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Secondary somatic embryogenesis and applications in plant breeding

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Summary

Secondary somatic embryogenesis is the phenomenon whereby new somatic embryos are initiated from somatic embryos. Such cultures have been described in at least 80 Gymnosperm and Angiosperm species. In the initial step (primary somatic embryogenesis) such cultures have to be started from plant explants. In general, primary somatic embryogenesis from vegetative plant explants is, indirect and mostly driven by auxin (AUX) or auxin and cytokinin (AUX/CYT) supplemented media, whereas, from zygotic embryos it is direct and driven, to a larger extent, by CYT or growth regulator free media. Primary somatic embryogenesis from floral plant explants is between these two extremes. Indirect and direct somatic embryogenesis should be seen as two extremes of one continuum: in indirect somatic embryogenesis the embryos develop up to the (pre)-globular stage and in direct somatic embryogenesis to mature stages before they are subjected to secondary embryogenesis. In general, secondary embryogenesis requires no growth regulators in species with CYT driven primary embryogenesis. Whereas, continuous exposure to growth regulators is needed in species with CYT/AUX or AUX driven primary embryogenesis.

In most species somatic embryos can be converted into shoots, although the frequencies are mostly low. In general, somatic embryos induced by growth regulator free or CYT supplemented media meet more difficulties in shoot development than embryos induced by AUX supplemented media. Applications of secondary somatic embryogenesis for plant breeding are discussed.

Introduction

Somatic embryogenesis has been described in at least 200 Gymnosperm and Angiosperm species (Evans et al., 1981; Tisserat et al., 1979; Tulecke, 1987). It is defined as the process in which a bipolar structure arises through a series of stages characteristic for zygotic embryo development and having no vascular connection with the parental tissue (Ammirato, 1987; Sharp et al., 1980; Terzi & Loschiavo, 1990). Sharp et al. (1980) and Evans et al. (1981) distinguished direct and indirect somatic embryogenesis. Direct somatic embryogenesis proceeds from already pre-embryogenic determined cells and indirect somatic embryogenesis from cells which require redifferentiation before they can express embryogenic competence. As a consequence callus formation precedes the formation of embryos. Cells capable of direct somatic embryogenesis are physiologically similar to those in zygotic embryos. They are frequently found either in tissue before the onset of embryogenesis (i.e. in the flower organ) or in the developing zygotic embryo. In these cells the genes necessary for zygotic embryogenesis are active in varying degrees (Carman, 1990; Sharp et al., 1980). The ease at which induction of somatic embryogenesis occurs, can be seen as a 'memory' of pathways either previously or just after (Carman, 1990). Cells of tissue which are in time or space more diverged from zygotic embryo explants need a greater amount of reprogramming of previously active developmental pathways before they reach the embryogenic ground state (Carman, 1990; Sharp et al., 1980; Tulecke, 1987). The ability to express embryogenesis is often restricted to a small developmental window. In many species immature have and mature zygotic embryos have not the ability to express somatic embryogenesis

(Finer, 1987, Gingas, 1991; Jia & Chua, 1992; Southworth & Kwiatkiowski, 1991; Tenning et al. 1992; Tulecke & McGranaham, 1985). Direct and indirect somatic embryogenesis should be considered as two extremes of a continuum (Carman, 1990; Wann, 1988; Williams & Maheswaran, 1986). It is not always clear which type occurs or both direct and indirect can be observed. Emonds (1994) argued that in many systems where embryogenesis has been described as indirect, the embryogenic callus is organized in young embryos (pre-embryogenic masses or (pre-)globular embryos) and that the type of embryogenesis is depending on the time which the inductive growth regulator is applied. If that period is short the process will be direct and if it is long than indirect.

Somatic embryos have shown to be an excellent source for secondary embryos. It is associated with loss of integrated group control of cells organized in the somatic embryos. Some cells break away from group control and initiate new somatic embryos (Williams & Maheswaran, 1986). In most cases somatic embryos develop up to pre-embryogenic masses (PEM's) or globular embryos, without differentiations into organs, before they are subjected to secondary embryogenesis (indirect embryogenesis). Such cultures are difficult to synchronize and it is difficult or impossible to distinguish between different cycles of secondary embryogenesis. In other cases embryos develop up to maturity (direct embryogenesis). In these cases it is often more easy to distinguish between different cycles of secondary embryogenesis. In such cultures secondary embryos are formed from cotyledons (Maheswaran & Williams, 1986), hypocotyls (Kato, 1989; Plata & Vieitez, 1990), roots (Vieitez & Barciela, 1990) or combinations of these organs (Gui et al., 1991; Polito et al. 1989; Tenning et al., 1992). In most cases the embryos originated from epidermal and/or subepidermal cells; either from single cells (Polito et al., 1989; Thomas et al., 1976), from multiple cells (Maheswaran & Williams, 1986; Plata & Vieitez, 1990; Raemakers, 1993) or from both (Liu et al., 1992; Pence et al., 1980). A mesophyll origin was observed in *Dactylis glomerata* (Trigiano et al., 1989) and *Manihot esculenta* (Raemakers, 1993).

This review tries to evaluate the different tissue culture procedures used to obtain somatic embryogenesis in Gymnosperm and Angiosperm species. It further describes the methods used to obtain cultures which continuously proliferates new embryos by secondary somatic embryogenesis. The tissue culture procedures in the different species have been grouped to make a comparison within and between species possible. The review does not go in to detail on other important subjects as biochemistry and molecular biology of somatic embryogenesis (Choi & Sung, 1989; Engelen & de Vries, 1992; de Jong et al., 1993; Komamine et al., 1992), relation between somatic embryogenesis and adventitious shoot formation (Ranchi, 1990) or the genetics and heritability of somatic embryogenesis (Evans et al., 1981; Parrott et al., 1991). In Table 1 some characteristics of primary and secondary somatic embryogenesis in Gymnosperm and Angiosperm species is given. Primary embryogenesis is the process in which embryos are formed from plant explants and secondary embryogenesis is the process in which embryos are formed from embryos.

Primary and secondary somatic embryogenesis in Gymnosperms

In the Gymnosperm species listed in Table 1, primary embryos were solely initiated from zygotic embryos. Although vegetative explants (Ruaud et al., 1992) and floral explants (Nagmani & Bonga, 1985) have also been reported to be embryogenic. Mostly media supplemented with cytokinin plus auxin (CYT/AUX) were used. *Abies nordmanniana* required CYT supplemented media. In the genus Pinus also AUX supplemented medium had the capacity to induce primary embryogenesis (Gupta & Durzan, 1991). The origin of the primary embryos had been described as indirect (consisting of pre-embryogenic masses or pre-globular embryos). These young stages are subjected to secondary embryogenesis. In *Picea abies* (Mo et al., 1989) and *Pinus taede* (Gupta & Durzan, 1991) transfer of these young embryos to medium with ABA ensured maturation which will give secondary embryos after transfer to CYT/AUX medium. A detailed description of somatic embryogenesis in *Picea* and *Pinus* species has been given in several reviews (Adu-Ampomak et al., 1988; Durzan, 1988; Tautorius et al., 1991).

