge Department of Biotechnology in ACSEN HYVEG Pvt. Ltd. Kullu, H.P. for providing facilities to carry out this work and GLA University, Mathura U.P. for providing guidance and encouragement whenever required.

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