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Haploids in the improvement of Crucifers

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Abstract

The production of doubled haploids (DH) in plants is a biotechnological tool useful in producing homozygous breeding lines and varieties. The production of haploids can be achieved either by gynogenesis or by androgenesis. The formation of haploids in the first case proceeds from the embryo sack (megagametophyte), in the second case microspores are used as target tissue. The use of haploids in producing new cultivars of crucifers (*Brassicaceae*) has widespread use. Biotechnological DH line production offers various advantages for plant breeders, including the possibility to obtain homozygous lines rapidly, as well as easy selection due to the absence of heterozygosity. It also facilitates genetic studies, particularly regarding quantitative traits. Furthermore, the use of DH progeny as mapping population(s) for the development of molecular markers is very advantageous since it enhances the efficiency of detecting markers, particularly for quantitative traits. Within the genus *Brassica*, most work for the development of DH lines has been devoted to *B. napus*. This is not surprising since it is one of the most important oilseed crops worldwide. Furthermore, *B. napus* is much easier to handle in tissue culture than other *Brassicaceae* (Weber et al. 2000). In this review the steps of producing DH lines using the microspore culture system will be described. Furthermore, the highlights regarding the creation of doubled haploids in crucifers and the role of haploids, more precisely of doubled haploids in breeding programs of crucifers will be explained detailly.

Keywords: haploid, Brassica, rapeseed, biotechnology

Brassica Islahında Haploid Bitkilerin Kullanılması

Özet

Bitkilerde dihaploid (DH) geliştirme homozigot ıslah hatları ve çeşitleri geliştirmede kullanılan oldukça yararlı bir biyoteknolojik vasıtadır. Haploid bitkicik ledesi ya ginogenesis ya da androgenesis yoluyla elde edilebilmektedir. İlk verilen örnekte haploid bitki gelişimi embriyo kesesinden (megagametofit) ikinci örnekte ise mikrosporlar hedef dokular olarak karşımıza çıkmaktadır.

Brassicaceae familyasında yeni çeşitlerin geliştirme çalışmalarında haploid bitkilerin kullanılması oldukça yaygın kullanıma sahiptir. Biyoteknolojik vasıtalar ile dihaploid hatların geliştirilmesi bitki ıslahçılarına çeşitli avantajlar sağlamaktadır; bunlar arasında hızlı olarak homozigot hatların elde edilmesi, ve heterozigotluk bulunmadığından kolay seleksiyon imkanı sayılabilir. Ayı zamanda özellikle kantitatif karakterlere yönelik genetic araştırmaları kolaylaştırmaktadır. Bundan başka, moleküler marker geliştirmede DH soyağaçlarının haritalama populasyonu olarak kullanılması oldukça avantajlıdır, çünkü özellikle kantitatif özellikler için marker geliştirmedeki etkinliği artırmaktadır. *Brassica*, cinsi içersinde dihaploid hat geliştirme çalışmaları daha çok *B. napus* ile ilgilidir. Bu sürpriz değildir, çünkü kolza dünya üzerindeki en önemli yağ bitkilerinden bir tanesidir. Ayrıca, *B. napus* doku kültürü çalışmalarına diğer *Brassicaceae*' e göre daha yatkındır. Bu derlemede mikrospor kültürü kullanılarak dihaploid bitki geliştirme işlemi açıklanacaktır. Bundan başka, Haçlıgiller familyasında dihaploid bitki geliştirmede önemli konular ve dihaploid bitkilerin Haçlıgiller familyasındaki ıslah programlarında kullanılması detayıyla açıklanmaya çalışılacaktır.

Anahtar Kelimeler: haploid, Brassica, kolza, biyoteknoloji

Introduction

The importance of haploid induction in diploid and polyploid species has been realised for a long time in genetics and plant breeding. Haploids usually occur in nature, with an extremely low frequency of 0.001-0.01% (Bhojwani and Razdan, 1996).

The induction of haploid plants by *in vitro* cultuvation of gametophytic cells, particularly male gametophytes, are of enormous importance in plant breeding programs. A comprehensive utilization of doubled-haploid (DH) production has been involved in *Brassica* breeding and also in gene transfers, biochemical and physiological studies, and other manipulations (Takahata et al., 2005). Doubled haploids (DH) are presently used in breeding of a number of crop species. This method enables breeders to develop completely homozygous genotypes from heterozygous parents in one single generation. Doubled haploids allow to fix recombinant gametes directly as fertile homozygous lines. Time saving is the most obvious advantage, because yield and other traits can be tested much earlier than with conventional lines (Kucera et al., 2002).