Primary and secondary somatic embryogenesis in Angiosperms

Monocot species

In Angiosperm monocots, primary embryogenesis was exclusively induced by AUX supplemented media. Furthermore, mostly synthetic auxins with strong

Table 1. Examples of secondary somatic embryogenesis in Gymnosperm and Angiosperm species

	Primary embryogenesis			Growth regulators used					Ref
	Explant	Origin	Sec. emb. explant	No	CYT	CYT AUX	AUX	Shoots (%)	
Gymnosperms									
Abies nordmanniana	z.emb	I	g.emb	-	+	-	-	no	Norgaard & Krogstrup, 1991
Larix decidua × *leptolepis*	z.emb	I	emb	-	-	+	-	30	Klimaszewska, 1989
Picea abies	z.emb	I	g.emb/m.emb	-	-	+	-	5	Arnold, 1987; Mo et al., 1989
P. glauca	z.emb	I	g.emb	-	-	+	-	low	Kong & Yeung, 1992
P. mariana	z.emb	I	g.emb	-	-	+	-	low	Tremblay & Tremblay, 1991
P. glauca × *engelmanni*	z.emb	I	g.emb	-	-	+	-	low	Eastman et al., 1991
P. rubens	z.emb	I	g.emb	-	-	+	-	low	Tremblay & Tremblay, 1991
P. sitchensis	z.emb	I	g.emb	-	-	+	-	no	Roberts et al., 1991
Pinus strobus	z.emb	I	g.emb	-	-	+	-	no	Finer et al., 1989
P. taeda	z.emb	I	g.emb/m. emb	-	-	+	-	low	Gupta & Durzan, 1991
Angiosperms (monocots)									
Asparagus officinalis	male apex	I	emb	+[c]	-	-	+	yes	Delbreil & Marc, 1992
Hemerocallis sp.	ovary	I	g.emb	-	-	-	+	yes	Krikorian & Kann, 1981
Dactylis glomerata	leaf	B	emb	-	-	-	+	95	Conger et al., 1983
Oryza sativa	z.emb	I	emb	-	-	-	+	yes	Jones & Rost, 1989
Panicum maximum	z.emb	I	g.emb	-	-	-	+	yes	Lu & Vasil, 1981
Pennisetum americanum	inflor.	I	g.emb	-	-	-	+	yes	Tremblay & Tremblay, 1991
Triticum aestivum	z.emb	I	g.emb	-	-	+	+	yes	Carman, 1988
Zea mays	apex, z.emb	B	emb	-	-	+	+	80	Emonds & Kieft, 1991; Zhong et al., 1992
Angiosperms (dicots)									
Acanthopanax senticosus	z.emb (c,e)	D	t.emb (c,e,h)	-	-	+[c]	+	75	Gui et al., 1991
Aesculus hippocastanum	z.emb (r)	D	c.emb (r)	-	-	+	-	1	Kiss et al., 1992
	filament	B	c.emb (r)	-	-	+[c]	+		
Apium graveolens	leaf	I	g.emb	-	-	+	+	100	Nadel et al., 1989; Nadel et al. 1990
Arachis hypogaea	z.emb (c,e), leaf	I	g.emb	-	-	-	+	5	Baker & Wetzstein, 1992; Durham & Parrott, 1992; Sellars et al., 1990
A. paraguariensis	z.emb (c,e)	I	g.emb	-	-	-	+	yes	Sellars et al., 1990
Atropa beladonna	anth.	D	emb (h)	+	-	+	-	yes	Rashid & Street, 1974
Beta vulgaris	z.emb (h)	I	emb (h,c,r)	-	-	-	+	yes	Tenning et al., 1992
Brassica campestris	z.emb (h)	D	emb (c,h)	+[p]	+	-	-	yes	Maheswaran & Williams, 1986
B. napus	m.sp./anth.	D	emb (c)	+[c]	+[c]	+[c]	+[p]	yes	Thomas & Wenzel, 1975
	z.emb (h)	D	emb (h)	+[c]	+	-	-	yes	Pretova & Williams, 1986
B. juncea	m.sp.	D	emb	-	-	+	-	yes	Prabhudesai & Bhaskaran, 1993
Camellia japonica	z.emb (c,e), root	D	emb (h,e,c)	+	+	+	+	72	Vieitez & Barciela, 1990; Vieitez et al. 1991
C. reticulata	z.emb (c,e)	D	emb (h)	+	+	+	+	yes	Plata & Vieitez, 1990; Plata et al., 1991
C. sinensis	z.emb (c)	D	emb (h)	+	+	+	+	21	Jha et al., 1992
Carica papaya	ovule	I	g.emb	-	-	-	+	yes	Litz & Conover, 1983
Carum carvi	petiole	I	g.emb	+	-	-	-	yes	Ammirato, 1977
Citrus microcarpa	nucellus	D	g.emb	+	+	-	-	yes	Rangaswamy, 1961

