Chapter 18
Current Status of Doubled Haploids in Medicinal Plants

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Abstract Although herbs, spices, medicinal, and nutraceutical plants have been used in human health for millennia, there has been renewed interest over the past number of years. In the past, people have relied on landraces or "wild" plants as there has been very little breeding or genetics done on these species. This is changing as consumers are demanding scientific evidence for the medical claims (clinical trials) as well as uniformity in the products. Doubled haploidy would be one way in which to achieve homozygous, true-breeding lines. Haploidy response, i.e. callus, embryos, regenerated haploid/doubled haploid plants, have been reported in a number of medicinal/nutraceutical species, however the frequency of response is low compared to other species. This review will focus on a few of the major plant families with medicinal properties.

Keywords Apiaceae, Compositae, Labiatae, medicinal plants, nutraceuticals, Solanaceae

Introduction

There are many potential benefits for developing a doubled haploidy protocol in a species, as outlined in this book and many other reviews. This is especially so for the medicinal plants. Compared to crops like wheat, barley, *Brassica* species etc., there has been very little breeding or genetics done on the medicinal plants. This results in variation in plant populations which can cause problems if the plants are grown for commercial production to produce active compounds, conduct agronomic research, or do clinical trials. The development of doubled haploidy protocols

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would yield uniform, true-breeding lines. These homozygous lines could be incorporated into a breeding program as starting material for varietal development or parental lines in F_1 hybrids. Doubled haploidy would also allow selection of lines yielding high levels of the important medicinal compound. However, there has been very little haploidy work done on the medicinal plants (Ferrie 2006; Ferrie et al. 2005), although micropropagation and *de novo* plant regeneration has been successful in some of these species. There are several methods available for development of doubled haploids, such as androgenesis (anther culture, isolated microspore culture), gynogenesis, and wide crosses as described in other chapters of this book. Most of the success has been with androgenesis, although this depends on the species. Table 1 is a listing of reports of production of callus, embryos or haploid/doubled haploid plants for species classified as having medicinal benefits.

It would be impossible to review all medicinally important species in this chapter, therefore the focus is made on a few plant families.

Compositae

The Compositae is the largest plant family and the most diverse. There are over 1,100 genera and 20,000 species. Many of the species are economically important as foods, oilseeds, ornamentals, industrial products, and medicinals (e.g. yarrow, chamomile, feverfew). The plants within this family contain many novel secondary metabolites, fatty acids, insecticides, and industrial chemicals. Milk thistle is known for the treatment of liver disorders. The active component is silymarin, which functions as an antioxidant. *Echinacea* is one of the top five selling medicinals and is known as an immune-stimulant. *Artemisia annua* contains the antimalarial compound, artemisinin.

The Compositae are considered recalcitrant in terms of doubled haploidy. Although anther culture, isolated microspore culture, and ovule culture protocols have been reported, there are no efficient, reliable protocols available for this family. Fifteen Compositae species were screened for microspore culture response using the *Brassica napus* microspore culture protocol (Ferrie et al. 2005). There was no response from 14 of the 15 species. Swollen microspores were observed in *Eupatorium perfoliatum* (boneset). Further experimentation has resulted in callus and haploid/doubled haploid plants in several other compositae species (Ferrie 2007).

Haploid plants were regenerated from anther culture of purple coneflower (*Echinacea purpurea* L.) (Zhao et al. 2006). The authors evaluated basal media (N6, MS) and growth regulators (BA, NAA, 2,4-D). Callus was observed after 2 weeks and reached maximum production after 4 weeks. Plants were regenerated from this callus. Of the 30 plants analyzed, 19 were haploid, indicating an androgenic origin.

