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REVIEW

Towards artificial seeds from microspore derived embryos of *Brassica napus*

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Abstract

Microspore derived embryos are haploid and their immediate diploidization generates doubled haploid homozygous plants, whereas a normal breeding process would take 7–8 generations to attain homozygosity. However, the flexibility available in conventional seeds—storage, transport, variable planting time, and handling—is not possible with microspore derived embryos in breeding programs; they are continuously growing from induction of embryogenesis to planting in the soil, without a pause. Artificial seed technology can by-pass the expensive and time-consuming process of acclimatizing and loss of in vitro derived embryos in the green house. The doubled haploid technology in Brassica species has advanced considerably on many fronts—reliable induction of embryogenesis, in vitro diploidization, desiccation and conversion of embryos to plantlets. Although microspore derived embryos are bipolar, they are not considered within the scheme of artificial seeds. The development of artificial seeds in brassica, however, needs the input from other biotechnologies to develop vigorous embryos capable of conversion to plants. It is now necessary for empirical research on microspore derived embryos of Brassica to expand the applications available to the plant breeder by including the biotechnologies of encapsulation and embryo priming to develop artificial seeds. The objective of this review is to draw the attention of researchers to make the transition from microspore-derived embryos to artificial seeds in Brassica crop species, by bringing together relevant studies from the relevant biotechnologies. This would expand the present scope of artificial seeds and thus provide more options and flexibility to the Brassica plant breeder.

Key message

Artificial seeds from microspore derived embryos enhance breeding of Brassica species.

Keywords Artificial seeds · Microspore-derived embryos · Conversion of embryos · Brassica species · Encapsulation · Artificial endosperm · Priming embryos

Abbreviations

ABA Abscisic acid
BAP 6-Benzylaminopurine
B5 Gamborg's medium
DH Doubled haploid
GA3 Gibberellic acid
IBA Indole butyric acid
MS Murashige and Skoog medium
NAA Naphthalene acetic acid

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Introduction

The in vitro induction of haploid embryogenesis from microspores, for the instant production of completely homozygous doubled haploid (DH) lines, is now routinely applied in many plant breeding programs (Germanà 2011a). The main advantage of doubled haploids is a significant saving of time and labour to produce homozygous inbred lines, compared to conventional breeding, which requires 7 to 8 generations of selfing for nearly homozygous lines. The doubled haploid technology has particularly developed in the *Brassica* genera with rapid progress in the efficient

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induction of embryogenesis, chromosome doubling in vitro, and direct conversion of embryos to plants. Embryos derived from somatic or gametic tissues (somaclonal, androgenic and gynogenic embryos) differ from zygotic embryos in (i) the absence of an endosperm, (ii) the absence of a seed coat to provide protection from desiccation, pathogens and physical damage, and (iii) a resting or quiescent phase (Fig. 1). These differences should be overcome for the survival and germination of in vitro derived embryos when transferred to soil. This, however, has remained a major challenge in the development of a viable system for artificial seed production. Embryogenesis in zygotic and microspore-derived embryos are schematically compared in Fig. 1.

Natural seeds can be stored, transported, handled and planted at variable times. However, this flexibility is not available for plant breeders with microspore-derived doubled haploid embryos/plantlets, since they are continuously in the growth phase from induction of embryogenesis to planting in the soil (Fig. 1). A survey among winter rapeseed breeding companies in Germany showed that obtaining planting material was a problem for direct planting in the field in August/September to have a better visual assessment of the plant's performance (Möllers and Iqbal 2009). This can be overcome if artificial seeds are available. It can further help to synchronize the production of doubled haploid genotypes for the winter season by vernalization in cold greenhouses, before transferring the plantlets to the field in spring (Cegielska-Taras et al. 2002). Alternatively, plantlets transferred to the field in autumn are vernalized under natural conditions. Increasing the duration of storage of dried embryos—quiescent period in normal seeds—can improve germination and conversion to plantlets (Senaratna et al. 1991). There is a substantial loss of plantlets during the transition from in vitro conditions in the laboratory to in vivo field establishment (Haddadi et al. 2008; Ahmadi et al. 2012). The development of artificial seeds from microspore-derived embryos can avoid this loss and cost of transition.

Developing artificial seeds is a costly process. It should be considered for elite material from DH breeding programmes. Similar to natural seeds, artificial seeds are convenient to store a large volume of planting material, since storing and providing growth conditions for a large number of plantlets can be costly. They also provide flexible planting times, which is not possible with in vitro derived plantlets in a continuous state of growth. Due to limitations in laboratory capacities, only a limited number of embryos are regenerated to plantlets. However, for quantitative traits the number of DH lines regenerated per cross cannot be large enough. A functional artificial seed system would allow plantlet regeneration of a much larger number of embryos in the soil.

In recent years, researchers have reviewed different aspects on the development of artificial seed technology. The potential of non-embryogenic in vitro derived explants as artificial seeds were reviewed by Standardi and Piccioni (1998). Reviewing the status of artificial seeds technology and the limitations for commercial exploitation, Ara et al.

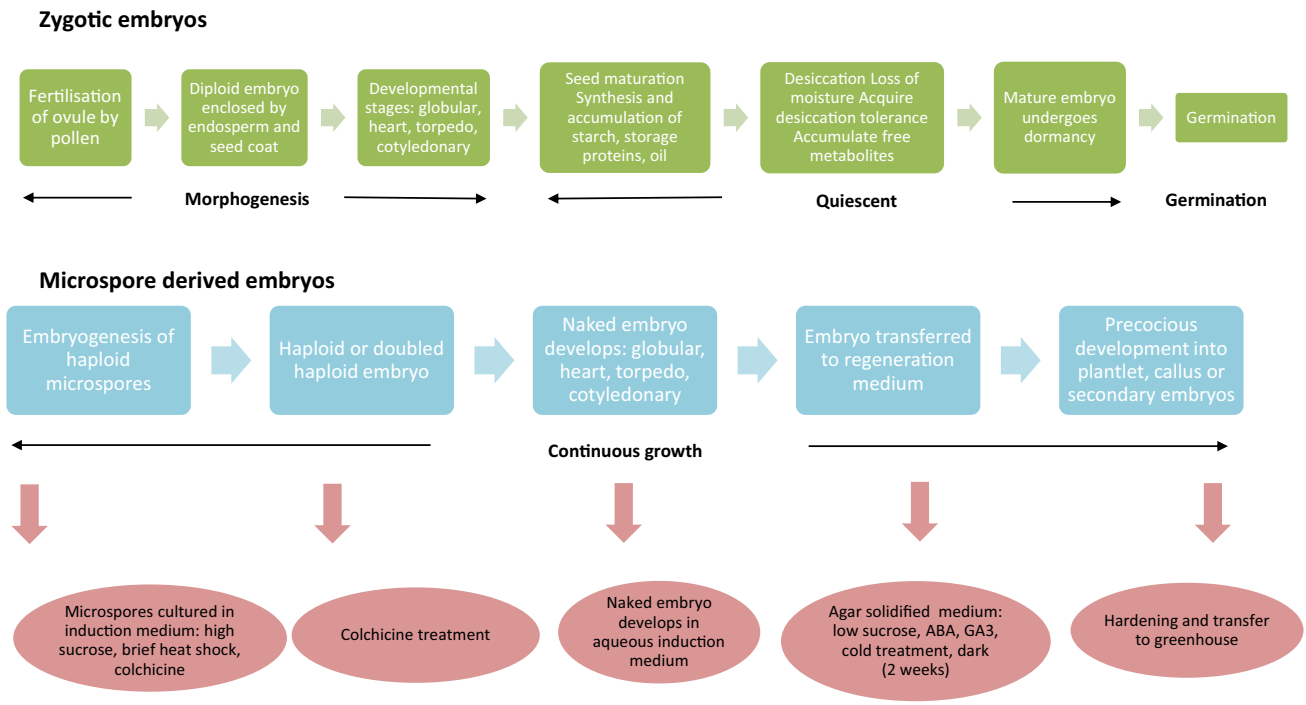

Fig. 1 Schematic comparison of embryogenesis in zygotic and microspore derived embryos of brassica