Table 1. continued

	Primary embryogenesis			Growth regulators used					Ref
	Explant	Origin	Sec. emb. explant	No	CYT	CYT AUX	AUX	Shoots (%)	
C. paradisi	nucellus	D	emb	+	+	+	-	yes	Kochba et al., 1972
Citrus sinensis	ovule	D	emb	+	+	+	-	yes	Kochba et al., 1972
Clitoria ternatea	z.emb (r,h,c)	B	emb (r,h)	+	+	+	-	72	Dhahnalakshmi & Lakshmanan, 1992; Lakshmanan & Dhahnalakshmi, 1990
Cucurbita pepo	z.emb	I	emb	-	-	+	+	yes	Jelaska, 1972
Daucus carota	z.emb (c,r,h)	B	emb (r,c,h)	+	-	-	+	94	Smith & Krikorian, 1989
Elaeis guineensis	leaf	I	g.emb	-	-	-	+	18	De Touchet et al., 1991
Euphoria longan	leaf	I	c.emb (r)	-	-	+	-	yes	Litz, 1988
Eucalyptus citriodora	z.emb	D	t.emb	-	-	-	+	50	Muralidharan & Mascarenhas, 1987; Muralidharan et al., 1989
Euphorbia pulcherrima	apex	I	g.emb	-	-	+	-	yes	Preil et al., 1982
Fagopyrum esculentum	z.emb	B	emb (c,h)	-	-	+	-	low	Neskovic et al., 1987
Fagus sylvatica	z.emb	I	g.emb	-	-	+	-	10	Vieitez et al., 1992
Glycine max	z.emb (c)	B	g.emb	-	-	-	+	90	Liu et al., 1992; Sellars et al., 1990
G. canescens	z.emb (c)	B	g.emb	-	-	-	+	90	Sellars et al., 1990
Helianthus annuus	z.emb (c)	D	emb	-	-	-	+	low	Finer, 1987
Ilex aquifolium	z.emb (c,e)	D	c.emb (c)	+	-	-	-	yes	Hu & Sussex, 1972
Juglans hindsii	z.emb	D	c.emb (r,c)	+[c]	+	-	-	low	Tulecke & McGranahan, 1985
J. major	z.emb	D	c.emb (r,c,h)	+[c]	+	-	-	low	Cornu, 1988
J. nigra	z.emb	D	c.emb (r,c,h)	+[c]	+	-	-	low	Cornu, 1988; Deng & Cornu, 1992
J. nigra × *regia*	z.emb	D	c.emb (r,c,h)	+[c]	+	-	-	low	Cornu, 1988; Tulecke & McGranahan, 1985
J. regia	z.emb	D	c.emb (r,c,h)	+[c]	+	-	-	low	Tulecke & McGranahan, 1985
Limnantes alba	z.emb (c,h)	D	emb (h)	-	+	+	-	no	Southworth & Kwiatkiowski, 1991
Liquidambar styraciflua	z.emb	I	emb (h,c)	+	+	-	-	yes	Sommer & Brown, 1980
Magnolia spp	z.emb	B	t.emb (r)	-	-	+	+	25	Merkle & Wiecko, 1990
Malus domestica	nucellus	D	emb (c)	+	-	-	-	no	Eichholz et al., 1979
M. pùmila	nucellus	D	emb (c)	+	+	+	-	no	James et al., 1984
Manihot esculenta	z.emb (c,e), leaf	D	emb (c,e)	-	-	-	+	32	Raemakers, 1993; Stamp & Henshaw, 1987; Szabados et al., 1987
Mangifera indica	nucellus	I	g.emb	-	-	+[c]	+	yes	DeWald et al., 1989; DeWald et al., 1989
Medicago sativa	z.emb (h), leaf	I	t.emb (c,e,h)	+[c]	-	+	-	yes	Lupotto, 1982; Lupotto, 1986; Parrott & Bailey, 1993
Myrciaria cauliflora	ovule	I	g.emb	-	-	-	+	yes	Litz, 1984
Pharbitis nil	z.emb (h)	D	emb (r)	-	-	-	+	yes	Jelaska, 1972
Poncirus trifoliata	nucellus	D	g.emb	-	+	+	+[c]	yes	Matsumoto & Yamagychi, 1983
Populus ciliata	leaf	I	g.emb (h,c)	-	-	-	+	yes	Cheema, 1989
Prunus persica	z.emb (r)	D	emb	-	-	+	-	yes	Bhansali et al., 1990
P. incisa × *P. serula*	z.emb	I	emb	+[c]	+	-	-	yes	Druart, 1990
Pterocarye spp	emb	D	emb (r,c)	+[c]	+	-	-	low	Tulecke & McGranahan, 1985
Querqus alba	z.emb	B	emb (c)	+[c]	+	+	-	3	Gingas & Lineberger, 1989
Q. bicolar	o flower	I	emb (c)	+[c]	-	-	+	yes	Gingas, 1991
Q. rubra	z.emb	B	emb (c)	+[c]	+	+	-	yes	Gingas & Lineberger, 1989

Table 1. continued

	Primary embryogenesis			Growth regulators used					Ref
	Explant	Origin	Sec. emb. explant	No	CYT	CYT AUX	AUX	Shoots (%)	
	o flower	I	emb (c)	+[c]	-	-	+	yes	Maâtaoui et al., 1990
Q. suber	stem	I	emb (r)	+[c]	-	+	-	no	Maâtaoui et al., 1990
Ranunculus sceleratus	floral buds	I	emb (h), plant	-	-	-	+	44	Konar & Ntaraja, 1969
Rauvolfia vomitoria	leaf	I	c.emb	-	-	-	+	yes	Trémouillaux-Guiller & Chénieux, 1991
Ribes rubrum	ovule	?	c.emb	+	+	-	-	low	Zatykó et al., 1976
Theobroma cacao	z.emb	B	emb (c,e)	+	+	+	+	yes	Adu-Ampomah et al., 1988; Aguilar et al., 1992; Pence et al., 1980
Trifolium repens	z.emb (h)	D	t.emb (a,c)	-	+	-	-	yes	Maheswaran & Williams, 1986
Vitis longii	z.emb (c,e)	D	g.emb	-	-	+	+		Stamp & Meredith, 1988
	anth.	I	emb (r,h)	+[c]	-	+	-	50	Gray & Mortensen, 1987
V. rupestris	anth.	D	g.emb	-	-	+	+	41	Stamp & Meredith, 1988
	leaf	I	g.emb	-	-	+	+[c]	yes	Martinelli et al., 1993
V. rupestris × *vinifera*	anth.	I	g.emb	-	-	+	-	yes	Stamp & Meredith, 1988
V. vinifera	z.emb, leaf, anth.	D	g.emb	-	-	+	-	36	Stamp & Meredith, 1988; Stamp & Meredith, 1988

Abbreviations: Explant: anth.: anther, c: cotyledon, c.emb: cotyledonary embryo, e: embryogenic axis, g.emb: globular embryo or younger stages of development, h: hypocotyl, inflor.: inflorescence, m.sp.: micro spores, r., root, t.emb: torpedo-shaped embryo and z.emb: zygotic embryo.
Origin of embryogenesis: B: both direct and indirect, D: direct, I: indirect, ?: unknown.
Growth regulators used: [c]: only in secondary embryogenesis, [p]: only in primary embryogenesis.

effects such as Picloram, Dicamba or 2,4-D were used. An exception is *Asparagus officinalis* (Conger et al., 1983), where also the natural auxin NAA had that capacity. Mostly primary embryos were formed indirect. In *Dactylis glomerata* (Conger et al., 1983) and *Zea mays* (Zhong et al., 1992) the origin was both direct and indirect. In most species a continuous exposure to growth regulators is needed for secondary embryogenesis. However, in *Asparagus officinalis* (Delbreil & Marc, 1992) secondary embryogenesis was driven by growth regulator free medium.

Dicot species

In 51% of the dicots, listed in Table 1, primary somatic embryos were initiated from zygotic embryos in 23% of floral explants and in 3% of vegetative explants. In the remaining 23% both zygotic embryos with either vegetative or floral explants were used. Usually this was accomplished on the same media, except in *Aesculus hippocastanum* (Kiss et al., 1992), *Brassica napus* (Pretova & Williams, 1986; Thomas & Wenzel, 1975; Thomas et al., 1976) and *Querqus rubra* (Gingas & Lineberger, 1989; Gingas, 1991) where floral explants required AUX supplemented and zygotic embryos AUX-free medium. In most species primary somatic embryos can be induced on a wider range of explants than shown in Table 1 (for references see cited literature) and most likely all these embryos can be used for secondary embryogenesis.