Hieracium pilosella (mouse-ear hawkweed) is native to Europe and North Asia and is classified as a weed and as a noxious weed in some parts of the USA. This plant contains umbelliferone, which is used in sunscreens. An anther culture

Table 1 List of nutraceutical/medicinal plant species in which doubled haploidy response has been observed. (A = anther culture, G = gynogenesis, IMC = isolated microspore culture, C = callus, E = embryos, P = plant) (modified from Ferrie 2006)

Species	Common name	Method	Results	Reference
Aconitum carmichaeli	Chinese aconite	A	C	Hatano et al. (1987)
Allium sativum	Garlic	A	C, P	Suh and Park (1986)
Ammi majus	Laceflower	IMC	E, P	Ferrie et al. (2005)
Anemone sp.		A	E	Johansson et al. (1982)
Anethum graveolens	Dill	IMC	E, P	Ferrie et al. (2005)
Angelica archangelica	Angelica	IMC	C	Ferrie et al. (2005)
Atropa belladonna	Deadly nightshade	A	E, P	Zenkteler (1971)
Azadirachta indica	Neem	A	C, P	Chaturvedi et al. (2003)
Borago officinalis	Borage	IMC	C, E	Ferrie et al. (2005)
Boswellia serrata	Indian boswellia	A	C	Prakash and Chand (1999)
Bupleurum falcatum	Bupleurum	A	C, P	Shon and Yoshida (1997); Shon et al. 2004
Camellia sinensis	Tea	A	C	Seran et al. (1999)
Carum carvi	Caraway	IMC	E, P	Ferrie et al. (2005)
Catharanthus roseus	Madagascar periwinkle	A	C	George (1985)
Digitalis sp.	Foxglove	A	C, P	Perez-Bermudez et al. (1985);
			C	Corduan and Spix (1975)
				Diettrich et al. (2000)
				Badea et al. (1985)
Datura metel	Devil's trumpet	A	E, P	Iqbal and Wijesekara (2007)
Echinacea purpurea	Purple coneflower	A	C, P	Zhao et al. (2006)
Fagopyrum esculentum	Buckwheat	A	C, P	Bohanec et al. (1993)
		G	C, P	Bohanec (1997)
Foeniculum vulgare	Fennel	A	C	Matsubara et al. (1995)
		IMC	E, P	Ferrie et al. (2005)
Gingko biloba	Gingko	IMC	E	Laurain et al. (1993)
Hepatica nobilis	Sharp-lobed hepatica	A	E, P	Nomizu et al. (2004)
Hieracium pilosella	Mouse-ear hawkweed	A	C, P	Bicknell (1996)
Hyoscyamus sp.	Henbane	A	E, C, P	Raghavan and Nagmani (1989)
		G	E, P	Chand and Basu (1998)
Hypericum perforatum	St. John's wort	A	C, P	Schulte et al. (1996)
Levisticum officinale	Lovage	IMC	E, P	Ferrie et al. (2005)
Mentha spp.	Mint	A	C	Van Eck and Kitto (1990)
Oenothera hookeri	Evening primrose	A	C, P	Martinez and de Halac (1995)

(continued)

Table 1 (continued)

Species	Common name	Method	Results	Reference
Panax ginseng	Ginseng	A	C, P	Qiquan and Anscheng (1986)
Papaver somnifernm	Opium poppy	A	C, P	Dieu and Dunwell (1988)
Physalis ixocarpa		A	E, P	Bapat and Wenzel (1982)
Psoralea corylifolia	Scurf pea	G	C, P	Chand and Sahrawat (2007)
Pimpinella anisum	Anise	IMC	E, P	Ferrie et al. (2005)
Salvia sclarea	Clary sage	A	C	Bugara (1986)
Saponaria vaccaria	Cow cockle	IMC	E, P	Kernan and Ferrie (2006)
Sesamum indicum	Sesame	A	E	Govil and Singh (1982)
Silene latifolia	Bladder campion	A	E, P	Safarova et al. (2005)
Zingiber officinale	Ginger	A	C, P	Samsudden et al. (2000)

method was developed for an apomictic biotype of *H. pilosella* (Bicknell 1996). Callus developed from both the somatic tissue and the microspores. The uni-nucleate stage of microsporogenesis was the most responsive in terms of callus development. The regenerated plants showed segregation for apomixis as well as a range of ploidy levels.

Labiatae

The Labiatae (Lamiaceae) family is another large family with 224 genera and over 3,200 species. This family is known for their aromatic qualities. The essential oils found in many of the Labiatae are used for pharmaceutical and cosmetics purposes. The important commercially grown species include mint, basil, oregano, rosemary, sage, and thyme. Rosemary is used in food, cosmetics, and as a medicinal. The German government has approved internal use of rosemary for indigestion and as a supportive treatment for rheumatism. External use of rosemary is approved for circulation problems. Essential oils include cineole, B-pinene, camphor, and limonene. Lemon balm contains polyphenols and is used for insect repellents, cosmetics, foods, and medicines. Oregano is also used in foods and medicines. Oregano contains carvacrol and thymol, which have anti-helmintic and anti-fungal properties.