(2000) identified the inability to simulate the accessory tissues in natural seeds—the endosperm and protective coatings—as a primary bottleneck to develop artificial seeds. Sharma et al. (2013) have reviewed the presently available methods for encapsulation of artificial seeds in different species and the types of propagules available for encapsulation. Artificial seeds in the ornamental plant sector is now extended to trees, shrubs, and cut-flowers. Protocols for encapsulation are available for various explants with high rates of conversion, shorter period to germination and production of high quality plants (see review by Lambardi et al. (2006)). Artificial seeds in medicinal plants were reviewed by Gantait et al. (2015) and in fruit plants by Rai et al. (2009). Thus, artificial seeds are available for many species and plant types. Recent reviews of doubled haploids in crop species in general (Ferrie and Caswell 2011; Ferrie and Möllers 2011; Forster et al. 2007; Germanà 2011b) and in *Brassicaceae* in particular (Cardoza and Stewart 2004; Park et al. 2012) have failed to address the possibility of utilising microspore derived embryos as artificial seeds in any *Brassica* crop.

This paper reviews the recent advances in microspore-derived embryos and in artificial seeds for the purpose of combining these biotechnologies to develop artificial seeds in *Brassica*. We first review the empirical advances in the regeneration of plantlets from microspore derived embryos in Brassica from induction to conversion of embryos to plantlets, and the importance of inducing desiccation tolerance and embryo maturation. Thereafter examples are provided from other species on the artificial encapsulation of embryos to develop artificial seeds. Finally, we examine how seed priming, a recent development in seed science, could improve the quality of germination and overcome limitations of artificial seed technology and augment the vigour, germinability and conversion of microspore derived embryos to plantlets in the greenhouse.

Definitions and synonyms

Many synonyms describe the use of in vitro somatic embryos and propagules as alternatives to natural seeds. Among these are synthetic seeds, synseeds, dry artificial seeds, artificial seeds, and somatic seeds, which are a source of confusion in the literature. Microspore derived embryos are, however, absent in the definition of artificial seeds. They are bipolar and similar to somatic embryos but are distinct in their genome constitution (haploid or doubled haploid), their method of induction, and applications in plant breeding.

Murashige (1977, cited by Standardi and Piccioni 1998) defined artificial seeds as 'an encapsulated single somatic embryo' capable of realising the convenience of conventional seeds in terms of transport, storage and sowing to eventually germinate into a plantlet (Murashige 1977). This and other definitions by Gray et al. (1991) restricted artificial seeds to only somatic embryos. Aitken-Christie et al. (1995) expanded the definition of synthetic seeds to 'artificially encapsulated somatic embryos, shoots or other tissues, which can be used for sowing under in vitro or ex vitro conditions. We suggest that doubled haploid embryos be included to Aitken-Christie's definition (1995) above, to expand their definition to include DH embryos: 'Artificial or synthetic seeds are artificially encapsulated somatic and doubled haploid embryos, shoots or other tissues, for sowing under in vitro or ex vitro conditions'.

The terms germination and conversion are often used to mean the same on in vitro derived embryos. McKersie and Brown (1996) provide an unequivocal separation of these terms. In zygotic seeds, germination is a single process where root and shoot formation are tightly coupled. However, in in vitro derived embryos the emergence of the radicle precedes the plumule and development of the apical meristem can be abnormal preventing normal development of shoots. Hence, germination of in vitro derived embryos is the emergence of the radicle and conversion is the formation of both shoots and roots, leading to a plantlet.

Artificial seeds in *Brassica* species

Artificial seeds in *Brassica* were first attempted with microspore-derived embryos of *B. napus* (Senaratna et al. 1991) and *B. oleracea* (Takahata and Keller 1991). Microspore derived embryos of *B. napus*, at the cotyledonary stage, were treated with abscisic acid (ABA) and desiccated to less than 15% moisture and stored for at least 7 days; a conversion of 40 to 50% to plantlets was achieved. In *B. oleracea*, desiccation was induced in cotyledonary stage embryos placed on filter papers resting on agar solidified Gamborg's medium (Gamborg et al. 1968). Desiccation tolerance was induced by treating the embryos with 100 µM ABA for one or 7 days; embryos maintained their viability for 3 months without control of humidity or temperature (Takahata et al. 1993). Germination of these embryos was 62% compared to 41% of the embryos not placed on filter paper. Thus, if desiccated embryos had almost the same capacity as natural seeds, naked desiccated embryos would be a simple form of artificial seeds with similar advantages of storage and handling (Takahata et al. 2005).

Rihan et al. (2011, 2012) produced artificial seeds from non-embryogenic tissues of *Brassica*. They aseptically shaved and cultured the outermost meristematic layer of cauliflower (*B. oleracea* var. *botrytis*) in liquid MS medium (Murashige and Skoog 1962) for 2 weeks with IBA and kinetin. The developing micro-shoots were *divided into three main stages:* (a) very low growth rate (0–11 days), (b) accelerated growth rate (11–15 days), and (c) rapid growth rate after 15 days. The optimal age for micro-shoot encapsulation

in sodium alginate was 13–14 days during the stage of accelerated growth. The composition of the moistening/irrigation solution was also critical for conversion of the encapsulated micro-shoot. An MS medium with 3% sucrose, kinetin and NAA was optimal for conversion of the micro-shoots to plantlets.

Towards artificial seeds from microspore derived embryos

There are fundamental differences in the morphogenesis, maturation and germination of zygotic and microspore derived embryos, illustrated schematically in Fig. 1. To utilise microspore derived embryos of *Brassica* species as artificial seeds, they need to approach the convenience of natural seeds, such as synchronous germination, conversion to vigorously growing seedlings, and good survival in the soil. We review below the extent to which microspore derived embryos of *Brassica* fulfil these requirements for artificial seeds.

Synchronous development of embryos

Synchronous development of microspore-derived embryos is essential to conduct subsequent in vitro manipulations in a single step. In *B. napus*, a uniform development of microspore-derived embryos is routinely obtained by using morphological markers such as size of the buds, and petal to anther length ratio, which are correlated to the late uninucleate stage of microspore development. For valuable genotypes recalcitrant to microspore culture, an experimentally demanding method to isolate the correct stage for induction of embryos is the percoll gradient method developed by Bhowmik et al. (2011) to separate microspores at different stages of development.

In *B. juncea*, reducing the sucrose in the modified Nitsch and Nitsch (1969) induction medium (NLN) from 17% (w/v) to 10% after 48 h of culture increased the production of embryos by six- to seven-fold in 12 out of the 13 genotypes tested (Lionneton et al. 2001). They also report a strong genotype effect on embryogenesis. The authors also reported a species-specific effect also: the embryos from the highly responsive *B. napus* cv. Drakkar did not regenerate from the NLN-17/10 sucrose change. Similar increase in the frequency of embryogenesis were obtained by Baillie et al. (1992) with *B. campestris*, and by Ferrie et al. (1999) with *B. oleracea*. Thus, there is a species and genotypic effect, which needs to be specifically evaluated.

