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Conifer somatic embryogenesis – an efficient plant regeneration system for theoretical studies and mass propagation

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Abstract: Since the first report of somatic embryogenesis in Norway spruce in 1985, the *in vitro* process has been initiated for a number of conifer species belonging to different genera. The process of somatic embryogenesis involves initiation, proliferation, maturation and plantlets (emblings) regeneration. The initiation of somatic embryogenesis is restricted mostly to juvenile explants, although recently explants taken from adult trees produced embryogenic tissues. The successful initiation, maturation and emblings regeneration are affected by factors as developmental stage of primary explants, genotype, plant growth regulators content in the culture medium, light conditions. Optimisation of mentioned factors resulted in regeneration of emblings capable of growing after transfer to soil.

Additional key words: *in vitro*, plant biotechnology, plant growth regulators (PGRs)

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Introduction

Somatic embryogenesis (SE) is an *in vitro* process of embryo differentiation without sexual fusion of gametes from somatic cells of a plant body. Owing to the origin, the differentiated structures are called somatic embryos and, in morphology as well as internal organisation of tissues, they resemble their counterparts in seeds resulted from gamete fusion. In appropriate conditions the differentiated structures develop and their development results in complete plant formation.

The process was first described for carrot in 1958 and later for numerous angiosperm species. For conifers, SE was first reported for Norway spruce (Hakman et al. 1985; Chalupa 1985) and has since been achieved for other conifer species belonging to genera *Abies*, *Picea*, *Pinus*, *Larix*, *Pseudotsuga* and *Taxus*. It is worth to mention that in suspension cultures of *Pinus banksiana,* Durzan and Chalupa (1976) observed cell aggregates, long vacuolised cells resembling the suspensor cells as well as polarised structures that did not develop further into bipolar structures described later as somatic embryos and no plantlet regeneration occurred. For conifers, the process of SE represents an excellent plant regeneration system available for the theoretical study of early plant development and is convenient for large-scale trees vegetative propagation. Conifer SE as a plant regeneration system plays also an important role in biotechnological approaches as genetic transformation and cryopreservation.

