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Efficient Androgenic Embryo Induction and Plant Regeneration in Different Genotypes of Sweet Pepper via Anther Culture

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Creation of pure lines is one of the basic requirements in plant breeding. Doubled haploid (DH) lines are completely homozygous lines and anther culture is an efficient method for DH line production. Induction of androgenic embryos in anther culture is the main restricting factor. In this study we investigated androgenic embryo induction in different F1 hybrid peppers including Cadia, Magno, Plato, King Arthur and Maratus. Excised anthers were cultured in C medium containing 2 mg L^{-1} 2-4D and 2 mg L^{-1} Kinetin and incubated at 35°C for eight days and followed by four days at 25°C in darkness, then anthers sub cultured to R medium containing 0.1 mg L^{-1} Kinetin and incubated at 25°C and 16h photoperiod. The effect of genotype, cold pretreatment (4°C, 24 h) and heat shock on the efficiency of anther culture in pepper (*Capsicum annuum* L.) was evaluated. Cold pretreatment applied to excised buds improved microspore embryogenesis efficiency as compared to control. Among genotypes tested, Cadia, Magno, and Maratus were shown to be more responsive than King Arthur and Plato. Plants produced in responsive genotypes were approximately three-fold higher (2.5, 2.33 and 2.33 regenerated plant per petri dish, respectively) than King Arthur and Plato (0.83 and 0.66, respectively). Results showed that heat shock (35°C) applied to cultured anthers was effective on microspore embryogenesis, as heat shock (35°C) treatment for eight days had the best results in Cadia with 0.16 plant per petri dish.

Keywords: Androgenesis; breeding; *Capsicum annuum* L.; cold pretreatment; heat shock.

Pepper (*Capsicum annuum* L.) because of its nutritional effects and pharmaceutical application is one of the most important vegetables around the world. Nowadays, F1 hybrids demonstrating unique characteristics are mainly applied seeds, especially in greenhouses. Conventional breeding methods to produce inbred lines through selfing is time and labor consuming. DH technology sheered disadvantages of traditional breeding and made possible achievement to parental lines in one generation. This technology consists of stimulation and germination of haploid plants via anther or microspores culture. Haploids are sporophytes containing gametophytic chromosomes so are valuable for not only inbreeding programs but also cytogenetic and genetic studies (Touraev *et al*. 1997). Gaining to haploids was made possible through application of female and male gametic cells. Androgenesis (anther or microspores culture) is the most interested method because of its higher efficiency and number of male gametes existing in each bud. As reported in the literature, pepper is one of the recalcitrant crops in haploid and DH

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production because of its various nature (Nowaczyk & KisiaBa 2006; Kothari *et al.* 2010). At the first time, Wang *et al*., (1973) and George and Narayanaswamy (1973) reported plant regeneration from *C. annuum* anthers (Wang *et al*. 1973; George & Narayanaswamy 1973). Afterward, many other researchers presented lots about the different methods of androgenesis including anther, shed-microspore and isolated microspore cultures (Sibi *et al*. 1979; Dumas de Vaulx *et al*. 1981; E. D. J. Supena *et al.* 2006b; Kim *et al.* 2008). The genotype has the primary role and often restricting aspect in the pepper androgenesis (Buyukalaca *et al*. 2004; Wang & Zhang 2001; Rodeva *et al*. 2004; Koleva-Gudeva *et al*. 2007; Liu *et al*. 2007). It cannot be overcome by controlling of growth conditions of parental plants and androgenic cultures (Kristiansen & Andersen 1993). So finding responsive genotypes for androgenic programs in plant breeding is one of the crucial factors. Embryo induction both in plants and animals is more or less equal and requires stress treatment(Zimmerman & Cohill 1991). To deviate from the gametophytic to the sporophytic pathway, stresses such as low or high temperature, osmotic pressure or starvation stress are needed (Maraschin *et al*. 2005; Shariatpanahi *et al*. 2006; Koleva-Gudeva *et al*. 2009). Olszewska *et al*., (2014) investigated the combined effect of anther incubation time on CP induction medium (12, 14, and 16 days) and 2 concentrations of kinetin in R1 regeneration medium (0.1 and 0.3 mg L^{-1}) on the effectiveness of androgenesis in 17 genotypes of *Capsicum* grown in Poland(Olszewska *et al*. 2014). Abdollahi *et al*., (2015) studied the anther culture response of *Citrullus lanatus* L. to evaluate the effect of growth regulators and wheat ovary-conditioned medium in Charleston Gray cultivar and the effect of temperature pretreatment in Crimson sweet cultivar. Their results showed that the highest frequency of mean number of embryos per anther was obtained from cultured anthers incubated at 32°C for 2 days. Embryos germinated into plantlets on MS medium containing 2.22 µM BAP, 3 % sucrose, and 0.8 % agar. The root tips of 12 regenerated plantlets were analyzed for ploidy level, of which 10 have been haploids (Abdollahi *et al*. 2015). So, in this study, we examined effects of different genotypes and temperature pretreatments on pepper anther culture.