Colchicine is now routinely included in the induction medium itself to diploidize haploid microspores. This is in contrast to the earlier tedious process of treating the regenerated haploid plants. This provides uniformity in development, since only diploidized microspores go on to produce microspore derived embryos (Chen et al. 1994; Iqbal et al. 1994; Möllers et al. 1994; Hansen and Andersen 1996).

Conversion of embryos to plants

Bipolar microspore derived embryo converts to a plantlet by the development of secondary leaves from the apical meristem and elongation of the root. Unlike in zygotic embryos, there is a tendency for secondary embryos to arise from the hypocotyl of microspore-derived embryos in the culture medium (Huang et al. 1991). This sets back conversion, since further cycles of sub-cultures of the secondary embryos are necessary to convert them to plantlets (Tian et al. 2004, Cegielska-Taras et al. 2002). To overcome this and shorten the period to conversion to a plant, various physical and chemical treatments on the microspore derived embryos have been investigated before and during in vitro culture, summarised in Table 1 and discussed below.

Embryo stage and size

The size and age of the embryos is a key determinant in the conversion of embryos to plantlets. However, size of the microspore-derived embryos is a poor indicator of maturity or germination potential. The embryogenic potential of a genotype determines the number of embryos in a Petri dish. This can interact with the nutrient availability in the medium. Thus, cultures with low embryogenic potential would have fewer and larger embryos with convoluted cotyledons in a Petri dish; cultures with high embryogenic potential have a high density of smaller globular embryos from competition for available nutrients. Thus, the embryogenic potential of a genotype can confuse the age and development stage of the embryos. For this reason, it is necessary to routinely refresh the culture medium and dilute the density of embryos, particularly in embryogenic cultures, after 3 weeks of culture. Conversion of 40 to 60% of microspore derived embryos to plants was achieved by the simple step of exchanging the microspore induction medium, after 2 days of culture, with fresh medium (Gu et al. 2003); this is also necessary if colchicine was included in the induction medium at culture initiation. This, however, involves culture intervention at an early stage of microspore culture.

Embryo size is important for later seedling and plant development. The frequency of plant development was higher with microspore-derived embryos of at least 4 mm at the late torpedo stage of development (Zhou et al. 2002; Agarwal et al. 2006; Zhang et al. 2006; Ahmadi et al. 2012; Mohammadi et al. 2012). This stage is now adopted for conversion of embryos, corresponding to 4 weeks after culture of microspores. Related studies are summarised in Table 1.

Table 1 Regeneration of microspore derived embryos, in selected Brassica species, to plantlets

Species, age and size of MDE	Regeneration medium	Treatments to convert embryos to plants	Efficiency of regeneration	Remarks	Callogenesis, Secondary embryogenesis	Reference
B. napus 2–4 mm and 4–6 mm MDE	B5-2%, 0.7% agar followed by B5-1%, 0.9% agar	4 °C for 1 week followed by 16 photoperiod for 5–7 days	Normal regeneration mostly in 4–6 mm MDE with BA and IAA ≥ 0.1 mg/L	Embryo size, GA3 affects normal regeneration, callogenesis and SE	Highest SE: 0.15 GA3 + 0.3 BA Highest callogenesis: 0.15 GA3 + 0.1 BA + 0.1 IAA	Ahmadi et al. (2012)
B. napus cotyledonary embryos	½ MS-2%, 0.1 GA3, 0.7% agar	2 °C for 10 days followed by 24 °C with 16 h photoperiod	Highest frequency with 125 and 250 mg/L colchicine for 12 and 24 h	Cotyledonary embryos treated with colchicine were transferred to soil 8–9 weeks after microspore isolation	Callusing and SE not reported	Mohammadi et al. (2012)
B. rapa 20 days cotyledonary embryos; 3–5 mm	MS − 3%, 0.1 g/L active carbon, 0.55% agar. 25 °C, 16 h photoperiod	PCIB 40 µM on NLN; After 21 days MDE treated for 5 days at 4 °C	58% directly to plants (9.6-fold increase)	PCIB is an anti-auxin; optimal concentration 40 µM; lower embryo age	SE at lower PCIB concentrations and callogenesis at higher concentration	Zhang et al. (2011)
B. juncea, MDE 21–30 days (< 35 days)	MDE on B5-2% + 0.1 mg/L GA3 medium for 30 days, 0.7% agar.	Incubation at 4 ± 1 °C for 10 days; air drying on sterile filter paper on laminar flow hood for 15 min	82.3% conversion to plantlets	Cold treatment was important; most of the plants were haploid	Not reported	Prem et al. (2008)
Brassica spp.	Solid B5 medium free of growth regulators and maintained at 22 °C with a 14-h photoperiod for 3–4 weeks	Polyethylene glycol 4000 (PEG). Solid B5 medium	Spontaneous diploids: 68–92% Regeneration: 97% of PEG treated were normal compared to 76% of sucrose treated	Addition of 3 ml of 13% sucrose to PEG medium improved embryo quality and quantity. PEG increased spontaneous doubled haploids	Not reported	Ferrie and Keller (2007)
B. napus	½ MS + 2% sucrose, 0.9% agar, 2 mg/L 6-BAP	Cold: 4 °C, 3–5 days, followed by 16 h photoperiod at 24 °C. Partial desiccation for 1–2 days and excision of cotyledons	Good response by all 5 genotypes Germination 60–90%, Regeneration: 23–58%	Desiccation for 1-day improved germination and > 4 days, MDE died. Cotyledon excision significantly improved germination. Good conversion if MDE < 4 mm	Not reported	Zhang et al. (2006)
B. juncea	B5- 1%	Fully developed dicot embryos on B5-1% incubated at normal culture room conditions for 3 weeks	70% germination	Microspore cultures treated with colchicine up to 50 mg/L for 24 h. PCIB 0—80 µM	Not reported	Agarwal et al. (2006)
B. napus	MS-2%	Colchicine 50 mg/L	18–48%	Function of genotype; crosses performed better	Colchicine favoured direct conversion to plantlets	Weber et al. (2005)

Table 1 (continued)

Species, age and size of MDE	Regeneration medium	Treatments to convert embryos to plants	Efficiency of regeneration	Remarks	Callogenesis, Secondary embryogenesis	Reference
B. napus cv. Topas	½ MS with 900 mg/L $CaCl_2$	Ca supplement	Regeneration: > 80% 7 mm MDE showed better regeneration	Ca is important for regeneration; may replace desiccation and growth supplements. If MDE remain in liquid medium > 60 days, regeneration is reduced.	MS medium: > 90% developed via secondary embryos or organogenesis. ½ MS: 31% of 7 mm MDE developed directly to plants	Tian et al. (2004)
B. napus ssp. *oleifera* Winter cv. DH line. 21-day MDE	MDEs were plated on petri dishes, on B5-2%, 0.8% agar	Cold: 24 °C, 4 °C, 1 °C. Combination of photoperiod and low temperature	MDE grown at 1 °C/14 days and 24 °C for next 21 days formed 74–86% shoots	No significant effect by GA3	MDE grown at 24 °C/35 days, 4 °C/14 days, developed SE	Cegielska-Taras (2002)
B. napus, late torpedo, large embryos	½ MS (macro)-2%, 0.1 mg/L GA3, 0.9% agar	10 days at 2 °C; 24 °C with 16 h photoperiod, low light	High regeneration; without colchicine treatment callus and SE	Colchicine 500 mg/L added to isolation medium for 15 h	Irregular callus like embryoids without change of NLN and colchicine treatment; more sub-cultures required	Zhou et al. (2002)
B. napus 28 day MDE	B5-2, 1.25% agar. large embryos transferred to regeneration medium	Colchicine, Trifluralin, Oryzalin, Amiprophos methyl	40%	Treatments had no significant effect on regeneration	Not given	Hansen and Andersen (1996)