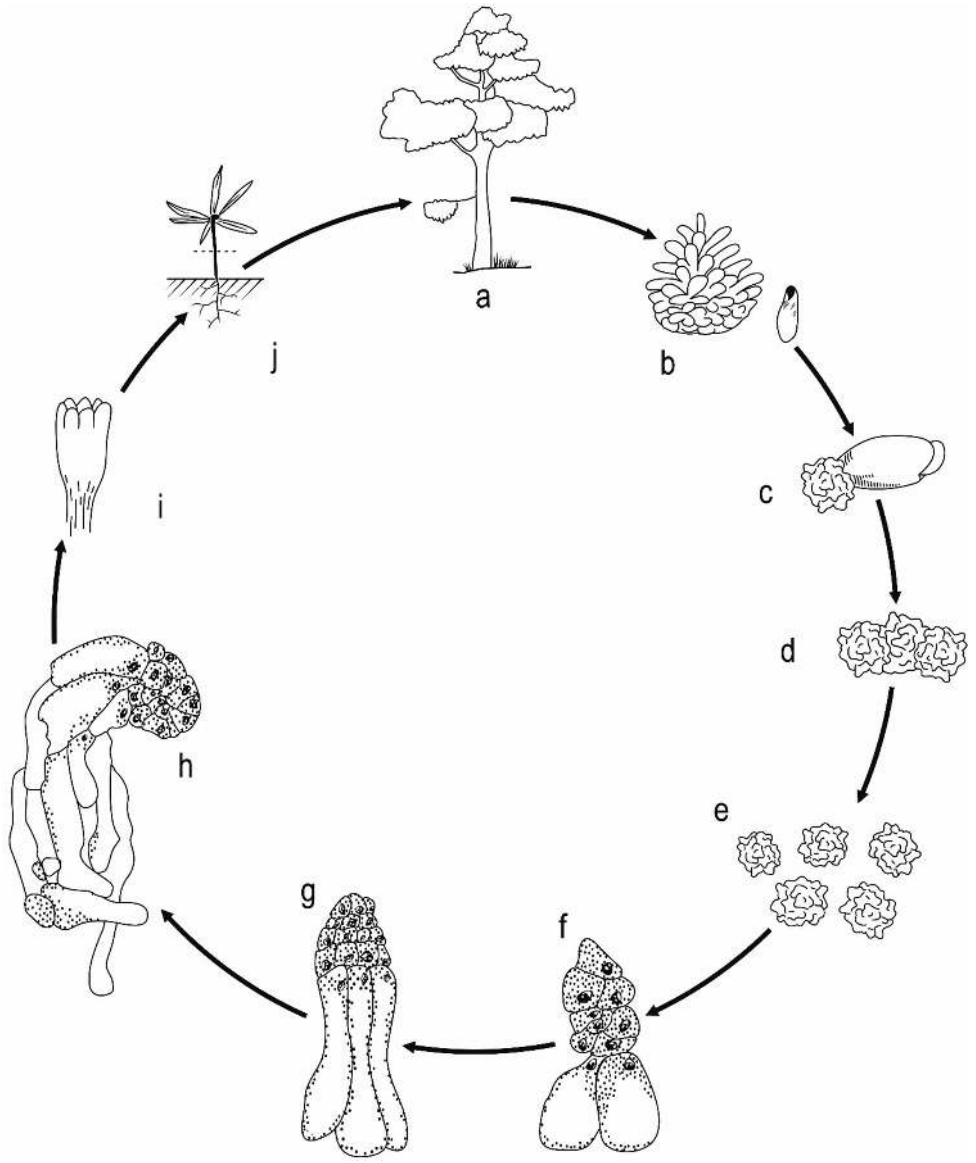

Fig. 1. Embling (somatic seedling) regeneration *via* somatic embryogenesis (an example of *Pinus nigra*): from an adult tree (a) green cones are collected (b) and the excised megagametophytes of immature seeds as explants are placed on the culture medium. Approximately after 12–15 days of cultivation embryogenic tissue is produced on explants (c). Reaching 0.5–1 cm in size (d) the tissue is separated from megagametophyte explants and divided into several pieces (e) and cultivated. Microscopic examination reveals bipolar strucures – somatic embryos in the tissue (f, g, h). In appropriate conditions the bipolar structures develop into mature somatic embryos with apparent cotyledons (i). The mature somatic embryos give rise to small plantlets – emblings capable of growth in soil (j). The emblings have the potential to grow and develop into adult tree (a). With permission from Salaj and Ostrolucká (2010)

The *in vitro* process of SE can be divided into several steps as a) initiation, b) proliferation, c) maturation, d) plantlet regeneration (Fig. 1).

Initiation of somatic embryogenesis

The effect of plant growth regulators – plant growth regulators play key role in the initiation of somatic embryogenesis. Different concentration and combination of auxins and cytokinins were used and these requirements have to be tested for each explant type as well as species. The auxin most frequently used is 2,4-D (dichlorophenoxyacetic acid, a synthetic auxin) and BA (6-benzyladenine) is the most common cytokinin. As a rule concentrations of 2,4-D in culture media are higher in comparison to BA concentrations. Although the most frequently used plant growth regulators are 2,4-D and BA, sometimes BA can be replaced by kinetin and 2,4-D by NAA. For some species solo PGRs (BA, 2,4-D) were efficient to initiate embryogenic tissues (for details see review Tautorus et al. 1991; Attree and