Tissue culture regeneration has been reported in some of the Labiatae species, however, no reliable doubled haploidy protocols are available for any of the species within this family. Fourteen Labiatae species were screened for microspore culture response (Ferrie et al. 2005); swollen microspores were observed in only three

[anise-hyssop (*Agastache foeniculum*), mint (*Mentha piperita*), marjoram (*Origanum marjorana*)] of the 14 species. Haploid embryos have been observed in *Salvia sclarea* (Bugara 1986) and callus was observed from anthers of *Mentha* species (Van Eck and Kitto 1990), but no plants were regenerated.

Apiaceae

The Apiaceae (carrot family) consists of vegetables, herbs, and spices. Although there are tissue culture protocols for the Apiaceae, this group of plants is also considered recalcitrant for doubled haploidy methodology. Nineteen Apiaceae species were screened for microspore culture response using the *Brassica napus* standard protocol (Ferrie et al. 2005). Response was observed and with further modifications to the protocol, microspore-derived embryos were observed in 10 of the 19 species, with haploid/doubled haploid plants generated in 8 of these. Haploid and doubled haploid plants were regenerated in dill (*Anethum graveolens*), caraway (*Carum carvi*), carrot (*Daucus carota*), fennel (*Foeniculum vulgare*), lovage (*Levisticum officinale*), parsnip (*Pastinaca sativa*), anise (*Pimpinella anisum*), and laceflower (*Amni majus*). Field evaluation of dill, caraway, and fennel doubled haploid lines has resulted in the identification of lines with different agronomic characteristics and beneficial biochemical profiles (Ferrie, in preparation).

Bupleurum falcatum is used in Chinese medicine as a painkiller, and as an anti-inflammatory, anti-allergy, and anti-pyretic. Anther culture studies have resulted in callus formation and haploid plantlet regeneration (Shon and Yoshida 1997; Shon et al. 2004). Differences in anther culture response were observed among the genotypes and the habitats in which the genotypes were grown.

Solanaceae

The Solanaceae (potato family) is an economically important family which includes herbs, shrubs, trees, fruits, vegetables, and medicinals. The first *in vitro* haploid embryos and plantlets derived from culturing anthers was observed in *Datura* in the 1960's (Guha and Maheshwari 1964; Guha and Maheshwari 1966). Several of the Solanaceae species have exhibited embryogenic properties (i.e. tobacco).

Atropa belladonna (deadly nightshade) is a member of the solanaceae that contains a number of alkaloids including hyoscyamine. This compound is used to dilate the eye pupils, as an antispasmodic for respiratory problems, and for rheumatic and muscular pain. Microspore-derived embryos and haploid plants have been produced from this species (Zenkteler 1971). Microspore-derived embryos from *A. belladonna* have also been cryopreserved and regenerated (Bajaj 1978). This could be beneficial in germplasm conservation.

Hyoscyamus (henbane) species contain tropane alkaloids. Some of the early developmental work was done on *H. niger* (Nagmani and Raghavan 1983; Raghavan 1978; Sunderland and Wildon 1979). Embryos of *H. niger* were produced, however plantlets were not obtained (Nagmani and Raghavan 1983; Raghavan 1978). Embryogenesis and plant regeneration was obtained from unpollinated ovaries of *H. muticus* L. (Chand and Basu 1998). Plant regeneration occurred from callus cultures.

Some *Physalis* species contain steroids that are of medicinal importance. Haploid plants have been regenerated via microspore embryogenesis in *P. ixocarpa* Brot. A cold treatment (3°C for 2 days) of the anthers was required. NN basal medium supplemented with IAA and Kinetin resulted in embryo formation. For further embryo development, coconut milk was essential. Haploid plants were regenerated from these embryos. Steroidal levels of the regenerated plantlets were not evaluated (Bapat and Wenzel 1982).