Hormone concentrations in mg/L

MDE microspore-derived embryo, *GA3* gibberellic acid, *SE* secondary embryogenesis, *B5* Gamborg's medium, *MS* Murashige and Skoog's medium, *PEG* Poly ethylene glycol, *IAA* indole acetic acid, *BA* benzyl adenine, *x %* sucrose %, *PCIB* p-chlorophenoxyisobutyric acid, *NLN* modified Nitsch and Nitsch medium (1969)

Regeneration medium for conversion to plantlets

Microspore derived embryos of Brassica need a low sucrose content and nutrients in the culture medium for germination and conversion to plantlets; the nutrient rich NLN medium (Nitsch and Nitsch 1969; Lichter 1982), for induction of microsporogenesis, induces callusing and secondary embryogenesis on the embryos. Regeneration media generally contain low sucrose (2–3%), ½ strength nutrients of the basal medium and agar (Tables 1, 2). This is supplemented with hormones, such as BAP (6 benzyl aminopurine) and GA3 (gibberellic acid) and physical treatments (temperature and light). In a study with five Chinese genotypes of *B. napus*, Zhang et al. (2006) found the best conversion of microspore derived embryos was with ½ MS supplemented with 2 mg/L BAP. This produced large healthy embryos converting to vigorous shoots and plantlets. In *B. napus*, the level of GA3 and embryo length significantly affected regeneration and secondary embryogenesis (Ahmadi et al. 2012); the addition of 0.1 mg/L GA3 gave a poor conversion to plantlets (Zhang et al. 2006). Secondary embryogenesis (43.7%) and callogenesis (50.8%) was high with 4–6 mm embryos cultured with 0.15 mg/L GA3. Highest conversion was possible with 0.05 mg/L GA3 with 4–6 mm embryos or 0.1 mg/L GA3 with 2 to 4 mm embryos. Most media for conversion have a sucrose content of 2% and half strength MS medium (Murashige and Skoog 1962) or B5 (Gamborg et al. 1968), solidified with 0.7 to 0.9% agar and supplemented with 0.1 mg/L gibberellic acid (Table 1). This is the basic medium for conversion of embryos to plantlets. Other treatment variations that have improved the conversion of embryos are summarised in Tables 1 and 2.

Desiccation tolerance of embryos

The developmental process of zygotic embryos is largely anabolic with the synthesis of storage reserves. This ceases when the seed enters a phase of maturation drying or desiccation, followed by a quiescent state; this is the mature seed. Upon hydration, germination begins, and the seed enters a catabolic state (Bewley 1997). Microspore derived embryos are, however, continuously in an in vitro aqueous environment, develop through the stages of globular, heart shape, torpedo and cotyledonary embryos and develop shoots and roots from the apical and root meristems without undergoing desiccation or quiescence (Fig. 1). Since in vitro derived embryos do not readily undergo germination and conversion to plantlets (Kott and Beversdorf 1990; Baillie et al. 1992), desiccation tolerance was investigated with in vitro derived somatic embryos of alfalfa and microspore derived embryos of *B. napus*, so that they may undergo maturation to promote their conversion to plantlets (Senaratna et al. 1990).

Tolerance to desiccation is a fundamental attribute of seeds: they can lose moisture and acquire desiccation tolerance while in vitro derived embryos lose their viability if they lose moisture. This requires that desiccation tolerance should be induced in in vitro embryos, which is also necessary for their subsequent encapsulation.

Previous studies have induced desiccation tolerance in microspore derived embryos of *B. napus* by slow air drying on dry filter paper and embryo germination was positively correlated with the number of days of desiccation (Kott and Beversdorf 1990), thermal stress (Anandarajah et al. 1991), and also in *B. oleracea* and *B. campestris* (Senaratna et al. 1991; Wakui et al. 1999). The authors suggest these positive effects of desiccation on germination is a response to phenomena similar to that occurring in developing zygotic embryos; artificial drying or chilling of mature microspore derived embryos are realigning the morphological and physiological patterns of embryo development and thus promoting germination. The stage of embryo development for desiccation was critical for subsequent survival of *B. napus* microspore derived embryos: embryos should at least be at the late torpedo stage (Brown et al. 1993).

Microspore-derived embryos of *B. napus* partially desiccated on sterile filter paper for 1 to 4 days were cultured on ½ MS medium with 2% sucrose at 24 °C (Zhang et al. 2006). The germination from partial desiccation for 1 to 2 days was 26 to 41% and conversion to plantlets was 32 to above 50%. However, desiccation for more than 4 days was fatal to the embryos. Microspore derived embryos from *B. juncea* were air dried on sterile filter paper for 15 min on a laminar flow hood and transferred to B5 medium (Prem et al. 2008). The conversion frequency was 81% for 21 to 35-day old embryos, which declined for embryos older than 35 days. Cotyledonary embryos from five subspecies of *B. rapa* were slow dried by placing on filter paper overlaid on 0.8% and 1.6% agar solidified B5 medium (without filter paper) resulted in four to eight-fold higher normal plant regeneration than embryos cultured on 0.8% agar medium without filter paper; highest frequency of normal plant development was on 1.6% agar (Takahashi et al. 2012). Slow air drying on sterile filter paper of microspore derived embryos of *B. napus* by Prem et al. (2012) gave a three-fold higher embryo germination ($50.3 \pm 19.8\%$) compared to direct culture of embryos ($14.0 \pm 4.7\%$). Slow air desiccation was followed by culture of the embryos on solid MS medium with 2% sucrose at 18.0 ± 1 °C in dark conditions until the plumule and radicle were activated, gave an optimum germination frequency of $86.5 \pm 3.7\%$.

Desiccation of the microspore-derived embryos of *B. napus* reduced the incidence of callogenesis and secondary embryo formation (Haddadi et al. 2008). Desiccation treatments for 10 and 15 min regenerated 60% and 52% normal plantlets, respectively, compared to the 22% from the

Table 2 Desiccation with Abscisic acid and by air-drying of microspore derived embryos of selected Brassica species to enhance direct embryo to plant conversion