There is a large variation of growth regulators used to induce somatic embryogenesis in dicot species. In 17 of the 65 dicot Angiosperm species listed in Table 1, primary somatic embryogenesis was induced on growth regulator free medium and in 13 of these species also CYT and/or CYT/AUX had that capacity. CYT supplemented media were used in 25 species. BA was used most frequently (57%) followed by kinetin (37%), zeatin (3%) and TDZ (3%). AUX supplemented media were applied in 29 dicot species. 2,4-D was most frequently used (49%) followed by NAA (27%), IAA (6%), IBA (6%), Picloram (5%) and Dicamba (5%). AUX/CYT media were used in 31 species and in 18 of them also either AUX or CYT media had that capacity. Combinations of growth regulator free and/or CYT media with AUX media were only found in *Camellia*

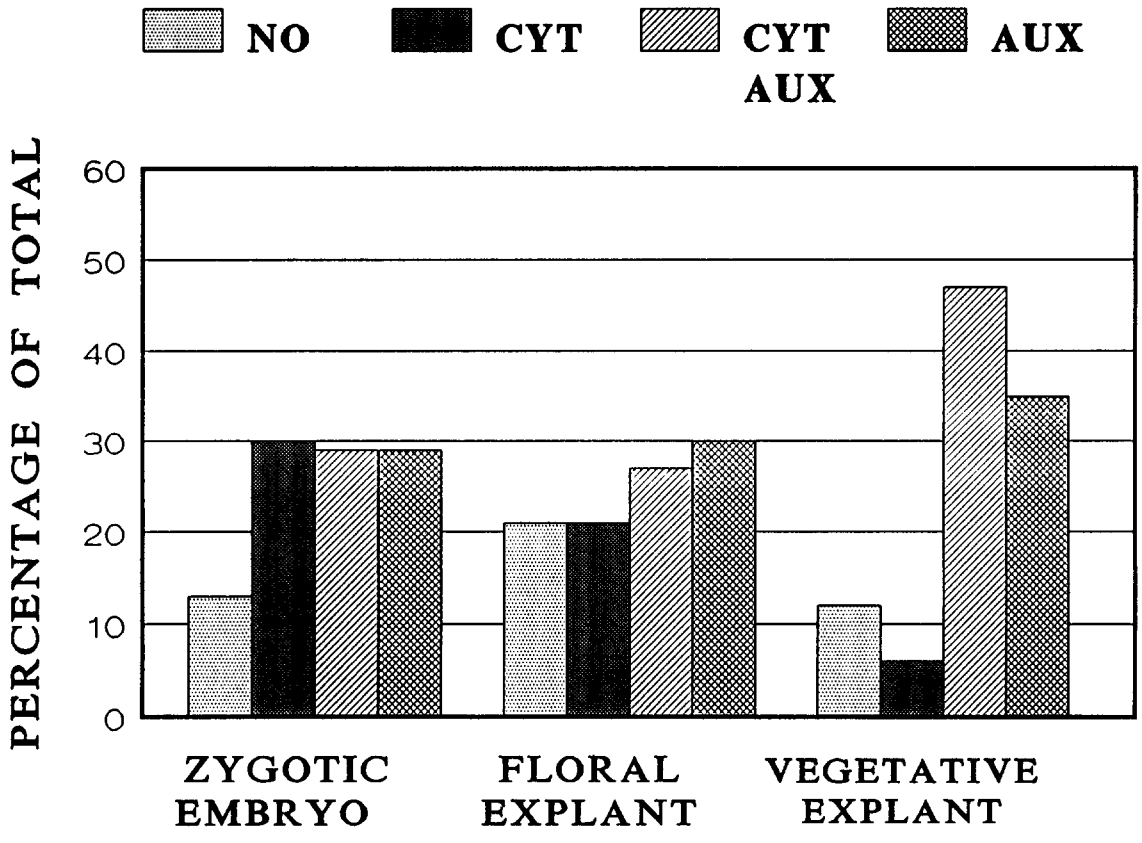

Fig. 1. Generalisation of primary and secondary somatic embryogenesis the Angiosperm dicot species mentioned in table. a) Relation between growth regulators used and explant type in primary embryogenesis.

sps. (Plata & Vieitez, 1990; Vieitez et al., 1991), *Daucus carota* (Smith & Krikorian, 1989) and *Theobroma cacao* (Pence et al., 1980). Figure 1 gives some general characteristics of primary and cyclic somatic embryogenesis of the dicot species mentioned in Table 1. Figure 1a shows that the different growth regulators were not equally used to initiate primary somatic embryogenesis in different explant types. Zygotic embryos and flower associated explants initiated embryos in a greater proportion of species on CYT supplemented or growth regulator-free media than vegetative explants. In the latter explant type AUX and AUX/CYT supplemented media are used to a greater extent. Figure 1b shows that there is a relation between direct or indirect embryogenesis and the explant used. Zygotic embryos initiated embryos directly in 63% of the species, in 23% indirect and in 14% both direct and indirect. For vegetative explants this figure was respectively 15, 77 and 8%. The reaction of floral explants was in between these two extremes. There is also a relation between the frequency of direct or indirect embryogenesis and growth regulators used during the induction of embryos (Fig. 1c). Direct embryogenesis was observed in about 70% of the species where growth regulator free or CYT supplemented media were used, indirect in 13%. AUX or AUX/CYT media gave a shift to indirect embryogenesis. The type of embryogenesis is not only dependent on the explant or growth regulators used. In *Fagus sylvatica* (Vieitez et al., 1992) and *Populus ciliata* (Cheema, 1989) embryos developed in liquid culture up to the (pre)-globular stage and in solid culture to maturity before they are subjected to secondary embryogenesis. In *Daucus carota* (Smith & Krikorian, 1989) the occurrence of direct or indirect embryogenesis was depended on the NH_4 concentration.

In *Acanthopanax senticosus* (Gui et al., 1991), *Manihot esculenta* (Raemakers, 1993; Szabados et al., 1987), *Ribes rubrem* (Zatykó et al., 1976) and *Trifolium repens* (Maheswaran & Williams, 1986) mature embryos can be used for secondary embryogenesis. In *Brassica napus* (Thomas & Wenzel, 1975; Thomas et