Dihaploids in Brassica Breeding

The development of homozygous genotypes from heterozygous parents in a single generation is possible with DH breeding and this method allows the fixing of recombinant gametes directly as fertile homozygous lines as mentioned earlier. These lines may be used for developing mapping populations for linkage maps using molecular markers, in addition to their use in mutation breeding and genetic engineering. Above all, *in vitro* screening for complex traits such as drought, cold, and salinity tolerance can be done during the culture process (Pratap et al., 2007; Fig. 1). Breeders recognized the advantages of DH technology globally and more than 280 varieties were produced with the use of various haploid production methods in many crops (Szarejko and Forster, 2006).

Brassica oilseeds are the world's third most important source of vegetable oils after palm and soybean, and contribute significantly to the economy of many countries (Gupta and Pratap, 2007). In addition to improvements in the nutritional profile of the *Brassica* oil and its meal, conventional breeding in combination with modern biotechnological tools such as the DH technology has led to improvements in various agronomically important quantitative and qualitative characters in rapeseed (*B. napus* L.).

Kameya and Hinata (1970) reported first anther culture in *Brassica* and thet described callus and haploid plants from cultured anthers of *B. oleracea*. Microspore embryogenesis reports in *B. napus* (Thomas and Wenzel, 1975) and *B.campestris* (Keller et al., 1975) followed.

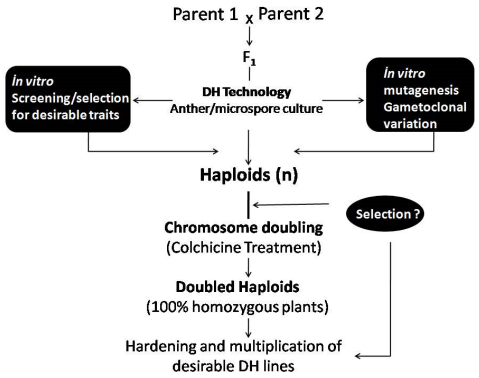

Fig. 1: Pathway showing rapid development of doubled haploid (DH) plants in *Brassica* spp. through anther/microspore culture. Selection for desirable traits can be done at any stage of the process. (*Adapted f*rom Pratap et al., 2007.)

Later due to improvisation of the technique, haploids from several *Brassica* species were reported through anther culture (Jain et al., 1989; Keller and Armstrong, 1978, 1979; Sharma and Bhojwani, 1985). For DH production, the first success with the production of microspore-derived embryos from *Brassica* anthers was reported by Keller et al. (1975) and Thomas and Wenzel (1975), and later from microspores by Lichter (1982), which provided breeders with a new tool for breeding improved cultivars of rapeseed (*B. napus* L.).

Xu et al. (2007) stated that the microspore culture technique is relatively simpler and easier than anther culture and it has widespread applications in *Brassica* breeding due to its great efficiency in haploid and doubled haploid production, mutation and germplasm regeneration, and gene transformation. Well known microspores provide uniform, synchronous, and easily accessible populations of cells, which are helpful in the understanding of the basic biochemical and physiological aspects of embryogenesis (Kott, 1996; Palmer et al., 1996). Using the isolated microspore cultures, yields in excess of 150,000 embryoids per 100 anthers have been reported (Swanson et al., 1987). Siebel and Pauls (1989) found that microspore culture was ten times more effective than anther culture for embryo production in *B. napus*. Due to its described potential, the microspore culture technique has been used in generating haploid and DH plants in many *Brassica* species (Takahata and Keller, 1991; Ferrie et al., 1995; Gu et al., 2003a, b, Zhang et al., 2003). Of course based on this walse of information,

extensive research has been carried out to investigate embryogenesis in anther- and microspore cultures. As a result, DH technology has been developed to its present form (Wang et al., 2002; Shi et al., 2002 etc).

Technologies to Obtain Doubled Haploids

We know that haploids (Hs) and doubled haploids (DHs) can be obtained from male or female gametic cells. İn some monocots and potato both ways can be used, but in most dicotyledonous species the current choice is limited to just one way. In androgenesis sporophytic growth of the male gametic cell, the microspore, is induced. This requires the complete re-programming of the developmental plan, to embryo rather than pollen grain formation. The microspore is originally programmed to produce pollen consisting of two cells: one vegetative, one generative. Eventually the generative cell gives rise to two male gametes (sperm) during pollen maturation or later, during pollen germination on the stigma. Each pollen cell is highly specialised and performs specific biological function thus, the induction of sporophytic development is only possible at early developmental stages, when the gametic cell appears to be totipotent (Touraev et al., 2001).