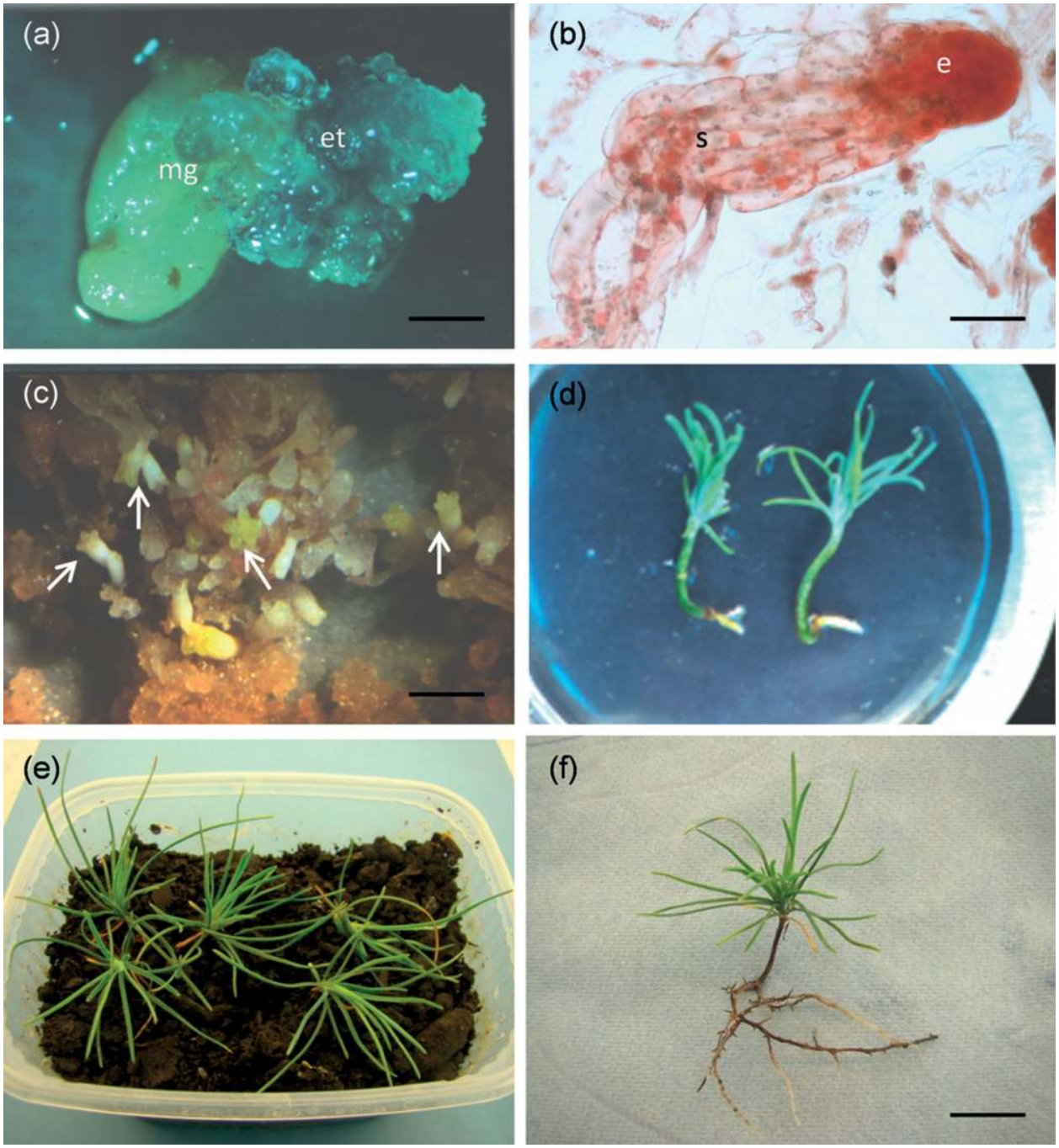

Fig. 2. Developmental sequences in conifer somatic embryogenesis (an example of *Pinus nigra*): (a) – protruding embryogenic tissue (et) from the megagametophyte explant (mg), (b) – early bipolar somatic embryo at stage 1 (e – embryonal cells, s –suspensor), (c) – cotyledonary somatic embryos (arrows), (d) – emblings, (e) – emblings transferred to soil, (f) – embling growing in the soil for 3 months (a, c, d – with permission from Salaj and Ostrolucká 2010). Bars: (a) – 1.2 mm, (b) – 200 μm, (c) – 5 mm, (f) – 15 mm

Fowke 1993; Klimaszewska and Cyr 2002; Lelu-Walter et al. 2013).