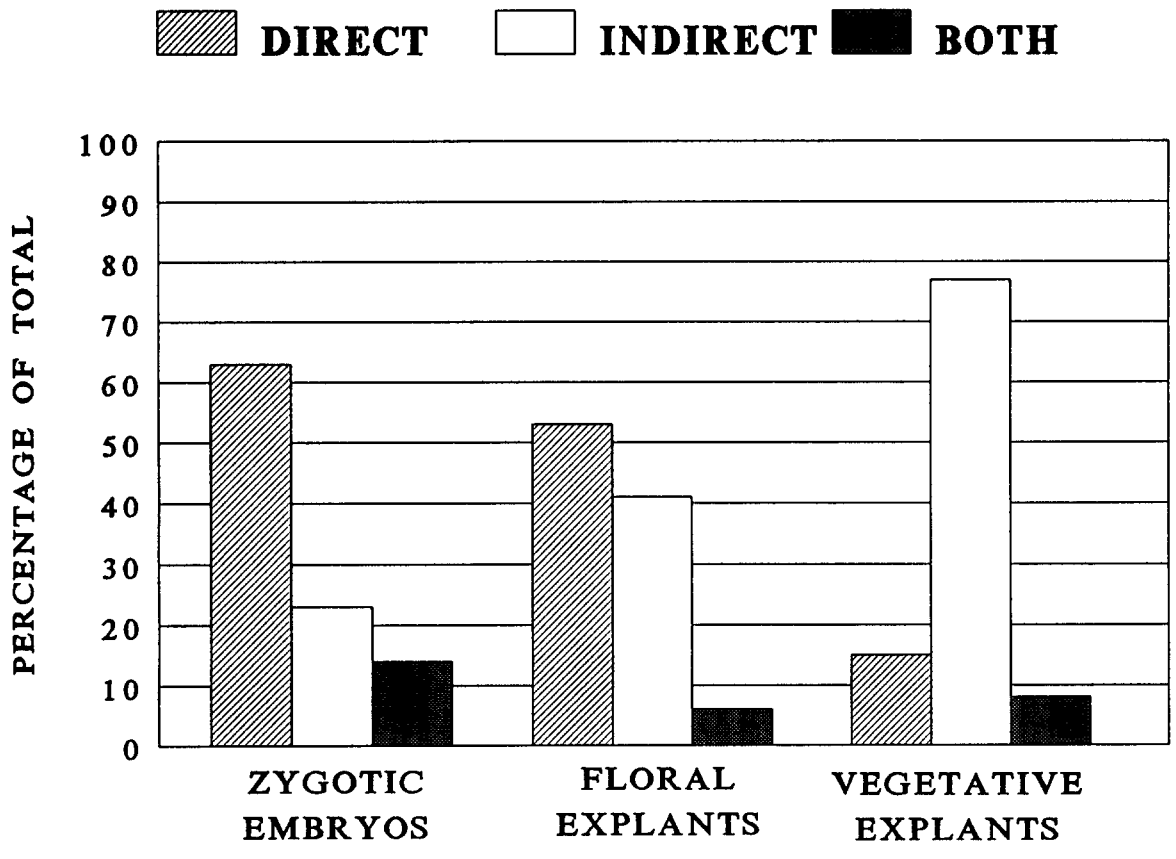

Fig. 1. b) Relation between explant type used in primary embryogenesis and direct or indirect embryogenesis.

al., 1976), *Medicago sativa* (Lupotto, 1986), *Ranunculus sceleratus* (Konar & Ntaraja, 1969) embryo derived plants were even subjected to this process.

Usually, the same induction medium was used for primary and secondary embryogenesis. However, in *Trifolium repens* (Maheswaran & Williams, 1986) higher concentrations of BA and in *Arachis hypogaea* (Durham & Parrot, 1992) lower concentrations of 2,4-D were required. In *Helianthus annuus* (Finer, 1987) and *Hemerocallis* sps. (Krikorian & Kann, 1981) Dicamba was not capable of inducing primary embryogenesis, but did so in secondary embryogenesis. In *Manihot esculenta* (Raemakers, 1993), 2,4-D induced both primary and secondary embryogenesis and NAA only secondary embryogenesis.

All species with growth regulator free driven primary somatic embryogenesis, except *B. campestris*, required also no growth regulators for further cycles of embryogenesis (Fig. 1d). *Brassica campestris* (Maheswaran & Williams, 1986) where CYT supplemented media were necessary for secondary embryogenesis. In 83% of the species with CYT induced primary embryogenesis, secondary embryogenesis required no growth regulators (Fig. 4). For the AUX and AUX/CYT groups this was respectively 31 and 23%. All the 26 dicot species listed in Table 1 where primary embryogenesis was restricted to AUX supplemented media also secondary embryogenesis required AUX supplemented medium.

Efficiency of secondary compared to primary embryogenesis

Maintenance of embryogenic capacity for more than 2 years by repeated secondary embryogenesis has been reported in *Asparagus officinalis* (Delbreil & Marc, 1992), *Citrus sinesis* (Rangaswamy, 1961), *C. microcarpa* (Rangaswamy, 1961), *Camellia japonica* (Vieitez & Barciela, 1990, Vieitez et al., 1991), *Clitoria ternatea* (Dhahnalakshmi & Lakshmanan, 1992), *Eucalyptus citriodora* (Muralidharan et al., 1989), *Heme-*

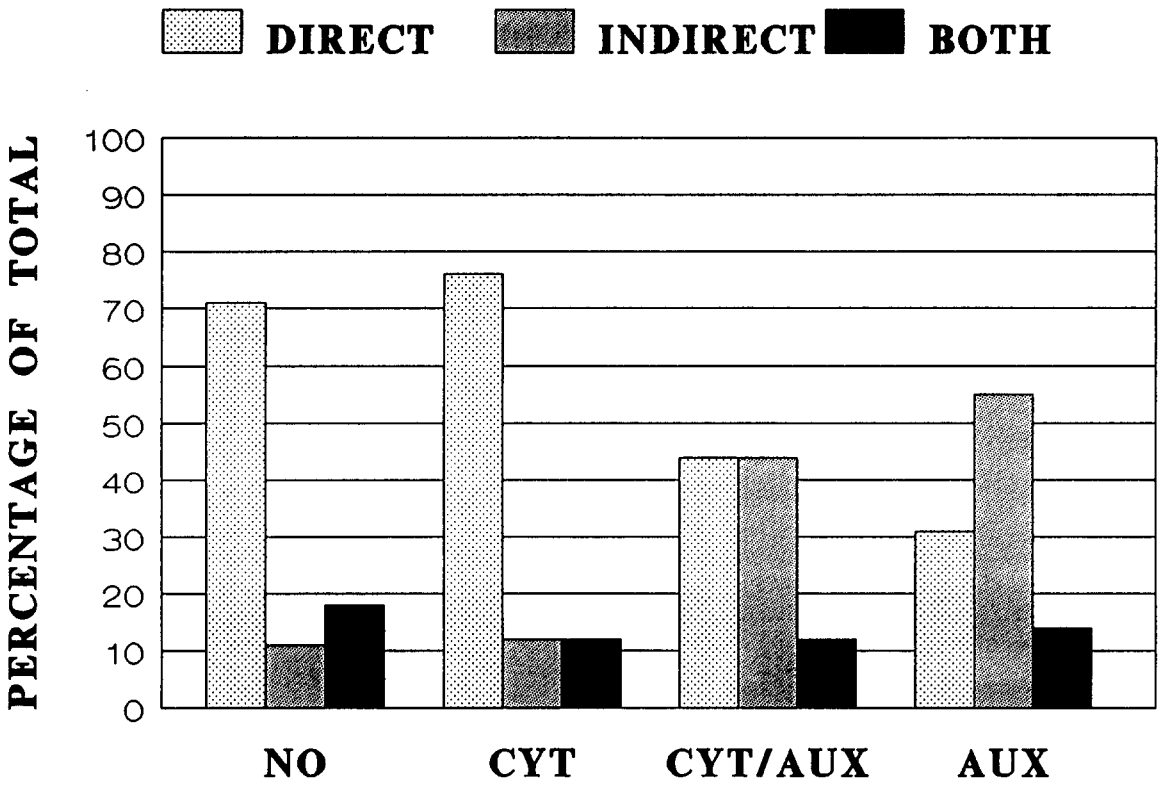

Fig. 1. c) Relation between growth regulators in primary embryogenesis and direct or indirect embryogenesis.

rocallis sp. (Krikorian & Kann, 1981), *Juglans nigra* (Deng & Cornu, 1992), *Manihot esculenta* (Raemakers, 1993), *Picea glauca* (Kong & Yeung, 1992) and *P. glauca* ×*engelmannii* (Eastman et al., 1991).