The microspores are the first generation of male gametic cells and have a haploid chromosome complement. They are produced in plant anthers in large numbers thus, they are relatively easy to access and manipulate. In gynogenesis, sporophytic development is induced in the unfertilised egg cell (female gamete). An advantage here is that re-programming is not required; the embryogenic pathway just needs to be switched on without proper fertilisation. Gynogenesis is usually not very efficient, partly because plants produce far fewer egg cells than pollen grains, but it is deployed in species where androgenic methods are not effective.

Haploid plants can also be obtained in some genera by “wide crossing”. This involves pollination with a genetically distant male partner or a pollinator of the same genus having special genetic properties, e.g. “haploid inducing genes”. In this technique, fertilisation usually takes place but male genetic material is eliminated from cells of the developing embryo at early stages of its growth. Since the endosperm fails to develop properly, the resulting haploid embryos originating from the female partner in the cross needs to be rescued and cultured *in vitro* to produce haploid plants. Wide crossing is a common technique in cereal and potato breeding, but there is the potential to develop the technique in other species. The microspore culture technique has its wide applications in plant genetic research and breeding programmes in oilseed Brassicas due to its relative simplicity, efficiency in haploid and doubled haploid production, mutation and germplasm generation, and gene transformation. (Xu et al., 2007).

Various factors like donor plant genotype, donor plant physiology, microspore developmental stage, culture conditions, culture environment and pretreatments could influence microspore embryogenesis and haploid production. Stress is also an essential component during embryogenesis induction in microspore culture. Efficient plant regeneration from microspores mostly occurs through direct embryogenesis ensuring minimal occurrence of cytogenetic abnormalities. Appropriate stress conditions such as chilling, partial desiccation, cotyledon excision, and successive subculture of microspore-derived embryos could promote plant development in oilseed rape. Medium renovation, phytohormones and plant growth regulators, and chromosome doubling agents such as colchicine treatment also affect plant regeneration in Brassica species. Compared to colchicine treatments of microspore-derived embryos and plants, immediate colchicine treatment of isolated microspores results in high embryogenesis and diploidisation and low chimeric percentages. The ploidy level of microspore-derived plants of Brassica species could be estimated by different methods at various stages. Mutation breeding techniques are widely used in plant breeding for producing useful mutants and variants. Microspore culture also provides an ideal method for mutation because the mutated traits can be fixed in homozygous condition by chromosome doubling, which can enforce to obtain target mutation traits efficiently. Ultraviolet irradiation, mutagenic agents ethyl methane sulphonate and sodium azide could be applied to isolated microspores and the derived embryos of rapeseed.

Current Applications of *Brassica* Doubled Haploidy

Microspore culture and anther culture provide the opportunity of producing haploid embryos at high frequencies in many *Brassica* species and their commercial cultivars and, combined with other biotechnologies such as marker assisted selection and induced mutations, can speed up breeding programmes (Maluszynski et al., 1995; Morrison and Evans, 1988). Some possible uses of DH technology combined with mutation are the production of *Brassica* lines with desirable traits such as disease resistance, herbicide resistance and altered fatty acid content. There are two main mutation methods for isolated microspores: chemical mutation using mutagenic agents such as

ethyl methane sulphonate (EMS) or sodium azide (NaN3) and physical mutation ultraviolet (UV) or gamma radiation. Many mutated varieties have been obtained using these mutation methods. Mutants exhibiting herbicide resistance were obtained in *B. napus* and Chinese cabbage (*B. campestris* subsp. *pekinensis*) by Swanson et al. (1988, 1989). Other studies obtained *B. napus* mutant lines showing resistance to black spot disease (*Alternaria brassicicola*) (Ahmad et al., 1991) and white rot (*Sclerotinia sclerotiorum*) (Liu et al., 1997), and Chinese cabbage (*B. campestris* subsp. *pekinensis*) with soft-rot resistance (*Erwinia carotovora*) (Zhang and Takahata, 1999).