Explants for initiation of somatic embryogenesis – Juvenile explants as immature or mature zygotic embryos as well as segments of seedlings or emblings origin and recently also explants taken from adult trees have been used.

Immature zygotic embryos: the zygotic embryos in this developmental stage are of extremely small size and usually are enclosed in megagametophytes. The megagametophyte tissue protects the embryo from undesirable environmental factors e.g. as dessiccation and it is supposed it also serves as a nutritive tissue for immature embryo. According to visual observation, the embryogenic tissue has been extruded from micropylar end of the megagametophyte (Fig. 2a). Using megagametophytes with immature embryos as explants is advantageous owing to the

easy manipulation. However, immature embryos are available only in a very short period of the year. Immature embryos enclosed in megagametophytes have been used as explants in several conifer species as *Abies alba* (Gajdošová et al. 1995; Krajňáková et al. 2013a),

Abies nordmanniana (Nörgaard and Krogstrup 1991), *Juniperus communis* (Helmersson and von Arnold 2009), *Taxus brevifolia* and *T. cuspidata* (Ewald et al. 1995) but are typical for the species of the genus *Pinus*, as *Pinus sylvestris (*Aronen et al. 2009), *Pinus radiata* (Montalbán et al. 2012; Hargreaves et al. 2009), *Pinus pinaster* (Humanez et al. 2012), *Pinus nigra* (Salajova and Salaj 1992), *Pinus palustris* (Jones and van Staden 1995), *Pinus halepensis* (Montalbán et al. 2013) *Pinus oocarpa* (Lara-Chavez et al. 2011). Immature zygotic embryos excised from megagametophytes have been used in *Picea glauca* and *Picea mariana* (Hakman and Fowke 1987), *Picea abies* (Nagmani et al. 1987), *Picea sitchensis* (Krogstrup et al.1988).

The initiation frequencies have been variable depending on the species, year of cones harvest, developmental stage of zygotic embryos, nutrient medium composition reaching values in average 20% for *Pinus pinaster* (Humanez et al. 2012), 5–50% for *Juniperus communis* (Helmersson and von Arnold 2009), 5% for *Pinus taeda* (Becwar et al. 1990), 2.1–10.3% for *Pinus nigra* Salaj et al. (2014) and 20.6% for *Pinus radiata* (Montalbán et al. 2012).

Mature zygotic embryos: for *in vitro* cultivation the mature zygotic embryos are excised from surface sterilised seeds and placed on culture medium. Their advantage as explants is that the seeds can be stored for longer time period, even for several years. The disadvatage of using of mature zygotic embryos has the risk of microbial contamination during the manipulation of the initial explant. The mature embryos as explants have been used for *Picea abies* (Hazubska-Przybyl and Bojarczuk 2008), *Abies alba* (Zoglauer and Rheuter 1996), *Abies fraseri* (Guevin and Kirby 1997), *Abies* hybrids (Korecky and Vitamvas 2011) and it has been less sucessful for *Pinus* species.

Seedlings explants: the cultivation of immature or mature zygotic embryos resulted in initiation of embryogenic tissue in numerous species of conifers, although in this case the genotyp of explants was unproven. Efforts have been made to extend the „initiation window" to explants in more advanced developmental stage. For this purpose mostly segments dissected from seedlings or emblings have been cultured.

In *Picea abies* embryogenic tissues have been initiated on cotyledon explants from 7-day-old seedlings (Lelu et al. 1987). Similarly, success in initiation from cotyledons has been achieved in *Picea glauca, Picea mariana* (Attree et al. 1990) and *Abies* hybrids (Salajová and Salaj 2001). In some of these studies informations related to somatic embryo origin on structural level have been obtained (Lelu et al. 1987; Salajová and Salaj 2001).