The production of embryos is determined by the number of responding explants and the number of embryos produced per responding explant. In *Arachis hypogaea* (Baker & Wetzstein, 1992; Durham & Parrott, 1992; Sellars et al., 1990), *Beta vulgaris* (Tenning et al., 1992), *Camellia japonica* (Vieitez & Barciela, 1990), *C. reticulata* (Plata & Vieitez, 1990), *Glycine max* (Liu et al., 1992; Sellars et al., 1990), *Picea abies* (Mo et al., 1989) and *Vitis* sp. (Stamp & Meredith, 1988) less than 50% of the primary explants produced embryos wereas more than 75% of the somatic embryo formed secondary embryos. In *Medicago sativa* (Parrott & Bailey, 1993) only 5 of the 300 zygotic embryos produced primary embryos, whereas all somatic embryo explants initiated secondary embryos. In *Glycine max* (Liu et al., 1992) between 0.2 and 1.3 primary embryos per explant are formed and between 8 and 30 secondary embryos per explant. Although no exact numbers were given, it was evident that also in other species the production of embryos in primary embryogenesis was lower than in secondary embryogenesis (Delbreil & Marc, 1992; Gingas & Lineberger, 1989; Kiss et al., 1992; Kochba et al., 1972; Maâtaoui et al., 1990; Makeswaran & Williams, 1986; Muralidharan & Mascarenkas, 1987; Sellars et al., 1990; Tulecke & McGranahan, 1985).

Influence of non hormonal factors on embryogenesis

Besides growth regulators several other factors are either controlling or obligatory for somatic embryogenesis. A few will be discussed here. In *Daucus carota* (Smith & Krikorian, 1989) whole embryos cultured on growth regulator free medium grew into shoots; wounding of embryos was necessary for secondary embryogenesis. In *Picea abies* (Arnold, 1987), *Arachis hypogaea* (Baker & Wetzstein, 1992) and *Manihot esculenta* (Raemakers, 1993) wounding was not obligatory but it increased the number of embryos formed. In *Magnolia* spp. (Merkle & Wiecko, 1990)

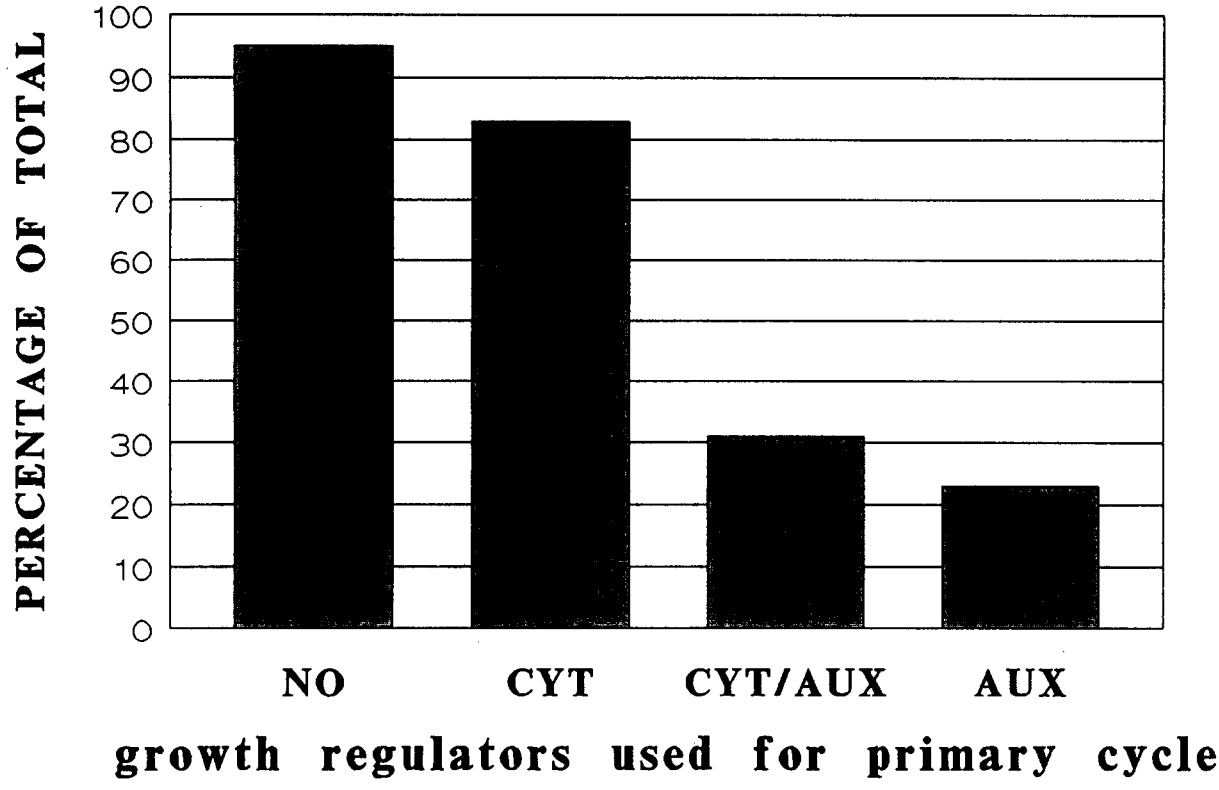

Fig. 1. d) Relation between the growth regulators used in primary embryogenesis and the occurrence of growth regulator free secondary embryogenesis (habituation).

light and in *Ilex aquifolium* (Hu & Sussex, 1972) dark conditions inhibited embryogenesis. Yeast extract was obligatory in *Medicago sativa* (Lupotto, 1982) and *Trifolium repens* (Maheswaran & Williams, 1986) and casein hydrolysate in *Citrus microcarpe* (Rangaswamy, 1961). Genetic control of the embryogenic response has been shown in many species (Adu-Ampomah et al. 1988; Jia & Chua, 1992; Raemakers, 1993; Zhong et al., 1992). Other factors which influence embryogenesis are temperature, pretreatment of donor plants, subculture duration, nitrogen source, type of agar, type of sugar and so on. For a more detailed description of these factors the following reviews (Sharp et al., 1980; Tisserat et al., 1979; Tulecke, 1987) are of importance.