Altered fatty acid lines have been developed by radiation mutation and chemical mutation. Using UV radiation, Barro et al. (2003) enhanced the content of erucic acid from 42.8 % to 49.5% in *B. carinata* lines. Ferrie (1999) also used radiation to obtain mutant variation in the content of fatty acids increasing oleic acid from 47.1 % to 50 %, decreasing linoleic acid from the normal 11.4 % to less than 8%, and decreasing the saturated fatty acids from 5.7 % to 5 %. On the other hand, using chemical mutation, oleic acid content was enhanced from 60 % to 85 % and linoleic acid content were decreased from 10 % to 3 % by Kott (1996) in *B. napus* mutant lines, while Barro et al. (2001) obtained *B. carinata* mutant lines with low erucic content (showing contents below 25%) and high erucic content (showing up to 52%) using EMS treatment as chemical mutant agent.

Discussion

Research on DHs accelerated in the last few decades with a growing number of successful applications (Forster et al., 2007). There has been a steady rise in the number of new plant species in which doubled haploidy has been report. Prior to the 1970s there was only a handful of reports on the production of DHs, this grew to about 50 publications in the 1970s, 185 in the 1980s and about 200 in the 1990s (Maluszynski et al., 2003). Currently there is much interest in applying DH technologies to neglected crops, particularly those of high value, e.g. pharmaceutical and aromatic species, where large gains can be achieved is a short space of time (Ferrie, 2007). A major component in the expansion of DH technologies to new species has been the adoption and adaptation of protocols from the model species, tobacco, rape-seed and barley. Published methodologies now cover about 200 species and represent a wide variability of modifications compared to the initial protocols. It is rarely the case that a protocol optimised for one genotype is optimal for another. Adaptation of methods from one species to another often requires substantial changes usually elaborated by empirical comparisons of protocols.

Although for the moment, further improvements can be expected mostly from empirical testing of new variants of known protocols, the new approaches in discovering gene function are expected to have an impact in the future. Although not perfect, DH systems are broadly applied in breeding (Thomas et al., 2003), genetic mapping (Forster and Thomas, 2005) and mutation studies (Barro et al., 2001; Szarejko, 2003; Szarejko and Forster, 2007). They also provide useful systems for transformation and fixing transformation events (Shim and Kasha, 2003), and for fundamental studies on plant embryogenesis and cell differentiation (Custers et al., 2001; Kumlehn et al., 2001; Bakos et al., 2005). Further progress in methods can be expected with the growing understanding of the processes involved.

Conclusion

Anther and microspore culture techniques are mostly used for haploid production in Brassicas. These techniques have been widely utilized for breeding commercially important species. In the Brassicas, plant microspore culture and haploid breeding have several advantages such as DH breeding, mutation breeding, and gene transformation breeding, and play an important role in crop improvement and germplasm generation. With the development of microspore culture technology and its related breeding method, more and more international seed companies and breeding workers are using this technology.

Microspore culture is an effective technology for the production of DH parental lines required for producing F1 hybrids of modern cultivars with desirable genetic recombinants, but the application of this technique for wide utilization as a routine breeding tool for these crosses still remains an obstacle because of the less efficient production of DH plants. Also, haploid plant regeneration directly and rapidly from the shoot apex of microspore derived embryos is an important step in DH production. Direct and quick regeneration ensures minimal occurrence of cytogenetic abnormalities, which is extremely important in a breeding program (Fletcher et al., 1998). The most widely used method of chromosome doubling is the use of the antimicrotubule agent colchicine. Colchicine treatment of isolated microspores increases the doubling efficiency of regenerated plants in B. napus obviously. The method of microspore and embryo culture, selection of the embryos resistant to herbicide at the embryo stage after the mutations are used for herbicide-resistant

breeding. The mutants with high oil contents and high oleic acid, low linolenic acid, and low erucic acid were obtained through mutations. Combining the rapeseed microspore induction and culturing together with pathogen selection, mutants with some rapeseed disease resistance were obtained in B. napus. The excellent agricultural characters can also be selected by using mutation techniques. Therefore, haploid microspore culture and DH technology have great potential for breeding crops due to their wide utilization and convenience, and at the same time they can be used as tools for more fundamental research in plant development and biotechnological processes.

References

Barker, S.B., Summerson, W.H., 1941. The colorimetric determination of lactic acid in biological material. *Journal of Biological Chemistry*, 138:535-554.

Ahmad, I., Day, J.P., Macdonald, M.V., Ingram, D.S., 1991. Haploid culture and UV mutagenesis in rapid-cycling *Brassica napus* for the generation of resistance to chlorsulfuron and *Alternaria brassicicola*. *Ann Bot*, 67:521–525.