Days after microspore culture, embryo size	Treatments to convert MDE to plants		Efficiency of MDE to plants	Callogenesis, Secondary embryogenesis	Reference
	ABA	Desiccation			
B. napus cotyledonary embryos 4–6 mm;	0.2 to 5.0 mg/L. B5-1% sucrose, 0.9% agar.		68% with 0.5 mg/L ABA for 12 h	ABA reduced SE. High ABA (5 mg/L) increased callusing to 67%	Ahmadi et al. (2014)
B. napus, 21–24 days MDE	None	Slow air desiccation followed by 18 °C dark.	90% of MDE to plantlets	Not reported	Prem et al. (2012)
B. rapa cotyledonary embryos;	None	MDE placed on filter paper on 1.6% agar	4–8 fold increase in regeneration	Not reported	Takahashi et al. (2012)
B. napus 30 days cotyledonary embryos	ABA 40 µM for 1 week	MDE air dried on open sterile glass plate on laminar flow hood.	ABA 40 µM 1 week: 68% ABA 40 µM + air dried: 63%	Desiccation (10—20 min) reduced callus; SE decreased with increasing desiccation time	Haddadi et al. (2008)
B. juncea 21–24 days MDE	None	Air drying on sterile filter paper for 15 min. Incubation of MDE at 4 ± 1 °C for 10 days in dark.	82% conversion to plants	Not reported	Prem et al. (2008)
B. juncea 20 days MDE, fully developed cotyledonary stage	ABA 1 and 5 mg/L in liquid NLN-13% for 24 h in dark.	Transferred to open sterile plates with filter paper exposed to sterile air in a laminar airflow 15 min. Partially desiccated MDE transferred to semisolid B5	Germination: B5-2%: 46—86% B5-2% + GA3: 40—80% B5-2% + ABA: 30—90%	Donor plants at 10 °C/5 °C were most responsive; field grown plants least responsive. Strong cultivar response	Chanana et al. (2005)
B. oleracea var. capitata 5 genotypes Size not given	ABA 5 mg/L, 25 °C dark shaken at 50 rpm	Transferred to sterile filter paper after 24 h. Germinated on B5 with 20 g/L sucrose at 20 °C	Germination of desiccated MDE: 54–70%. No germination without desiccation	Not given	Rudolf et al. (1999)
B. campestris Cotyledonary stage > 1 mm	10 µM ABA	Desiccation by transfer through desiccators from high to low RH. Desiccated MDE to B5 0.8% agar on filter paper 25 °C/16 h light	Germination: 87% Plant regeneration: 46% (maximum from 10 µM ABA treatment)	Moisture reduced to 10% after 6 days of desiccation; MDE not treated with ABA were not viable	Wakui et al. (1994)
B. oleracea var. *italica* (broccoli) Cotyledonary stage > 1 mm	100 µM ABA	Desiccated on filter paper over 6 days from high to low RH in desiccator; rehydrated for germination	Germination: 55%; Regeneration: 39%	Large embryos more responsive to ABA; higher ABA concentrations reduced conversion; water content reduced to 10%; desiccated MDE retained viability for 2–3 months	Takahata et al. (1993)

Table 2 (continued)

Days after microspore culture, embryo size	Treatments to convert MDE to plants		Efficiency of MDE to plants	Callogenesis, Secondary embryogenesis	Reference
	ABA	Desiccation			
B. napus 21—28 day MDE; elongated root/shoot axis with 2 enlarged cotyledons; 5 mm embryos selected	None	Chilling MDE for 9–12 days/4 °C; slow air-drying of MDE on filter paper.	Germination: > 90% of 35 days old MDE after air-drying for 8 days. 28 days embryos germinated better than 21 days independent of cold treatment	Germination declined after 35 and 49 days. Rapid air-drying killed MDE. Cold temperature improved conversion; less manipulation of MDE—suitable for large breeding programs.	Kott and Beversdorf (1990)

Regeneration medium was B5 with 2% sucrose solidified with 0.7% agar. Embryos were incubated at 20° to 25 °C

ABA Abscisic acid, *B5* Gamborg's medium, *GA3* gibberellic acid, *MDE* microspore-derived embryo, *MS* Murashige and Skoog's medium, *SE* secondary embryogenesis

non-desiccated control plants. Microspore derived embryos desiccated with 40 µM ABA were able to regenerate into normal plants in vermiculite or perlite moistened with B5 basic medium (Haddadi et al. 2008).

It is evident from the above results that artificial induction of desiccation tolerance is vital to improve the conversion of embryos to plantlets. The simple technique of slow air-drying of embryos on sterile filter paper on a laminar flow hood, at the late torpedo or cotyledonary stage of development for at least 3 days, can significantly improve the conversion of embryos to plantlets. Desiccation treatments and the efficiency of treatments to convert microspore derived embryos to plants, for selected Brassica species, are summarised in Table 2.

Abscisic acid and embryo maturation

Seed physiology studies have shown the significance of the phytohormone abscisic acid (ABA) for seed development and maturation. Besides key processes in the plant life cycle, it also regulates key events during seed formation such as deposition of storage reserves, prevention of precocious germination, acquisition of desiccation tolerance, and induction of primary dormancy (Kermode 2005). Dormancy in seeds, defined as the temporary incapacity of viable imbibed seeds to germinate under favourable conditions (Hilhorst et al. 2010), requires de novo synthesis of ABA in imbibed seeds to maintain seed dormancy (see review by Nambara et al. (2010) and citations therein). In zygotic seeds, ABA is involved in regulating the onset of dormancy, the control of seed germination and the achievement of desiccation tolerance. In addition, embryos are maintained in the developmental mode and ABA biosynthesis in the embryo and surrounding seed tissues ensures the maturation process and synthesis of storage proteins and storage lipids (Kermode 1995, 2005). ABA is thus a significant hormone in the development of seeds and during the maturation phase to ensure good germination and vigorous seedlings.

The plant hormones ABA and GA3 have antagonistic roles in seed germination: ABA promotes dormancy and GA3 promotes germination. Thus, their balance is important in the dormancy/germination process (Shu et al. 2016; Macovei et al. 2017). Based on these findings in zygotic embryos, ABA was used to manipulate embryo maturation and desiccation tolerance in microspore-derived embryos of *Brassica* to improve their conversion to plantlets. These studies are reviewed below, with an overview presented in Table 2.

In vitro derived embryos are not known to show desiccation tolerance. Microspore derived embryos of *B. napus,* at the cotyledonary stage, when treated with 50 µM ABA and dried to less than 15% moisture (desiccation) could be stored for at least 7 days (maturation) similar to natural

seeds with a conversion rate of 40 to 50% (Senaratna et al. 1991). The exogenous application of ABA in the culture medium of microspore derived embryos of *B. napus* cv. Westar increased the tissue levels of ABA tenfold after 1 to 2 days and declined thereafter. Desiccation of the microspore derived embryos after ABA treatment led to 71% germination; desiccation in the absence of ABA resulted in tissue death of the embryos. Less mature and smaller embryos (< 500 µm) when treated with ABA, without desiccation, also improved their germination to 99.5% compared to 75% in the untreated control embryos (Johnson-Flanagan et al. 1992). ABA (3.8 µM) had a positive effect on induction of desiccation tolerance of encapsulated somatic embryos of sugar cane; when combined with sucrose (0.5 M) as a protectant, the results were superior to that of ABA alone (Nieves et al. 2001).

The treatment of dried 21 day old microspore derived embryos of *B. napus* cv. Topas with 100 µM (10^{-4} M) ABA (to moisture levels in mature seeds), arrested further growth and stimulated synthesis of proteins (Pomeroy et al. 1994). Endogenous ABA levels declined in the untreated embryos and increased when ABA was added to the culture medium. Studies have further shown that these changes from controlled desiccation and rehydration enhanced embryo survival (Brown et al. 1993; Takahata et al. 1993). Callusing and secondary embryogenesis on the embryos sets back the process of regeneration to plants in *Brassica*.

In other *Brassica* species, exogenous application of ABA induced desiccation tolerance in microspore-derived embryos of broccoli *B. oleracea* L. var. *italica* (Takahata et al. 1993). The survival of the embryos depended on the concentration of ABA applied and developmental stage of the embryo, the cotyledonary stage being best suited. An application of 100 µM ABA induced desiccation tolerance with the dried embryos able to maintain viability of 57 to 63% for three to 6 months at room temperature; conversion was modest with 27 to 48%.

In *B. oleracea* ssp. *capitate*, desiccation tolerance was genotype dependent (Hansen 2000). Although one of the cultivars (cv. Hawke) showed desiccation tolerance independent of the ABA treatments, the untreated embryos did not survive beyond 30 days. Desiccation reduced the moisture content in the embryos from 80 to 12% in 28 days. While germination in the non-desiccated embryos was limited to a few, those treated with ABA produced shoots for a significantly longer period.