Explants from adult trees: success has been achieved in *Larix decidua, Larix eurolepis* (Bonga 1997). In these experiments embryogenic tissues have been obtained on explants from adult 42-years old trees. In initiated tissues early somatic embryos were formed but finally, the regeneration of somatic seedlings failed. In subsequent experiments, optimisation of nutrient medium led to higher initiation frequencies but complete plantlet regeneration remained the bottleneck of the process (Bonga 2004). Park et al. (2010) obtained embryogenic tissue from shoot bud cultures of *Pinus contorta* and Klimaszewska et al. (2011) obtained it from primordial shoot explants from 10-years-old *Picea glauca* somatic trees. In these studies putative embryogenesis specific genes were analysed and attempts were made to select some genes as a markers of SE.

Proliferation of embryogenic tissues

After successful initiation the embryogenic tissues are maintained by regular transfers to fresh media in 2–3 weeks intervals. The maintenance media usually have the same or similar composition as the media used for initiation and this composition is sufficient for most of species. Sometimes, the medium composition needs minor changes and the content of plant growth regulators and/or the inorganic componets are reduced. The embryogenic tissues of conifer species share the same or very similar features. They are of white color, mucilaginous consistence and relatively fast growing tissues. The characteristic feature of tissues is the presence of bipolar somatic embryos (Fig. 2b) although the specific structural organisation is cell line dependent. The media used for maintenance of embryogenic tissues can be solid (solidified with agar or other gelling agent as Phytagel, gelrite) or liquid – suspension cultures. The suspension cultures are established by resuspension of a defined amount of tissues growing on solid medium in liquid and maintained by regular changes of liquid in 7–10 days intervals (Silveira et al. 2003; Vágner et al. 2005; Salaj et al. 2007)

Maturation of somatic embryos

According to von Arnold and Hakman (1988) somatic embryo development can be classified in four different stages: stage 1 – embryos composed of small, densely cytoplasmic cells subtended by a sus-

pensor comprised of long, highly vacuolised cells, stage 2 – embryos (precotyledonary) with a more prominent and dense meristematic region, the embryos are still attached to tissues by long suspensor, stage 3 – embryos with cotyledons (Fig. 2c), stage 4 – green plantlets (Fig. 2d). The regenerated plantles are designated as emblings (somatic seedlings). On the culture media with plant growth regulators (2,4-D, cytokinins) the somatic embryos are in the stage 1. Their further development and maturation can be stimuated by transfer to medium lacking the mentioned PGRs and containing abscisic acid (ABA). The somatic embryo maturation is a complex developmental process characterised by internal histodifferentiation as well as morphological changes, the most apparent of them is appearance of cotyledons. The maturation is regulated by several factors; one of the most important is the genotype and therefore the maturation process is cell line dependent (Krajnaková et al. 2009; Alvarez et al. 2013; Fischerová et al. 2008; Montalbán et al. 2010; Vondráková et al. 2014). Another important factor is the medium composition used for maturation. ABA incorporated into the maturation medium stimulated storage material accumulation, synchronisation of somatic embryo development and prevents precoccious germination. The optimum ABA concentration used for somatic embryo maturation is different for each species and is defined empirically. In comparison to 2,4-D or cytokinins, the ABA concentration in nutrient media is higher reaching 32 μM to 120 μM.

The osmotic potential of culture medium is another important factor to take into account. For many species polyethylene glycol (PEG-4000) was the best osmoticum (Svobodová et al. 1999; Vooková et al. 2010; Mauelová and Vitámvas 2007). The adverse effect of PEG has also been demonstrated in *Picea abies* (Bozkhov and von Arnold 1998). Somatic embryo maturation is also stimulated by high concentration of gellan gum (1%) in the presence of ABA (Klimaszewska et al. 2000).