Shootconversion of somatic embryos

Interruption of secondary embryogenesis

For the development of somatic embryos into plants, first the process of embryo proliferation has to be stopped. With AUX induced embryogenesis this was easily accomplished by omission of auxin or lowering the concentration. In some species it continued for one or two cycles of secondary embryogenesis (Cheema, 1989; DeWald et al., 1989; DeWald et al., 1989; Dhahnalakshmi & Lakshmanan, 1992; Lu & Vasil, 1981; Tremblay & Tremblay, 1991; Vasil & Vasil, 1981), probably because of a carry-over effect of the auxin. It is a common observation of growth regulator free or CYT induced embryogenesis that the process of secondary embryogenesis is difficult to stop (Adu-Ampomak et al., 1988; Aguilar et al., 1992; Ammirato, 1977; Cornu, 1988; Deng & Cornu, 1992; Druart, 1990; Jha et al., 1992; Kochba et al., 1972; Parrott & Bailey, 1993; Plata & Vieitez, 1990; Rangaswamy, 1961; Rashid & Street, 1974; Southworth

& Kwiatkiowski, 1991; Thomas & Wenzel, 1975; Tulecke & McGranahan, 1985; Vieitez et al., 1991; Zatykó et al., 1976). In *Brassica napus* (Huang et al., 1991), *Carum carvi* (Ammirato, 1977) and *Medicago* spp (Lupotto, 1986; Parrott & Bailey, 1993) ABA stopped proliferation. In *Juglans* spp (Deng & Cornu, 1992; Tulecke & McGranahan, 1985) proliferation could be reduced by either a desiccation treatment, cold storage, liquid culture instead of solid and culture on GA_3 supplemented media. In *Theobroma cacao* (Adu-Ampomak et al., 1988) where new embryos were formed from the cotyledons and the embryogenic axis, proliferation can be interrupted by excision of the cotyledons. In *Camellia reticulata* (Plata & Vieitez, 1990) and *C. japonica* (Vieitez & Barciela, 1990) the use of GA_3 stopped embryo proliferation.

Maturation and germination of embryos

In some species no additional culture steps were needed for maturation of embryos, however, in many other species the development is blocked at the globular stage (Adu-Ampomah et al., 1988; DeWald et al., 1989; Jones & Rost, 1989; Kochba et al., 1972; Rangaswamy, 1961; Roberts et al., 1991). In *Glycine max* (Christou & Yang, 1989), *Mangifera indica* (DeWald et al., 1989), *Picea* spp (Tremblay & Tremblay, 1991) and *Zea mays* (Emonds & Kieft, 1991) maturation was improved by increasing the sucrose concentration in the medium, but in *Euphoria longan* (Litz, 1988) it had to be lowered. The transfer of globular embryos to a medium with ABA improved maturation in *Carum carvi* (Ammirato, 1977), *Pennisetum americanum* (Vasil & Vasil, 1981; Vieitez & Barciela, 1990), *Picea* spp (Arnold, 1987; Eastman et al., 1991) and *Pinus* spp (Finer et al., 1989; Gupta & Durzan, 1991). In *Mangifera indica* (DeWald et al., 1989) ABA had only a slight positive effect, whereas, the use of Gelrite instead of Difco agar had a distinct positive effect.

A consequence of insufficient maturation of embryos in the development of malformed somatic embryos and/or the formation of fleshy leaves with fasciated stems. This phenomenon has been observed in numerous species and has been named precocious germination (Ammirato, 1987; Litz, 1988; Vasil & Vasil, 1981; Vieitez & Barciela, 1990). In precocious germination, the developing embryo tends to skip the normal stages of embryogenesis and acquires the characteristics of a malformed seedling (De Touchet et al., 1991).

In *Abies nordmanniana* (Norgaard & Krogstrup, 1991), *Hevea brasiliensis* (Michaux-Ferrière & Carron, 1989), *Limnanthes alba* (Southworth & Kwiatkiowski, 1991), *Picea sitchensis* (Roberts et al., 1991), *Pinus strobus* (Finer et al., 1989) and *Querqus suber* (Maâtaoui et al., 1990) none of the somatic embryos developed into shoots. In none of these species embryogenesis was driven by AUX supplemented medium. On the other hand, in *Apium graveolens* (Nadel et al., 1989; Nadel et al., 1990), *Dactylis glomerata* (Conger et al., 1983), *Daucus carota* (Smith & Krikorian, 1989), *Glycine canescens* (Sellars et al., 1990) and *G. max* (Christou & Yang, 1989), all species with AUX induced embryogenesis, mature embryos can develop into shoots at frequencies higher than 90%. In auxin driven embryogenesis cytokinins were not necessary for shoot development, but they often increase the frequency (Gui et al., 1991; Jha et al., 1992; Szabados et al., 1987). For example, in *Manihot esculenta* (Raemakers, 1993) only 33% of the embryos developed into shoots on a medium with 0 mg/l BA, compared to 76% on a medium with 1 mg/l BA. In *Vitis longii* (Gray & Mortensen, 1987) a cold treatment of mature embryos was essential for development into shoots. Cold treatment also stimulated germination in *Brassica napus* (Huang et al., 1991), *Fagus sylvatica* (Vieitez et al., 1992), *Juglans* spp (Deng & Cornu, 1992; Tulecke & Granakou, 1985) and *Querqus* spp (Gingas & Lineberger, 1989; Gingas, 1991). Another frequently used treatment is desiccation. It enhanced germination in *Arachis hypogaea* (Durham & Parrott, 1992), *Brassica napus* (Huang et al., 1991), *Glycine max* (Christou & Yang, 1989), *Juglans regia* × *nigra* (Deng & Cornu, 1992), *Mangifera indica* (DeWald et al., 1989), *Manihot esculenta* (Mathews et al., 1993) and *Querqus alba* (Gingas & Lineberger, 1989).

Applications of secondary somatic embryogenesis

The continuous proliferation of somatic embryos of secondary embryogenesis has several possible applications. In crops with a long life cycle, as for example woody species, preservation of embryogenic lines can be a cost effective maintenance until the lines have been tested in field conditions. Selected lines can be multiplied in large quantities by secondary somatic embryogenesis. Furthermore, mature trees can be rejuvinalized for propagation purposes by somatic embryogenesis (Wann, 1988). In plant breeding wild species are

frequently used to introgress resistance genes in economically important cultivars. Post-fertilization barriers often prevent maturation of the embryo. Immature embryos of interspecific plants from incompatible crosses (Merkle et al., 1990) may be rescued by culturing them for secondary somatic embryogenesis and simultaneously the plant is multiplied. Finally, secondary embryogenesis can be used for the production of somatic embryos of species in which the zygotic embryos contains commercially important metabolites (Litz, 1988; Southworth & Kwiatkiowski, 1991). In the next section three other applications are discussed more in detail.