The consensus, from these studies is that microspore derived embryos of Brassica are capable of acquiring desiccation tolerance from exogenous ABA. This is best achieved by treating the embryos, at least 3 weeks old, with 10 to 100 µM of ABA (species dependent due to variable sensitivity to ABA) followed by slow air-drying on sterile filter paper under sterile conditions (Table 2). In the absence of protective tissues, the in vitro derived embryo is very sensitive to rapid moisture loss. Slow air desiccation is the preferred method to desiccate microspore derived embryos. This would contribute significantly to embryo maturation and improve the conversion of microspore-derived embryos to plantlets and also suppress secondary embryogenesis on the embryos.

Cold treatment of embryos

To improve the conversion to plantlets, young embryos were treated with less than 10 °C to determine their sensitivity to cold temperatures. These studies showed a positive effect on conversion and are reviewed below and in Table 1.

Cegielska-Taras et al. (2002) transferred physiologically mature 21 days old microspore derived embryos of winter oilseed rape *B. napus* ssp. *oleifera* to B5 medium and cultured them at 24, 4 and 1 °C for 14 days followed by 24 °C for 21 days. The control microspore derived embryos cultured continuously at 24 °C developed secondary embryos and abnormal cotyledons and hypocotyls. Embryos cultured at 4 °C for 14 days similarly developed abnormally as well as secondary embryos, with only 11–19% forming shoots and converting to plantlets. The microspore derived embryos cultured at 1 °C for 2 weeks, however, showed normal embryo development with shoot and root development from 74 to 86% of the cultured embryos. In another study by Zhang et al. (2006), embryos of *B. napus* at the cotyledonary stage were cold treated at 4 °C for 3 to 7 days followed by 24 °C under a 16 h photoperiod, showed a germination of 60 to 90% and a conversion to plantlet of 22 to 58%. The ideal embryo size was less than 4 mm, and these were cultured on ½ MS medium with 2 mg/L BAP.

In other *Brassica* species, the conversion of microspore derived embryos of *B. juncea* was significantly enhanced by a cold treatment of 4 ± 1 °C in the dark for 10 days; this increased conversion of the embryos to plantlets four-fold over the controls, which were continuously cultured at 25 °C (Prem et al. 2008). The conversion to plantlets of the cold treated embryos was unaffected by the genotype and age of the embryos. Similar results were shown for cold treated embryos of *B. juncea* by Chanana et al. (2005), who attributed this to a decrease in ABA levels. In ornamental kale *B. oleracea* var. *acephala*, cotyledonary microspore derived embryos cultured on solidified B5 medium at 4 °C for 2 or 5 days gave a conversion of 79%, which was further improved by the addition of 3 or 5 mg/L silver nitrate (Wang et al. 2011).

These studies provide evidence for significantly improving the conversion of 3 to 4-week old microspore derived embryos to plantlets by treating them at 0 to 4 °C on an agar solidified medium with B5 or MS with 2% sucrose. The

period of exposure to the cold temperature can vary from 2 to 14 days.

Encapsulation

In Brassica species, encapsulation was successfully demonstrated with micro-shoots of cauliflower *B. oleracea* var. *botrytis* (Rihan et al. 2011; Siong et al. 2012). A large number of microspore derived embryos are available from Brassica species—more than the number of potential seeds from a single plant. These embryos can be desiccated and converted to plantlets in a short period of 6 to 8 weeks by application of encapsulation technology to progress towards artificial seeds. Desiccated microspore derived embryos should be coated to isolate them from the external environment. The coating should be non-toxic, non-aqueous with a low melting point, and soft enough for emergence of the shoot and root. Relevant methods for encapsulation of the embryos are reviewed below and selected species are summarized in Table 3. The different coating agents available for encapsulation are reviewed by Ara et al. (2000) and Rai et al. (2009) and the different methods of encapsulation—single layered, double layered and hollow beads—are reviewed by Sharma et al. (2013).

Artificial seeds are encapsulated in a synthetic capsule, which is the equivalent of the seed coat. Besides providing mechanical support and protection to the embryo within, it should also contain the artificial endosperm, provide gaseous exchange with the outside environment while at the same time prevent moisture loss from the embryo. The capsule should also have moderate viscosity and quick gelation, with a high embryo to plant conversion (Swamy et al. 2009). It should also be inexpensive, readily available, non-toxic and easily solidified by treating with calcium, and perhaps most important it is bio-compatible (Tay et al. 1993).

The primary constituents of the capsule are sodium alginate and calcium chloride in correct proportions, whose concentrations should be optimized for the type of propagule and species. Most commonly, 3–4% (w/v) sodium alginate and 75 to 100 mM calcium chloride are used for 20–30 min (see review by Sharma et al. (2013). However, the alginate capsule also has disadvantages that need to be overcome. The hydrated capsules are easily permeable, sticky and need an additional hydrophobic layer; they can dehydrate rapidly and their porosity can lead to leakage of the enclosed artificial endosperm and moisture can diffuse out and compromise the germination and regrowth of the encapsulated propagules (Micheli et al. 2002).

The seed coat in conventional seeds is perforated by the radicle/root at the micropyle end for the embryo to emerge and establish in the soil. Capsules used in artificial seeds, however, are uniform, lack elasticity and the strength of the capsule does not facilitate easy emergence (Onishi et al. 1994). The commonly used alginate coat, while biologically compatible, is also a barrier to diffusion of gases (oxygen deficiency within) and moisture. To overcome this, a novel self-breaking gel bead and a sustained release microcapsule was developed by Onishi et al. (1994) for mass production of carrot somatic embryos. In this method, the embryo loaded alginate gel beads, are hardened by $CaCl_2$., After rinsing away the excess Ca, the beads are immersed in a monovalent cation solution (e.g. KNO_3) followed by another rinse—the self-breaking treatment. The K^+ partially replaces the Ca^{++}, which after pre-germination soaks readily and splits open.

The alginate capsule has a high concentration of sodium and calcium ions. In encapsulated somatic embryos of beech, germination was higher when the sodium alginate concentration was increased and period of exposure to $CaCl_2$ was reduced (Priscila et al. 2009). In this study, which included mature zygotic embryos and somatic embryos, the former had the vigor to emerge overcoming the capsule resistance and the lack of oxygen due to the high alginate content of the capsule matrix. Excessive exposure of the embryos to Ca can inhibit their growth, which is reflected in a decrease in germination. Similar observations were made by Prewein and Wilhelm (2003), and Malabadi and Van Staden (2005).

The embryos to be encapsulated can either be in the desiccated or hydrated form. The desiccated form is more appropriate for microspore derived embryos, which are bipolar and can undergo desiccation. This provides a maturation phase for the embryo (Sharma et al. 2013) and facilitates handling, storage, transport and protection from mechanical damage, similar to natural seeds.

Zhang and Khademhosseini (2017) synthesized a hydrogel from cellulosic nanofibers with a microporous structure, and hydrophilicity with a high capability for water absorption. This medium also had antifungal activity from the carboxylate content in the gel and was demonstrated as a soilless culture medium for sesame seeds. This medium could potentially substitute for the agar medium used for regeneration by incorporation of B5 nutrients and desiccated microspore derived embryos.

Thus, the maturation phase is essential for in vitro derived embryos prior to encapsulation. Maturation of embryos is an important phase in their eventual conversion to plantlets. Early germination of embryos leads to poor conversion to plantlets, due to insufficient accumulation of storage reserves and synthesis of storage proteins.