Other Medically Important Species

Opium poppy (*Papaver somniferum* L.) is a source of many alkaloids including morphine, codeine, and thebaine. Anther culture was reported (Dieu and Dunwell 1988) with differences observed among the genotypes evaluated. The authors also observed that growth regulators (2,4-D, NAA, Kinetin) were necessary for callus production and a cold treatment (7°C) of 7 days was beneficial. A total of 140 plants were regenerated, of those, 63 were analyzed for ploidy using chromosome counts. Of the 63 plants analyzed, only one was haploid, two had both haploid and diploid cells whereas 60 were diploid.

The Ranunculaceae (buttercup family) is found throughout the world. Many are used as ornamentals and as a source of alkaloids. Many species are highly toxic, whereas other have uses in traditional medicine (i.e. black cohosh). In some cases, overharvesting of these species for medicinal use has resulted in near extinction of the plant. Developing a doubled haploidy protocol would not only be beneficial for developing uniform lines but for germplasm preservation. *Hepatica nobilis* was once used as a medicinal herb to treat liver disorders, but is now marketed commercially as an ornamental. Anther culture techniques have been developed and have resulted in haploid plants (Nomizu et al. 2004). Activated charcoal was required for embryo induction although not for regeneration. A temperature shock of 35°C was also beneficial for embryo induction.

Catharanthus roseus L. (Madagascar periwinkle) belongs to the Apocynaceae family. This plant contains vincristine and vinblastine, which are extracted commercially from the leaves to be used as a cancer treatment, especially for leukemia and Hodgkin's disease. Anther culture has been reported (George 1985). Early globular stage embryos were observed, but no further development occurred.

The leaves of *Digitalis* species (e.g. *D. purpurea, D. lanata*) contain glycosides, especially digitoxin and digoxin. These compounds are used as standard medicines for treatment of heart disorders. Androgenesis studies have been conducted on these

species, however most response comes via callus proliferation and not direct embryogenesis (Perez-Bermudez et al. 1985; Corduan and Spix 1975; Badea et al. 1985). Very few plants have been obtained. Isozyme analysis has shown that callus/plants were obtained from the microspore (Corduan 1976). Cardenolides have also been analyzed from haploid *D. lanata* plants (Diettrich et al. 2000). Variation in total amount of cardenolides and in the levels of different cardenolides was observed.

Psoralea corylifolia (Papillionaceae) is used in Indian Ayurveda medicine for teeth care, diarrhoea, bronchitis, and inflammation. This species is also an endangered plant. Haploid plants were obtained via gynogenesis (Chand and Sahrawat 2007). Of the 13 regenerated plants that were evaluated, 11 were haploid, whereas 2 were diploid. There was no mixoploidy or albinism observed.

Conclusion

Over the past decade, there has been resurgence in the use of herbs, spices, and medicinal plants for prevention and treatment of medical problems. Consumers have many products from which they can choose. Studies have shown that uniformity of the product is not always reliable, which can result in conflicting clinical trial reports and perhaps less than optimum efficacy of the product. The majority of the medicinal plants used in product formulations are from wild plant populations which have inherent variability in the levels of active compounds. Very little plant breeding or genetics research has been conducted on these medicinal plant species. The development of plant breeding systems and protocols for doubled haploid plant production would be extremely beneficial for this group of plants.

As described in this chapter, there has been very little doubled haploid research conducted on these plants, although some progress has been made. Screening studies have been conducted, in which a number of species have been evaluated using standard microspore culture protocols. Optimization is then required in order to develop an efficient protocol that can be used for practical and basic research. Factors influencing embryogenesis include donor plant growth conditions, genotype, pretreatments, developmental stage of the explants, media composition, and culture conditions. Once an efficient protocol has been developed, breeders can use the methodology and the resulting doubled haploid lines for breeding, mutagenesis, and transformation. Doubled haploid plants would also be useful for studying biochemistry and physiology of the plants and the important biochemical pathways, similar to what the *Brassica, Hordeum*, and tobacco groups have been doing for years.