MATERIALS AND METHODS

Plant materials

Seeds of Five F1 hybrids including Cadia, Magno, Maratus (Enza-Zaden Co.), Plato and King-Arthur (Seminis Co.) were sown in the greenhouse of Agricultural Biotechnology Research Institute of Iran (ABRII) at November of 2015. They were within plastic pots containing a mixture of soil: peat: perlite(1:1:1). Plants grew at 25-30/18-22°C (day/night temperature) and natural light condition. Fertilization and irrigation were done according to standard procedure (Bosland *et al*. 2012).

Anther culture process

Buds with equal sepal and petal in size that were containing microspores at mid to late uninucleate developmental stages were harvested at early morning and pretreated at 4°C for 24 h. Pretreated buds were surface disinfected by immersion in 70% ethanol for 30 seconds followed by 2.5 %(v/v) sodium hypochlorite solution for 10 min and three times rinsing with cold sterile distilled water. The anthers were detached and placed in 80 mm petri dishes containing 12 ml C culture medium(Dumas de Vaulx *et al*. 1981) supplemented by 2mgL^{-1} 2,4-D, 2mgL^{-1} kinetin, 30 gL^{-1} sucrose, 0.7% plant agar and pH adjusted on 5.8. Each petri dish containing six anthers from a bud. Anther cultures were incubated at 35°C and darkness for eight days followed by incubation for four days at 25°C and darkness. Then anthers sub-cultured to R medium (Dumas de Vaulx *et al*. 1981) supplemented with 0.1 mgL^{-1} kinetin, 30 gL^{-1} sucrose, 0.7% plant agar and pH adjusted on 5.8 and incubated at 25°C and 16h photoperiod. In a separate part of the experiment, we examined the effect of different duration of heat stress on the efficiency of anther culture of the most responsive genotype, Cadia. So that anthers of two buds cultured in 80 mm petri dishes containing C medium (according to the previous experiment)(Dumas de Vaulx *et al.* 1981) were incubated at 35°C for 0, 4, 8, 12 and 16 days and darkness. Then they transferred to R medium (Dumas de Vaulx *et al*. 1981) and incubated at 25°C and 16 h light.

Plant regeneration

After five weeks, embryos were transferred to hormone-free B5 medium (Gamborg *et al.* 1968) with 20 gL^{-1} sucrose and solidified with 0.7% plant agar. In this study, we compared effects

of different genotypes and cold pretreatment applied to buds for pepper microspore embryogenesis efficiency during anther culture.

Statistical analysis

The present study performed in three replicates and each replication consisted of four petri dishes (each petri dish containing six anthers taken from a bud).ANOVA conducted via SAS 9 for Windows and means were compared using Duncan's multiple-range test ($p>0.05$).

RESULTS AND DISCUSSION

Effects of genotype on microspore embryogenesis induction and plant regeneration

The genotype had a significant effect on callusing and embryogenesis (Table 1). According to the results obtained, Cadia showed as the most responsive genotype among tested ones in which 9.5% of cultured anthers succeeded to embryo formation and approximately 0.57 embryo was produced per bud. This genotype had the best results in the number of regenerated plantlets per bud (0.15 plantlet), though there was no significant difference between Cadia, Magno, and Maratus. A similar phenomenon has been reported by other research groups (Dumas de Vaulx *et al*. 1981; Dolcet-Sanjuan *et al*. 1997; Ltifi & Wenzel 1994; Rodeva *et al*. 2004; E. D. J. Supena *et al*. 2006b). Recalcitrant nature of some genotypes in microspore embryogenesis could be referred to the ability of formation of the competent

Table 1. Effect of genotype on the embryo induction and development of embryos in anther culture of pepper (*Capsicum annuum* L.)