Priming of artificial seeds

Priming is a concept traditionally applied in commercial seed technology to enhance germination, seedling vigor and characteristics associated with successful establishment in

Table 3 Constituents of artificial endosperm (AE) for priming of artificial seeds in selected species to enhance germination and regeneration

Species	Artificial Endosperm					Germination	Regeneration	Encapsulation	Comments	Reference
	Carbohydrate source	Hormones	Nutrients	Anti-microbial	Other					
Citrus ZE, SE	Sucrose	ABA, GA3	none	none	none	100%	Irregular	Ca alginate	No growth regulators	Nieves et al. (1998)
Oryza sativa SE	Sucrose	IAA, NAA, BA, Activated Charcoal	MS, vitamins	Bavistin, Streptomycin (no effect on germination)	Self-breaking gel beads technology	Increased by 52% over control	Increased by 47% over control	KNO_3 dip for emergence	All primed seeds germinated	Kumar et al. (2005)
Nothofagus alpina (beech)	Sucrose	IAA, NAA, BAP	MS	none	AS cultured on MS + agar for 3 wks	ZE: 100% SE: 45%	ZE in 3 wks SE after 4 wks, irregular and asynchronous	Alginate: 2–4%. ZE have vigor to emerge	SE: immature and asynchronous development	Priscila et al. (2009)
Citrus reticulata (cv. Mandarino) SE	Sucrose 6.8 g/L	GA3, NAA	½ MS, malt extract, ascorbic acid	Germicide: Plant Preservative Mixture (PPM) Fungicide: Thiophanate-methyl	Storage at 4 °C for 60 days	Sprouting 83% Rooting 46% after storage at 4 °C for 60 days	PPM had negative effect	Ca alginate	PPM reduced rooting	Antonietta et al. (2007)
Quercus robur SE	Sucrose 3%	IAA, IBA, BAP	P_{24} medium (Teasdale 1992)	None	Germinated on P_{24}	Genotype dependent	Genotype dependent. One cell line: 26% over the control (naked SE)	Na alginate	With AE, germination and regeneration earlier	Prewein and Wilhelm (2003)
Acca sellowiana Feijoa, guava pineapple	Sucrose 3%	BA, GA3, activated charcoal	LP medium (von Arnold and Eriksson 1981) + Morel's vitamins	None	AE enhanced survival of SE		Encapsulation reduced regeneration, but increased survival +60 days of culture compared to SE	Na alginate 1%, $CaCl_2$ 50 mM. Torpedo stage SE.	70% plantlets from AS survived cf. 30% direct regeneration	Cangahuala-Inocente et al. (2007)

Table 3 (continued)

Species	Artificial Endosperm					Germination	Regeneration	Encapsulation	Comments	Reference
	Carbohydrate source	Hormones	Nutrients	Anti-microbial	Other					
Asparagus officinalis Asparagus SE	Micro-capsules of sucrose 30 g/L	IAA	MS	Fungicide: Thiophanate-methyl	Primed before encapsulation. MS, sucrose, hormones, ABA for 1 week; IAA to shorten roots	IAA necessary to suppress rooting before encapsulation	Pre-encapsulation culture of SE necessary for conversion. Fully encapsulated: 22%, Partially encapsulated 72%	Na alginate	No conversion without priming. Long roots from priming—difficult to encapsulate	Mamiya and Sakamoto (2001)
Quercus suber SE	Sucrose		Macro- and micro-nutrients + cofactors	None	Cotyledonary SE in basal medium + 1% AC for 3 mths, dark	Storage at 4 °C did not affect conversion.	AE with nutrients enhanced regeneration; no effect of sucrose	Na alginate	Storage for 2 mths at 4 C: no loss in regeneration; if > 100 days loss.	Pintos et al. (2008)
Sacharum spp. Sugar cane SE	90 g/L	IAA		Broad spectrum bactericide/fungicide (Vitrafural G1)	10 days after encapsulation, germinated on non-sterile substrate in the greenhouse	Not given	Initially thin long stems	Na alginate	Field planting with control: no difference in yield, quality, low flowering	Nieves et al. (2003)

AC activated charcoal, *ABA* abscisic acid, *AS* artificial seeds, *BAP* benzyl amino purine, *GA3* gibberelic acid, *IAA* indole acetic acid, *MS* Murashige and Skoog medium, *NAA* naphthalic acid, *SE* somatic embryos, *ZE* zygotic embryos

the field such as rapid and uniform seedling emergence, and ability to withstand biotic and abiotic stresses. These are characteristics found wanting in the establishment of in vitro derived propagules—somatic, microspore derived embryos and non-embryogenic explants. Priming is a water-based technique allowing controlled rehydration of the seed, which permits primary metabolic activities within the seed to initiate anti-oxidant and DNA repair mechanisms. This contributes to maintain genome integrity and seed vigor (Welbaum et al. 1998; McDonald 2000; Macovei et al. 2017), but prevent the transition towards full germination with radicle elongation (see Paparella et al. (2015) for an overview of seed priming).

Redenbaugh (1993), a pioneer in artificial seeds technology, identified the absence of a natural endosperm and a protective coating (seed coat) as a major drawback to store and handle artificial seeds. The performance of propagules from tissue culture—embryonic and non-embryonic—has met with much less success in terms of conversion to plants, vigor of growth, and establishment. In vitro derived propagules, although genetically similar to their seed counterparts, are physiologically and morphologically at a disadvantage in their preparedness to face the natural environment. In vitro artificial seeds or propagules are produced under plant tissue culture conditions, which is conducted in a controlled artificial environment, at low light intensities, in small vessels, with poor air exchange, on artificial growth media with sucrose, nutrients and growth regulators at concentrations exceeding natural conditions. These cause developmental distortions and changes in the metabolic pathways, morphology and physiology of the plant (see Nowak et al. (2003) for further references). When in vitro derived propagules are transferred to in vivo conditions, they are morphologically (no cuticle, tender root system) and physiologically (heterotrophic) at a disadvantage to meet the new micro- and macro-climate: a less humid atmosphere (more desiccation), higher light intensities (more transpiration), change from heterotrophic to autotrophic nutrition, exposure to soil pathogens etc. Thus, in vitro propagules routinely go through a process of acclimatization or hardening. This process can be enhanced or replaced by 'priming' the in vitro propagules. Primed artificial seeds can either avoid or reduce the expensive acclimation process required for the transfer of in vitro embryos to the soil.

Non-metabolizable osmoticum has improved microspore embryogenesis and conversion of embryos in *B. napus*. Using PEG 4000, Ilić-Grubor et al. (1998) replaced sucrose as an osmoticum to give comparable embryogenesis. Sucrose in the NLN medium was successfully replaced with PEG (25% w/v) in *B. napus* and many other Brassica species, without the mandatory heat shock (for microspore embryogenesis) besides also improving spontaneous chromosome doubling (Ferrie and Keller 2007). Seeds of *B. napus* osmo-primed with a PEG 6000 solution for 7 days at 25 °C in the dark had positive metabolic effects (Kubala et al. 2015). A transcriptomic and proteomic analysis was conducted during the two distinct phases of soaking and dehydration. There was an upregulation of the genes involved in mRNA synthesis, ribosome synthesis and translation. The regulation of distinct sets of genes during the two phases indicated that priming initiated metabolic pathways that eventually led to germination of the osmo-primed seeds. In addition, a positive impact was seen on the proteins involved in the management of oxidative stress during post-priming germination. Germination was improved and reduced the time taken for emergence of the radicle compared to unprimed seeds (Kubala et al. 2015). Thus osmo-priming microspore derived embryos with PEG would enhance conversion and quality of plantlets.