References

Badea E, Iordan M, Mihalea A (1985) Induction of androgenesis in anther culture of *Digitalis lanata*. Revue Roumaine de biologie. Serie de Biologie vegetale 30:63–71

Bajaj YPS (1978) Effect of super-low temperature on excised anthers and pollen-embryos of *Atropa*, *Nicotiana* and *Petunia*. Phytomorphology 2:171–176

Bapat VA, Wenzel G (1982) *In vitro* haploid plantlet induction in *Physalis ixocarpa* Brot. through microspore embryogenesis. Plant Cell Rep 1:154–156
Bicknell RABNK (1996) Isolation of reduced genotypes of *Hieracium pilosella* using anther culture. Plant Cell Tiss Org Cult 45:37–41
Bohanec B (1997) Haploid induction in buckwheat (*Fagopyrum esculentum* Moench). In Jain SM, Sopory SK, Veilleux RE (eds), *In Vitro* Haploid Production in Higher Plants. Kluwer, Dordrecht, 4:163–170
Bohanec B, Neskovic M, Vujicic R (1993) Anther culture and androgenetic plant regeneration in buckwheat (*Fagopyrum esculentum* Moench). Plant Cell Tiss Org Cult 35:259–266
Bugara AM (1986) Embryoidogenesis in anther culture of *Salvia sclarea*. Fiziologiia I Biokhimiia Kulturnykh Rastenii. Physiol Biochem Cultivated Plants 18:381–386
Chand S, Basu P (1998) Embryogenesis and plant regeneration from callus cultures derived from unpollinated ovaries of *Hyoscyamus muticus* L. Plant Cell Rep 17:302–305
Chand S, Sahrawat AK (2007) Embryogenesis and plant regeneration from unpollinated ovary culture of *Psoralea corylifolia*. Biologia Plantarum 51:223–228
Chaturvedi R, Razdan MK, Bhojwani SS (2003) Production of haploids of neem (*Azadirachta indica* A. Juss.) by anther culture. Plant Cell Rep 21:531–537
Corduan G (1976) Isozyme variation as an indicator for the generative or somatic origin of anther-derived plants of *Digitalis purpurea* L. Z. Pflanzenzuchtg 76:47–55
Corduan G, Spix S (1975) Haploid callus and regeneration of plants from anthers of *Digitalis purpurea* L. Planta 124:1–11
Diettrich B, Ernst S, Luckner M (2000) Haploid plants regenerated from androgenic cell cultures of *Digitalis lanata*. Planta Medica 66:237–240
Dieu P, Dunwell JM (1988) Anther culture with different genotypes of opium poppy (*Papaver somniferum* L.): effect of cold treatment. Plant Cell Tiss Org Cult 12:263–271
Ferrie AMR (2006) Doubled haploid production in nutraceutical species: a review. Euphytica 158:347–357
Ferrie AMR (2007) Developing double haploidy breeding methods for nutraceutical species. In Proceedings of The Natural Health Products Research Society of Canada and The Canadian Herb, Spice, and Natural Health Products Coalition Conference: Tradition to Technology, Saskatoon, Canada. May 10–13
Ferrie AMR, Bethune T, Kernan Z (2005) An overview of preliminary studies on the development of doubled haploid protocols for nutraceutical species. Acta Physiol Plant 27:735–741
George L (1985) Anther culture of *Catharanthus roseus* L. – development of pollen embryoids. Curr Sci 54:641–642
Govil CM, Singh VRR (1982) Induction of haploids in anther culture of *Sesamum indicum*. In Proceedings of the 5th International Congress Plant Tissue and Cell Culture. pp. 545–546
Guha S, Maheshwari SC (1964) *In vitro* production of embryos from anthers of *Datura*. Nature 204:497
Guha S, Maheshwari SC (1966) Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature 212:97–98
Hatano K, Shoyama Y, Nishioka I (1987) Somatic embryogenesis and plant regeneration from the anther of *Aconitum carmichaeli* Debx. Plant Cell Rep 6:446–448
Iqbal MCM, Wijesekara KB (2007) A brief temperature pulse enhances the competency of microspores for androgenesis in *Datura metel*. Plant Cell Tiss Org Cult 89:141–149
Johansson L, Andersson B, Eriksson T (1982) Improvement of anther culture technique: activated charcoal bound in agar medium and elevated CO2 concentration. Physiol Plant 54:24–30
Kernan Z, Ferrie AMR (2006) Microspore embryogenesis and the development of a double haploidy protocol for cow cockle (*Saponaria vaccaria*). Plant Cell Rep 25:274–280
Laurain D, Trémouillaux-Guiller J, Chénieux J (1993) Embryogenesis from microspores of *Ginkgo biloba* L., a medicinal woody species. Plant Cell Rep 12:501–505
Martinez LD, de Halac IN (1995) Organogenesis of anther-derived calluses in long-term cultures of *Oenothera hookeri* de Vries. Plant Cell Tiss Org Cult 42:91–96