Bakos, F., Jäger, K, Barnabás, B., 2005. Regeneration of haploid plants after distant pollination of wheat via zygote rescue. Acta Biol Crac Ser Bot 47: 167–171.

Barro, F., Fernandez-Escobar, J., De la Vega, M., Martin, A., 2003. Modification of glucosinolate and erucic acid contents in doubled haploid lines of *Brassica carinata* by UV treatment of isolated microspores. Euphytica 129: 1–6.

Bhojwani, S.S., Razdan, M.K., 1996. Plant Tissue Culture: Theory and Practice, Elsevier, Chapter 7, 177-201.

Ferrie, A.M.R., Epp, D.J., Keller, W.A., 1995. Evaluation of *Brassica rapa* L. genotypes for microspore culture response and identification of a highly embryogenic line. Plant Cell Rep. 14: 580–584.

Ferrie, A.M.R., 1999. Combining microspores and mutagenesis. In: PBI Bulletin National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada, pp. 17–19.

Ferrie, A.M.R., 2007. Doubled haploid production in nutraceutical species: A review. Euphytica 130: 347–357.

Fletcher, R., Coventry, J., Kott, L. S., 1998. Doubled haploid technology for spring and winter Brassica napus (revised ed.). Technical Bulletin p. 42. OAC Publication, University of Guelph, Ontario, Canada.

Forster, B.P., Thomas, W.T.B., 2005. Doubled haploids in genetics and plant breeding. Plant Breed Rev 25: 57–88.

Forster, B.P., Herberle-Bors, E., Kasha, K.J., Touraev, A., 2007. The resurgence of haploids in higher plants. Trends Plant Sci 12: 368–375.

Gu, H.H., Zhang, D.Q., Zhang, G.Q., Zhou, W.J., 2003a. Advances on *in vitro* microspore technology for mutation breeding in rapeseed. Acta Agric. Zhejiangen, 15: 318–322.

Gu, H.H., Zhou, W.J., Hagberg, P., 2003b. High frequency spontaneous production of doubled haploid plants from microspore culture in *Brassica rapa* ssp*. chinensis*. *Euphytica*, 134: 239–245.

Gupta, S.K., Pratap, A., 2007. History, Origin and Evolution, p. 1–20. In: Gupta, S.K. (Ed.), Advances in Botanical Research-Rapeseed Breeding, Vol. 45. Academic Press, London.

Jain, R.K., Brune, N., Friedt, W., 1989. Plant regeneration from *in vitro* cultures of cotyledon explants and anthers of *Sinapis alba* and its implications on breeding of crucifers. Euphytica, 43: 153–163.

Kameya, T., Hinata, K., 1970. Induction of haploid plants from pollen grains of Brassica. Jap. J. Breed. 20:82–87.

Keller, W.A., Rajhathy, R., Lacapra, J., 1975. *In vitro* production of plants from pollen in *Brassica campestris*. Can. J. Genet. Cytol. 17: 655–666.

Keller, W.A., Armstrong, K.C., 1978. High frequency production of microspore derived plants from *Brassica napus* anther cultures*. Z. Pflanzenzchtg.* 80: 100–108.

Keller, W.A. and Armstrong, K.C. 1979. Stimulation and embryogenesis and haploid production in *Brassica campestris* anther cultures by elevated temperature treatments. *Theor. Appl. Genet.,* 55:65–67.

Kott, L.S., 1996. Production of mutants using the rapeseed doubled haploid system. In: "Induced Mutation and Molecular Techniques for Crop Improvement. IAEA/FAO Proc. Int. Symp. Use of Induced Mutations and Molecular Techniques for Crop Improvement," p. 505–515, Vienna, Austria.

Kučera, V., Vyvadilová, M., Klíma**,** M., 2002. Utilisation of Doubled Haploids in Winter Oilseed Rape (*Brassica napus* L.) Breeding. Czech J. Genet. Plant Breed. 38, 2002 (1): 50–54.

Kumlehn, J., Kirik, V., Czihal, A., Altschmied, L., Matzk, F., Loerz, H., Bäumlein, H., 2001. Parthenogenetic egg cells of wheat: cellular

and molecular studies. Sex Plant Reprod 14: 239–243.

Lichter, R., 1982. Induction of haploid plants from isolated pollen of *Brassica napus*. Z. Pflanzenphysiol. 103: 229–237.

Liu, Y., Liu, H.Y., Zheng, Z.Y., 1997. The application of microspore culture screening disease resistance to *Sclerotinia sclerotiorum* in oilseed rape Southwest China. J. Agr. Sci. 10: 108–112.