Genotype	Embryogenesis (%)	No. embryos (per bud)	Callusing (%)	Regenerated plantlets(per bud)	Plant regeneration(%)
Cadia	9.5a	0.57a	90.0a	0.15a	2.5a
Plato	3.6b	0.22b	69.3c	0.04b	0.66b
Maratus	9.3a	0.56a	82.5b	0.14a	2.33a
King Arthur	2.0c	0.12c	53.0d	0.05b	0.83b
Magno	8.8a	0.53a	65.3c	0.14a	2.33a

*Column with same letter had no significant difference ($p\geq0.5$).

Table 2. Effect of cold pre-treatment on the embryo induction and development of embryos in anther culture of pepper (*Capsicum annuum L.*)

Genotype	Embryogenesis (%)	No. embryos (per bud)	Callusing (%)	Regenerated plantlets(per bud)	Plant regeneration(%)
4°C	8.0a	0.48a	74.8a	013a	2.2a
25°C	5.33b	0.32b	69.26b	0.076b	1.26b

*Column with same letter had no significant difference ($p\geq0.5$).

Table 3. Effect of heat shock duration on the embryo induction and development of embryos in anther culture of pepper (*Capsicum annuum L.*)

Heat shock duration (days)	Embryogenesis (%)	No. embryos (per bud)	Callusing (%)	Regenerated plantlets(per bud)	Plant regeneration(%)
0	0c	0b	87.75a	0c	0b
4	3.25b	0.17b	81.00b	0c	0b
8	11.62a	0.59a	89.50a	0.16a	2.42a
12	2.75b	0.06b	64.00c	0.04b	0b
16	0c	0b	27.50d	0c	0b

*Column with same letter had no significant difference ($p\geq0.5$)

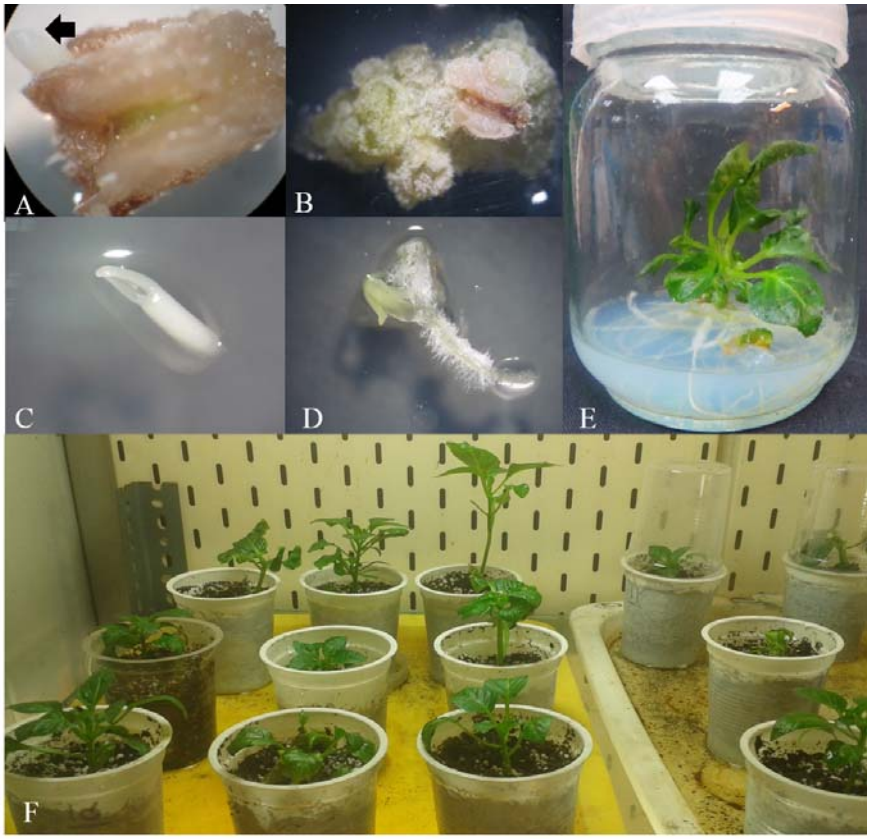

Fig. 1. Anther culture of pepper. (A) Embryogenesis (embryo indicated with arrow) (B) callusing (C) Cotyledonary embryo (D) Regenerated embryo (E) Regenerated plantlet (F) Adapted plants

microspores that is under the control of cytoplasmic and genomic genes and are under the influence of the environment (Testillano *et al*. 2000). Working on *Solanum tuberosum*, demonstrated that microspore embryogenesis capacity was a heritable trait controlled by several recessive genes (Chupeau *et al*. 1998; Rudolf *et al*. 1999; Smýkal 2000).