Plant transformation

There are some regeneration characteristics which favour the successful use of secondary somatic embryogenesis for plant transformation. An epidermal cell origin of somatic embryos is more suited to be used in conjunction with plant transformation than a mesophyll origin. It is evident that single cell origin is beneficial as compared to multiple cell origin. Another important characteristic is the developmental stage at which embryos are subjected to secondary embryogenesis. At such moments transformed cells have the opportunity to act independently from neighbouring cells, otherwise they have to divide in commitment with untransformed cells. In indirect embryogenesis very young embryos are subjected to secondary embryogenesis and this explains the successful application of indirect embryogenesis for plant transformation in *Apium graveolens* (Catlin et al., 1988), *Beta vulgaris* (D'Halluin et al., 1992), *Citrus sinensis* (Hikado et al., 1982), *C. reticulata* (Hidaka et al., 1990), *Daucus carota* (Scott & Draper, 1987), *Helianthus annuus* (Everett et al., 1987), *Medicago sativa* (Pezzotti et al., 1991), *Oryza sativa* (Christou et al., 1991) and *Zea mays* (Gordon-Kamm et al., 1990).

Direct embryogenesis has been used successfully in conjunction with plant transformation in *Brassica napus* (Swanson & Erickson, 1989), *Glycine max* (Finer & MacMullen, 1991) and *Juglans regi* (McGranahan et al., 1990). In these species mature somatic embryos are formed which are chimeric for the introduced characters. The chimeric nature of transformed embryos was changed after several cycles of secondary embryogenesis under selective conditions into solid transformed embryos.

In two other ways secondary embryogenesis can improve the efficiency of transformation procedures. Most protocols have been developed with the aim to obtain solid transformants in one step. Partly transformed embryos are discarded. However, they might give solid transformants after culturing them for a secondary embryogenesis. Secondly, primary somatic embryogenesis has to be started from explants isolated of plants. Especially if zygotic embryos or flowering organs are used, explants might not be available the year round. Zygotic embryos of crosspolinated crops are unique genotypes and because of that may react differently in tissue culture. Cultures maintained by secondary embryogenesis are available the year round and in a reproducible manner. Furthermore, in many species the efficiency of explants in primary embryogenesis is lower than in secondary embryogenesis.

Plant propagation

Secondary embryogenesis offers the possibility of large scale multiplication of plant material. The propagative potential depends on the duration of a cycle of secondary embryogenesis and on the number of embryos produced. In *Manihot esculenta* (Raemakers, 1993), where a cycle lasts 45 days and about 30 embryos are formed, 6.6 10^{11} embryos can be produced in one year. Compared with these numbers, micropropagation by cuttings has a relatively low multiplication rate. With somatic embryogenesis, in principle, discrete propagules are produced which possess the developmental program to grow into a complete shoot without additional shooting and/or rooting steps which are necessary in micropropagation by cuttings. Labour can further be reduced if somatic embryogenesis is conducted in liquid medium. Redenbauch et al. (1986) have proposed to encapsulate somatic embryos in an artificial seed coat and use this as a synthetic seed for direct deliverance to the greenhouse or field. For commercial application high rates of shoot development are required and as can be seen in Table 1 this has only be accomplished in a few species. It is further generally accepted that the efficiencies of the current procedures is not high enough to be competitive with normal seed development. Redenbaugh et al. (1986) estimated the cost of an *Medicago sativa* synthetic seed to be 40 times higher than that of a true seed. However, they argued that for crops as hybrid vegetables and flowers synthetic seeds might become competitive, if value-added components such as clonal uniformity and epistatic interactions that are normally not heritable through sexual seed are added (Redenbauch et al., 1986). Possible candidates for such

epistatic interaction may be inoculation of the artificial seedcoat with pesticides, or in the case of legiminosae, with Rhizobium bacteria. For more information about synthetic seeds the reader is referred to the following review (Gray & Purokit, 1991). One distinct advantage of true seed multiplication is the absence of virus. This has not been studied thoroughly in plants derived from somatic embryos. In *Euphorbia pulcherrima* (Preil et al., 1982) and *Citrus* spp (Button & Kochba, 1977), however, virus free plants were obtained from virus infected plants.

In all cases the interest of somatic embryogenesis for plant propagation depends on the absence of somaclonal variation. In most cases research on this was restricted to an evaluation of the morphology of a small number of plants and usually no obvious variation was found (Durham & Parrott, 1992; Gray & Mortensen, 1987; James et al., 1984; Klimaszewska, 1989; Lu & Vasil, 1981; Stamp & Meredith, 1988). In *Glycine max* (Barwale & Widholm, 1987; Freytag et al., 1989; Shoemaker et al., 1991) this was studied in detail and variation for leaf shape, leaf variegation, growth habit, sterility, iso-enzyme patterns and lipid composition of seed could be observed. In most cases, it was found that these altered traits were heritable, showing that the variation was of genetic origin.

Haploid breeding

Breeding at the haploid ploidy level offers advantages as a drastic reduction of time needed to produce homozygous plants and expression of recessive genes. Haploid plants can be obtained *in vivo* through gynogenesis, androgenesis, genome elimination, semigamie and by chemical and physical treatments such as heat shock, X-ray and UV light (Pierik, 1987). The frequency of these events is, however, low.

Theoretically, all steps necessary in a haploid breeding schema can be conducted by using somatic embryogenesis. In numerous species large numbers of haploid embryos can be obtained by culture of microspores or anthers (von Aderkas & Dawkins, 1993; Aslam et al., 1990; Loh & Ingram, 1983). The haploid embryogenetic lines can be multiplied and maintained by secondary somatic embryogenesis. Plants of the lines are evaluated in field trails. Embryos of the selected lines will be used to double the ploidy level. In *Brassica napus* (Loh & Ingram, 1983), *B. campestris* (Aslam et al., 1990) and *Citrus sinensis* (Hikado et al., 1982) this was accomplished by culturing somatic embryos for secondary embryogenesis in the presence of colchicine. In *Asparagus officinalis* (Feng & Wolyn, 1991) and *Camellia japonica* (Kato, 1989) the ploidy level of embryos doubled spontaneously in a low frequency. Selection of doubled somatic embryos in a state that they are still embryogenic, allows the rapid multiplication by secondary somatic embryos. At this moment the value of somatic embryogenesis for haploid breeding remains to be demonstrate. For more information on the use of tissue culture for haploid breeding the reader is referred to the following review (Morrison & Evans, 1988).

General conclusions

Secondary somatic embryogenesis has been reported in at least 80 species. It has, compared to primary somatic embryogenesis, advantages such as a high multiplication rate, independence of an explant source and repeatability. Furthermore, embryogenicity can be maintained for prolonged periods of time by repeated cycles of secondary embryogenesis.

Mostly developmentally young embryos are subjected to secondary embryogenesis. However, also mature embryos can be a source for secondary embryos.

In general auxin and or auxin/cytokinin supplemented media are used in somatic embryogenesis of Gymnosperm and Angiosperm monocot species. In certain dicots species also growthregulator-free and/or cytokinin supplemented media can initiate embryogenesis. Cytokinin and/or growthregulator free induced somatic embryogenesis has distinctly different characteristics compared to auxin induced somatic embryogenesis. Secondary somatic embryogenesis has potential applications for both plant breeding practice and research. The feasibility depends on the particular crop, alternative methods, and the specific characteristics of somatic embryogenesis in that particular species.

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