Nowak et al. (2003) suggest bio-priming, where beneficial micro-organisms are used in vitro and ex vitro after the artificial seed is planted. In *B. rapa*, seeds primed with the cauliflower mosaic virus (Kalischuk et al. 2015; Macovei et al. 2017) induced an increased response to biotic stress that was carried over to next generation(s); the authors suggest a trans-generational epi-genetic inheritance induced by priming.

Nieves et al. (1998) evaluated different artificial endosperms using zygotic embryos of citrus to determine their suitability to encapsulate somatic embryos. The embryos could metabolize only sucrose. Germination was 100% in the absence of growth regulators (ABA and GA3) and germination was possible if GA3 > ABA and delayed if ABA > GA3. The conversion of embryos to plantlets can benefit from priming the embryos with free metabolites such as amino-acids (glycine, proline) and the raffinose family oligosaccharides, which are associated with desiccation tolerance and longevity (Angelovici et al. 2010; Li et al. 2017). An overview of priming in selected species is given in the Table 3.

A critical phase in micro-propagation and in artificial seeds technology is the transition of explants from an aseptic state to septic state in the soil. The presence of sucrose and other nutrients in the artificial endosperm can also encourage infection by soil pathogens on transfer to soil, needing the inclusion of anti-microbials. The susceptibility of encapsulated artificial seeds to soil microbes requires that one or more anti-microbials are included in the artificial endosperm. Since sucrose and nutrients can influence the infections of the explant, they should be at a minimal level to maintain the embryo in the artificial seeds. What has not been attempted so far is a simulation of seed dressing on the external coat of the artificial seeds. In addition, the transplanting environment can be partially sterilized to reduce the microbial load for initial establishment of the artificial seed. While priming is still based on empirical findings, an

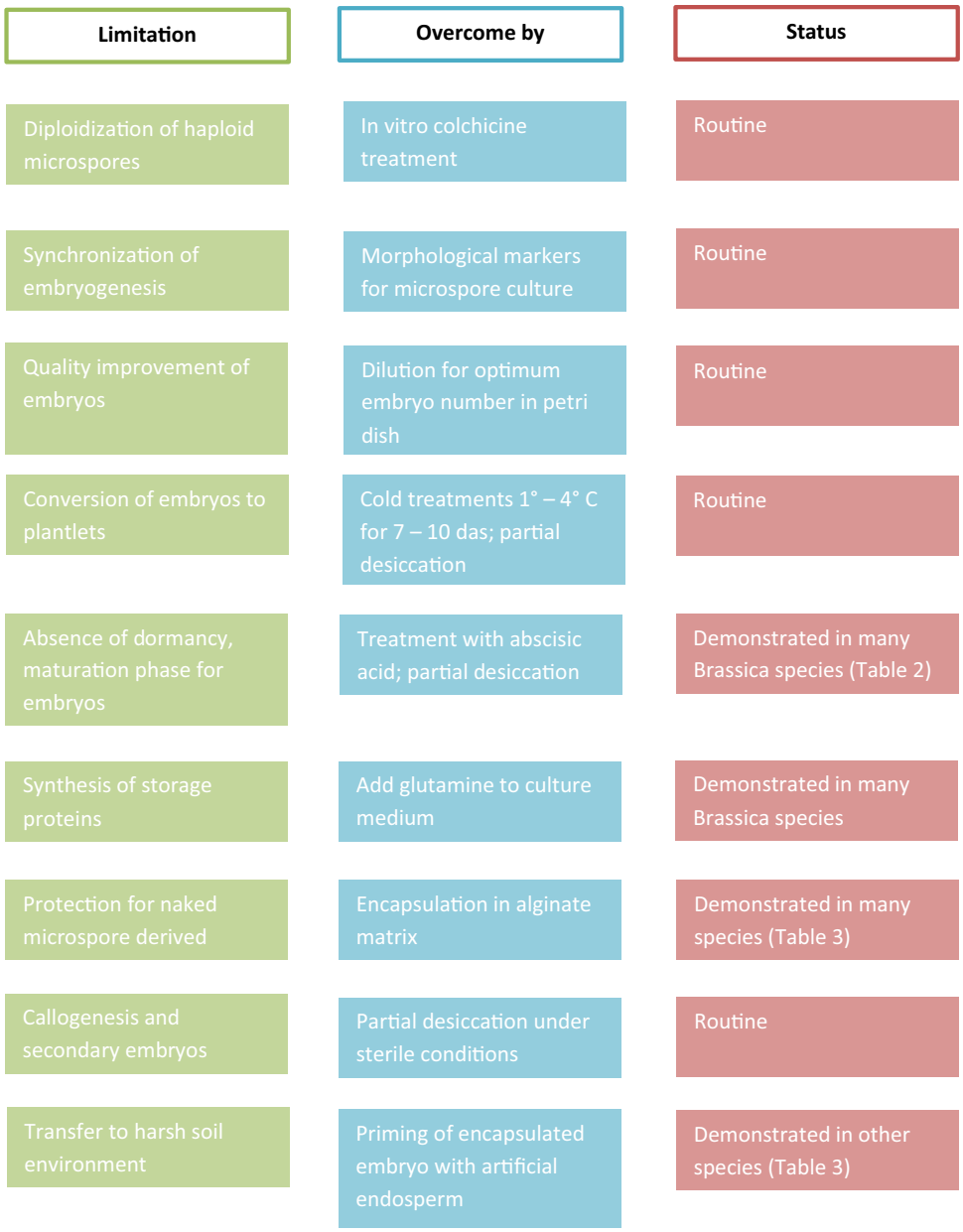

Fig. 2 Overcoming the limitations of microspore derived embryos as artificial seeds

understanding of the molecular responses in the embryo to priming can address genotype depended variability and treatment specific changes (Paparella et al. 2015; Macovei et al. 2017).

Future perspectives

Plant tissue culture has focused on manipulations of culture conditions—physical and chemical—to successfully improve the outcome of microspore derived embryos in Brassica species. There is a need to understand the fundamental mechanisms underlying critical phases in the development of microspore derived embryos—maturation, desiccation and germination—to improve the quality and vigour of plantlets. Whereas the conventional seed receives its nutritional and development requirements from the parent plant, in microspore derived embryos particularly (and less in somatic embryos), the embryo developing from the single cell stage is dependent on a synthetic cocktail of nutrients from the culture medium. Although some experimental studies have considered embryo quality (age, size), medium for conversion, ABA treatment, desiccation tolerance and cold treatment in isolation, some or combinations of all these have contributed together for successful germination and conversion of embryos to plantlets.

The major limitations to utilize microspore derived embryos as artificial seeds can be overcome from recent

progress in other species. In spite of biotechnological advances on many fronts necessary for artificial seeds (encapsulation, conversion, delivery methods), the potential foreseen in this technology is yet to be realised at the plant breeding level. Overcoming these limitations and their present status are summarised in Fig. 2.

A considerable body of research has accumulated in doubled haploids of Brassica species in general and *B. napus* in particular on the induction of embryogenesis, in vitro diploidization, desiccation and conversion of embryos to plantlets besides an understanding and mechanistic explanation of these events. While single experimental studies provide answers to specific questions, the development of artificial seeds, however, needs the input from different biotechnology processes (desiccation, encapsulation and priming) to develop uniform and vigorous in vitro embryos capable of germination and conversion to plantlets. It is now necessary for this empirical research to expand their applications by including the biotechnologies of encapsulation and embryo priming to make the transition from microspore-derived embryos to artificial seeds. We have reviewed the different relevant studies on the theme of embryogenesis and desiccation in Brassica haploids to provide future directions to answer the ambitious question of developing artificial seeds to provide some of the flexibility available from natural seeds, even if this is for a few weeks or months, to the Brassica plant breeder.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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