There exist considerable variations in mature somatic embryo yield/production among species and cell lines and maturation treatments. Some examples of mature somatic embryo yield (number) calculated per 1 g of fresh weight: 64 for *Abies fraseri* (Kim et al. 2009), 36 for *Abies cephalonica* (Krajňáková et al. 2009), 187 in *Pinus pinaster* (Alvarez et al. 2013), 321 in *Pinus sylvestris* (Aronen et al. 2009), 269 for *Picea glauca* (Kong and Yeung 1995), 65 for *Picea mariana* (Tremblay and Tremblay 1995), 300–960 for hybrid of *Larix* (Lelu et al. 1994). An undesirable phenomenon – loss of ability to produce mature somatic embryos – has been observed in embryogenic tissues of many species (Klimaszewska et al. 2009). This phenomenon can be avoid – at least partially – by cryopreservation and subsequent thawing of tissues.

The structural and morphological changes during maturation are accompained by biochemical changes as accumulation of storage reserves (Brownfield et al. 2007; Grigova et al. 2007; Kubes et al. 2014) in the form of protein and lipid bodies and the transport of protein bodies is mediated by Golgi apparatus (Hakman 1993). The storage proteins can also be markers of somatic embryo development (Teyssier et al. 2014).

Germination of somatic embryos

The germination of somatic embryos occurs on media without plant growth regulators and low content of carbohydrates. The success of germination process is also affected by several factors such as the environmental conditions in the previous maturation stage (Salaj et al. 2004; Krajňáková et al. 2008). Another factor involved is the genotype. In this sense, it was found, despite of the same maturation conditions, somatic embryos of different cell lines germinate by different frequencies (Lara-Chavez et al. 2011). The germination usually occurs in dark and after obtaining the small plants, they are transferred to light conditions. For germination low frequency light intensity is also recommended (Lara-Chavez et al. 2011). Improved germination of somatic embryos can be achieved by partial desiccation of somatic embryos immediately before germination (Vooková and Kormuťák 2002; Grigová et al. 2007).

Successful germination and somatic seedling regeneration as well as establishment in soil (Fig. 2e, f) have been reported for a number of conifer species as *Abies alba*, *Abies nordmanniana*, *Picea abies*, *Picea glauca*, *Picea mariana*, *Pinus caribaea*, *Pinus sylvestris*, *Pinus nigra*, *Pinus strobus*, *etc.* (see reviews Attree and Fowke 1993; Klimaszewska and Cyr 2002; Krajňáková et al. 2013b).

Grossnickle (1999) followed the growth and physiological parameters (photosynthesis, water relations, frost tolerance) of *Picea glauca* engelmannii emblings and compared these parameters to seedlings developed from zygotic embryos. During nursery development and field performance, all the mentioned parameters of emblings were comparable to zygotic seedlings. These observations indicate the emblings meet all the criteria required for use in plantation forestry. Cyr and Klimaszewska (2002) referred the numbers of somatic seedlings produced for commercial purposes as follows: *Picea glauca* engelmannii 1 million (1999), *Picea sitchensis* 50 000 (1998), *Picea glauca* 1.45 million (2000), *Pinus taeda* 0.2 million (2000–2001), *Pseudotsuga menziesii* 3.2 millions (2001). Grossnickle and Pait (2008) reported production of 10 million emblings (CellFor) and 500 000 to 1 million emblings of *Pinus taeda* (Arborgen).

Conclusion

SE in conifers represents an efficient *in vitro* regeneration system available for the study of early plant development and micropropagation as well as plays an important role in biotechnological approaches as genetic transformation and cryopreservation. The conifer species are recalcitrant to *in vitro* cultivation in general and also for SE but optimisation of plant growth regulators concentrations and combination in culture media as well as proper timing of pimary explant collection led to improvement of SE initiation. Successful emblings regeneration has been achieved in numerous conifer species and the regenerated plants have also been transferred to soil.

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