Effects of cold pretreatment on the induction of microspore embryogenesis in anther culture of pepper

Application of cold pretreatment (4°C) on excised flower buds for 24 h causes to a significant improvement in microspore embryogenesis efficiency in genotypes tested (Table 2). It has also been reported that physical pretreatment applied to harvested buds or whole inflorescences before culture stimulated sporophytic divisions in microspores(Sunderland & Roberts 1977; Lazar *et al*. 1985; Armstrong *et al*. 1987; Henry & Buyser 1990). Chilling pretreatment is one of the most effective practices in microspore embryogenesis. Sibi *et al* (1979) reported that cold (4°C) pre-treatment for 48h applied to flower buds enhanced embryogenesis and 1–3 plants per 100 anthers were obtained (Sibi *et al*. 1979). Cold pretreatment of flower buds from 24 to 100 h before anther culture provoked an androgenic response(Morrison *et al*. 1986; E. D J Supena *et al*. 2006b).

Working on wheat, Lazar *et al*., (1985) expressed callus yield and a frequency of spontaneous chromosome doubling increased in regenerated plants obtained with cold pretreatment. It assumed that cold pre-treatment slows down metabolism so suppresses normal gametophytic pathway and triggers sporophytic divisions. Another hypothesis suggests that starvation effects under low temperature had the primary role in anther culture response (Zheng 2003; Ziauddin *et al*. 1992; Kasha *et al*. 1990). Cold pretreatment

of flower buds from 24 to 100 h before excising anthers for culture stimulated the androgenic response(Morrison *et al*. 1986; E. D J Supena *et al*. 2006a).

Effects of heat shock duration on microspore embryogenesis in anther culture of pepper

The responses to heat shock duration on anther cultures were different (Table 3). Incubation of anther cultures at 25°C (without thermal shock), caused to no embryoid production. Heat shock treatment of anther cultivations from 4 to 12 days stimulated androgenesis response and showed the best results at eight days (Table 3). Increasing the duration of heat shock by more than 12 days had a detrimental effect and suppressed microspore embryogenesis as 16 days decreased callusing dramatically.

Stress treatment is the vital requirement for microspore embryogenesis (Shariatpanahi *et al*. 2006). Heat shock treatment has been used as a trigger to induce embryogenesis in pepper (Dumas de Vaulx *et al*. 1981; Gudeva 2003; Prayantini *et al*. 2006). Heat shock has been demonstrated to be an effective stimulating factor on microspore embryogenesis; it caused to change in microtubule and cytoskeleton (Hause *et al*. 1993; Cordewener *et al*. 1994; Simmonds 1994). Also, HSPs were reported to be synthesized in heat shock treated microspores (Segu1-Simarro *et al*. 2003) among which HSP70 was suggested to inhibit apoptosis(Jaattela *et al*. 1998). Application of proper period of heat stress is vital because a short period of heat temperature could not trigger enough ultra-structural changes and microspores tend to follow gametophytic pathway and longer duration had a detrimental effect.

As a result, during this study we achieved to an efficient protocol for production of pure lines by anther culture. Assessment of attained doubled haploid lines under greenhouse condition can help us to find appropriate lines for being applied in breeding programs as valuable plant materials.