Matsubara S, Dohya N, Murakami K (1995) Callus formation and regeneration of adventitious embryos from carrot, fennel and mitsuba microspores by anther and isolated microspore cultures. Acta Hortic 392:129–137

Nagmani R, Raghavan V (1983) Induction of embryogenic divisions in isolated pollen grains of *Hyoscyamus niger* in a single-step method. Z.Pflanzenphysiol 109:87–90

Nomizu T, Niimi Y, Han D-S (2004) Haploid plant regeneration via embryogenesis from anther cultures of *Hepatica nobilis*. Plant Cell Tiss Org Cult 79:307–313

Perez-Bermudez P, Cornejo MJ, Segura J (1985) Pollen plant formation from anther cultures of *Digitalis obscura* L. Plant Cell Tiss Org Cult 5:63–68

Prakash DVSSR, Chand S (1999) In vitro response from cultured anthers of *Boswellia serrata* Roxb. (Burseraceae). In Plant Tissue Culture and Biotechnology: Emerging trends. Universities Press, Hyderabad, India, pp. 226–231

Qiquan S, Anscheng L (1986) Somatic embryogenesis and plant regeneration from anther culture of *Panax quinquifolius* (Ginseng). Int Plant Biotech Network 6:2

Raghavan V (1978) Origin and development of pollen-embryoids and pollen calluses in cultured anther segments of *Hyoscyamus niger* (Henbane). Am J Bot 65:984–1002

Raghavan V, Nagmani R (1989) Cytokinin effects on pollen embryogenesis in cultured anthers of *Hyoscyamus niger*. Can J Bot 67:247–257

Safarova D, Kopecky D, Vagera J (2005) The effect of a short heat treatment on the *in vitro* induced androgenesis in *Silene latifolia* ssp. *alba*. Biol Plantarum 49:261–264

Samsudden K, Babu KN, Divakaran M, Ravindran PN (2000) Plant regeneration from anther derived callus cultures of ginger (*Zingiber officinale* Rosc.). J Hortic Sci Biotechnol 75:447–450

Schulte J, Büter B, Schaffner W, Berger K (1996) Gametic embryogenesis in *Hypericum* spp. In Pank F (ed) International Symposium on Breeding Research on Medicinal and Aromatic Plants. Quedlinburg, Germany, pp. 307–310

Seran TH, Hirimburegama K, Hirimburegama WK, Shanmugarajah V (1999) Callus formation in anther cultures of tea clones, *Camellia sinensis* (L.) O. Kuntze. J Natl Sci Found Sri Lanka 27:165–175

Shon T-K, Kim S-K, Acquah D, Lee S-C (2004) Haploid plantlet production through somatic embryogenesis in anther-derived callus of *Bupleurum falcatum*. Plant Prod Sci 7:204–211

Shon T-K, Yoshida T (1997) Induction of haploid plantlets by anther culture of *Bupleurum falcatum* L. Jpn J Crop Sci 66:137–138

Suh SK, Park HG (1986) Studies on the anther culture of garlic (*Allium sativum* L.). J Korean Soc Hortic Sci 27:89–95

Sunderland N, Wildon DC (1979) A note on the pretreatment of excised flower buds in float culture of *Hyoscyamus* anthers. Plant Sci Lett 15:169–175

Van Eck JM, Kitto SL (1990) Callus initiation and regeneration in *Mentha*. Hortscience 25:804–806

Zenkteler M (1971) In vitro production of haploid plants from pollen grains of *Atropa belladonna* L. Experientia 27:1087

Zhao F-C, Nilanthi D, Yang Y-H, Wu H (2006) Anther culture and haploid plant regeneration in purple coneflower (*Echinacea purpurea* L.). Plant Cell Rep 86:55–62