Maluszynski, M., Ahloowalia, B.S., Sigurbjornsson, B., 1995. Application of *in vivo* and *in vitro* mutation techniques for crop improvement. Euphytica 85: 303–315.

Maluszynski, M., Kasha, K.J., Szarejko, I., 2003. Published protocols for other crop plant pecies In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 309–336.

Pratap, A., Gupta, S.K., Vikas, 2007. Advances in doubled haploid technology of oilseed rape. Ind. J. Crop Sci. 2: 267–271.

Sharma, K.K., Bhojwani, S.S., 1985. Microspore embryogenesis in anther cultures of two Indian cultivars of *Brassica juncea* (L.) Czern. *Plant Cell Tiss. Organ Cult.* 4:235–239.

Shim, Y.S., Kasha, K.J., 2003. The influence of pretreatment on cell stage progression and the time of DNA synthesis in barley (*Hordeum vulgare* L.) uninucleate microspores. Plant Cell Reprod 21: 1065–1071.

Shi, S., W., Wu, J. S., Liu, H. L., 1995. In vitro selection of long pod and dwarf mutants in *Brassica napus* L. Acta Agriculturae Nucleat ae Sinica 9 (4), 252–253.

Siebel, J., Pauls, K.P., 1989. A comparison of anther and microspore culture as a breeding tool in *Brassica napus*. Theor. Appl. Genet*.,* 78: 473–479.

Swanson, E.B., Coumans, M.P., Wu, S.C., Barsby, T.L., Beversdorf, W.D., 1987. Efficient isolation of microspore-derived embryos from *Brassica napus*. *Plant Cell Rep.,* 6:94–97.

Swanson, E.B., Coumans, M.P., Brown, G.L., Patel, J.D., Beversdorf, W.D., 1988. The characterization of herbicide tolerant plants in *Brassica napus* L. after *in vitro* selection of microspores and protoplasts. Plant Cell Rep 7: 83–87.

Swanson, E.B., Herrgesell, M.J., Arnoldo, M., Sippell, D.W., Wong, R.S.C., 1989. Microspore mutagenesis and selection Canola plants with field tolerance to the imidazolinones. Theor Appl Genet 78: 525–530.

Szarejko, I., Forster, B.P., 2006. Doubled haploidy and induced mutation. Euphytica*,* 158: 359–370.

Szarejko, I., Forster, B.P., 2007. Doubled haploidy and induced mutations. Euphytica 130: 359–370.

Takahata, Y., Keller, W.A., 1991. High frequency embryogenesis and plant regeneration in isolated microspore culture of *Brassica oleracea* L. Plant Sci*.,* 74: 235–242.

Takahata, Y., Fukuoka, H., Wakui, K., 2005. Utilization of microspore-derived embryos. p. 153–169. In: Palmer, C.F., Keller, W.A., and Kasha, K.J. (Eds.), Biotechnology in Agriculture and Forestry Vol. 56.Haploid in Crop Improvement II, Springer-Verlag, Berlin Heidelberk.

Thomas, E., Wenzel, G., 1975. Embryogenesis from microspores of *Brassica napus*. *Z. Pflanzenzucht., 74:77–81.*

Thomas, W.T.B., Forster, B.P., Getersson, B., 2003. Doubled haploids in breedings. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 337–350.

Touraev, A., Vicente, O., Heberle-Bors, E., 1997. Initiation of embryogenesis by stress. Trends in Plant Science 2, 297–302.

Xu, L., Najeeb, U., Tang, G.X., Gu, H.H., Zhang, G.Q., He, Y., Zhou, W.J., 2007. Haploid and doubled haploid technology, p. 182–216 In: Gupta, S.K. (Ed*.),* Advances in Botanical Research-Rapeseed Breeding,Vol. 45. Academic Press, London.

Wang, M., Farnham, M. W., Nannes, J. S. P., 1999. Ploidy of broccoli regenerated from microspore culture versus anther culture. Plant Breeding 118, 249–252.

Zhang, F.L., Takahata, Y., 1999. Microspore mutagenesis and *in vitro* selection for resistance to softrot disease in Chinese cabbage (*Brassica campestris* L subsp. *pekinensis*). Breed Sci 49: 161–166.

Zhang, F.l, Aoki, S., and Takahata, Y., 2003. RAPD markers linked to microspre embryogenic ability in *Brassica* crops. *Euphytica*, 131:207–213.