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REFERENCES

1. Abdollahi, M.R. *et al*., The influence of phytohormones, wheat ovary co-culture, and temperature stress on anther culture response of watermelon (Citrullus lanatus L.). *Brazilian Journal of Botany*, 2015; **38**(3), pp.447–456.
2. Armstrong, T.A., Metz, S.G. & Mascia, P.N., Two regeneration systems for the production of haploid plants from wheat anther culture. *Plant Science*, 1987; **51**(2–3), pp.231–237. Available at: http://www.sciencedirect.com/science/article/pii/016894528790197X.
3. Bosland, P.W., Votava, E.J. & Votava, E.M., *Peppers: vegetable and spice capsicums*, 2012; Cabi.
4. Buyukalaca, S. *et al*., Effects of Silver Nitrate and Donor Plant Growing Conditions on Production of Pepper (*Capsicum annuum* L .) Haploid Embryos via., 2004; **69**(5), pp.206–209.
5. Chupeau, Y., Caboche, M. & Henry, Y., *Androgenesis and haploid plants*, Springer Science & Business Media 1998.
6. Cordewener, J.H.G. *et al*., Induction of microspore embryogenesis in Brassica napus L. is accompanied by specific changes in protein synthesis. *Planta*, 1994; **195**(1), pp.50–56.
7. Dolcet-Sanjuan, R., Claveria, E. & Huerta, A., Androgenesis in *Capsicum annuum* L. Effects of carbohydrate and carbon dioxide enrichment. *Journal of the American Society for Horticultural Science*, 1997; **122**(4), pp.468–475.
8. Dumas de Vaulx, R., D. de V., Chambonnet, D. & Pochard, E., In vitro anther culture in red pepper (*Capsicum annuum* L.): improvement of the rate of plant production in different genotypes by treatments at 35 C. *Agronomie*, 1981; **1**: pp.859–864.
9. Gamborg, O.L., R. A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research.
10. George, L. & Narayanaswamy, S., Haploid Capsicum through experimental androgenesis. *Protoplasma*, 1973; **78**(4): pp.467–470.
11. Gudeva, L.K., The effect of incubation treatmen on the pepper (*Capsicum annuum* L.) androgenesis 2003.
12. Hause, B. *et al*., Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of Brassica napus L. *Cell Biology*

International, 1993; **17**(2), pp.153–168.
13. Henry, Y. & Buyser, J. de, Wheat Anther Culture: Agronomic Performance of Doubled Haploid Lines and the Release of a New Variety "Florin." *Biotechnology in Agriculture and Forestry*, 1990; **13**: pp.285–352.
14. Jaattela, M. *et al*., Hsp70 exerts its anti apoptotic function downstream of caspase 3 like proteases. *The EMBO journal*, 1998; **17**(21): pp.6124–6134.
15. Kasha, K.J., Ziauddin, A. & Cho, U.-H., Haploids in cereal improvement: anther and microspore culture. In *Gene manipulation in plant improvement II*. Springer, 1990; pp. 213–235.
16. Kim, M. *et al*., Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. *Plant cell reports*, 2008; **27**(3), pp.425–434.
17. Koleva-Gudeva, L.R., Spasenoski, M. & Trajkova, F., Somatic embryogenesis in pepper anther culture: The effect of incubation treatments and different media. *Scientia Horticulturae*, 2007; **111**(2), pp.114–119. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304423806004237.
18. Koleva-Gudeva, T., F, D. & G, S.M., Androgenesis efficiency in anther culture of pepper (*Capsicum annuum* L.). *Acta Horticulture (ISHS)*, (2007), pp.183–190.
19. Kothari, S.L. *et al*., Chilli peppers — A review on tissue culture and transgenesis. *Biotechnology Advances*, 2010; **28**(1), pp.35–48. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0734975009001517.
20. Kristiansen, K. & Andersen, S.B., Effects of donor plant temperature, photoperiod, and age on anther culture response of *Capsicum annuum* L. *Euphytica*, 1993; **67**(1–2), pp.105–109.
21. Lazar, M.D., Schaeffer, G.W. & Baenziger, P.S., The physical environment in relation to high frequency callus and plantlet development in anther cultures of wheat (Triticum aestivum L.) cv. Chris. *Journal of plant physiology*, 1985; **121**(2), pp.103–109.
22. Liu, F. *et al*., [Embryogenesis of microspore derived multicells in *Capsicum annuum* L]. *Fen zi xi bao sheng wu xue bao= Journal of molecular cell biology/Zhongguo xi bao sheng wu xue xue hui zhu ban*, 2007; **40**(6), pp.371–379.
23. Ltifi, A. & Wenzel, G., Anther culture of hot and sweet pepper (*Capsicum annuum* L.): influence of genotype and plant growth temperature. *Capsicum Eggplant Newsl*, 1994; **13**: pp.74–77.
24. Maraschin, S.D.F. *et al*., Programmed cell death during the transition from multicellular structures to globular embryos in barley androgenesis. *Planta*, 2005; **221**(4), pp.459–470.
25. Morrison, R.A., Koning, R.E. & Evans, D.A., Anther culture of an interspecific hybrid of Capsicum. *Journal of plant physiology*, 1986; **126**(1), pp.1–9.
26. Nowaczyk, P. & KisiaBa, A., Effect of selected factors on the effectiveness of *Capsicum annuum* L. anther culture. *Journal of applied genetics*, 2006; **47**(2), pp.113–117.
27. Olszewska, D. *et al*., Study of in vitro anther culture in selected genotypes of genus Capsicum. *Turkish Journal of Biology*, 2014; **38**: pp.118–124.
28. Prayantini, D.C., Gniffke, P.A. & Woo, J.-G., Anther Culture in Hot Pepper. Available at: http://libnts.avrdc.org.tw/scripts/minisa.dll/144/VAVLIB/VAVLIB_SDI_REPORT/S.
29. Rodeva, V.N., Irikova, T.P. & Todorova, V.J., Anther culture of pepper (*Capsicum annuum* L.): comparative study on effect of the genotype. *Biotechnology & Biotechnological Equipment*, 2004; **18**(3), pp.34–38.
30. Rudolf, K., Bohanec, B. & Hansen, M., Microspore culture of white cabbage, *Brassica oleracea* var. capitata L.: Genetic improvement of non responsive cultivars and effect of genome doubling agents. *Plant Breeding*, 1999; **118**(3), pp.237–241.
31. Segu1-Simarro, J.M., Testillano, P.S. & Risueno, M.C., Hsp70 and Hsp90 change their expression and subcellular localization after microspore embryogenesis induction in *Brassica napus* L. *Journal of structural biology*, 2003; **142**(3), pp.379–391.
32. Shariatpanahi, M.E. *et al*., Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. *Physiologia Plantarum*, 2006; **127**(4), pp.519–534. Available at: http://doi.wiley.com/10.1111/j.1399-3054.2006.00675.x.
33. Sibi, M., Dumas de Vaulx, R. & Chambonnet, D., Obtaining haploid plants by in vitro androgenesis in red pepper (*Capsicum annuum* L.). *Annales de l'Amelioration des Plantes*, 1979; **29**: pp.583–606.
34. Simmonds, D.H., Mechanism of induction of microspore embryogenesis in Brassica napus: significance of the preprophase band of microtubules in the first sporophytic division. In *Biomechanics of active movement and division of cells*., 1994; Springer, pp. 569–574.
35. Smýkal, P., Pollen embryogenesis-the stress mediated switch from gametophytic to sporophytic development. Current status and

future prospects. *Biologia Plantarum*, 2000; **43**(4), pp.481–489.

36. Sunderland, N. & Roberts, M., New approach to pollen culture. *Nature*, 1977; **270**: pp.236–238.
37. Supena, E.D.J. *et al*., Evaluation of crucial factors for implementing shed-microspore culture of Indonesian hot pepper (*Capsicum annuum* L.) cultivars. *Scientia Horticulturae*, 2006a; **107**(3), pp.226–232. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304423805002840.
38. Supena, E.D.J. *et al*., Successful development of a shed-microspore culture protocol for doubled haploid production in Indonesian hot pepper (*Capsicum annuum* L.). *Plant Cell Reports*, 2006b; **25**(1), pp.1–10. Available at: http://link.springer.com/10.1007/s00299-005-0028-y.
39. Testillano, P.S. *et al*., Defined nuclear changes accompany the reprogramming of the microspore to embryogenesis. *Journal of structural biology*, 2000; **129**(2–3), pp.223–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10806072.
40. Touraev, A., Vicente, O. & Heberle-Bors, E., Initiation of microspore embryogenesis by stress. *Trends in Plant Science*, 1997; **2**(8): pp.297–302.
41. Wang, L.H. & Zhang, B.X., Advancement in the anther culture of *Capsicum annuum* L. *China Veg*, 2001; **3**: pp.52–53.
42. Wang, Y.Y. *et al*., induction of the pollen plantlets of Triticale and *Capsicum annuum* from anther culture. *Sci Sin* 1973.
43. Zheng, M.Y., Microspore culture in wheat (*Triticum aestivum*) - Doubled haploid production via induced embryogenesis. *Plant Cell, Tissue and Organ Culture*, 2003; **73**(3): pp.213–230.
44. Ziauddin, A. *et al*., Improved plant regeneration from wheat anther and barley microspore culture using phenylacetic acid (PAA). *Plant cell reports*, 1992; **11**(10), pp.489–498.
45. Zimmerman, J.L. & Cohill, P.R., Heat shock and thermotolerance in plant and animal embryogenesis. *The New Biologist*, 1991; **3**(7): pp.641–650.