Somatic embryogenesis of lodgepole pine (*Pinus contorta*) and subalpine larch (*Larix lyallii*.) for conservation purposes

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Abstract

This study was initiated with the aim of establishing somatic embryogenesis culture from lodgepole pine (for clonal propagation of superior genotypes with mountain pine beetle resistant genotypes) and subalpine larch (for conservation purposes), both of which have never been attempted prior. Cones were collected on Mt Frosty, Manning Provincial Park for larch and from Vernon Ministry of Forestry orchard (307) for lodgepole pine. Optimum stage of cone maturity for initiation success was tested by 4 collections, made at 1 week intervals for lodgepole pine and 3 collections made at 2 week intervals for subalpine larch. Three media were compared: modified Litvay medium, as standard and low and DCR. Pro-embryonal mass (PEM) from lodgepole pine developed as early as 4 weeks, which is twice as fast when compared to subalpine larch (8 weeks). Desired cone maturity was present in lodgepole pine collections 3 and 4, but never present for subalpine larch. In conclusion, no embryogenic tissue was obtained from either species.

Key Words

Somatic embryogenesis, zygotic embryogenesis, lodgepole pine, alpine larch, alpine ecosystems, germination, tissue culture.

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Introduction

Somatic embryogenesis (SE) is a method used for the vegetative propagation of plants. In trees, SE offers a means to mass produce trees of desired traits. The steps to establishing successful SE seems simplistic- an embryo from a seed is extracted from a seed and placed on a medium containing appropriate nutrients, hormones, sugars, amino acids and micronutrients. First a callus culture grows, then the culture becomes embryogenic and the somatic callus begin to differentiate on the callus. These somatic embryos can then be cryopreserved, while individual clones can be tested for desirable traits, or germinated and grown into somatic emblings. Simplistic as it may seem, in tree species, SE is difficult to achieve in tissue beyond the seedling stage; a consequence of the complex interplay of several physiological parameters and the appropriate regulation at the transcription level (reviewed in Bonga et al, 2009).

Somatic embryogenesis in gymnosperms was first reported in 1985 for norway spruce *(Picea abies* Karst.) (Hakman et al. 1985; Chalupa 1985) and european larch *(Larix decidua* Mill.) (Nagmani and Bonga 1985), and has since been achieved for several other conifers (Cheliak and Rogers 1990; Tautorus et al. 1991; Klimaszewska et al. 2006). Gymnosperms are generally more recalcitrant to propagate *in vitro* than most angiosperm trees and herbaceous plants (von Aderkas and Bonga, 2000). As a consequence, successful SE has most often been achieved from immature embryos and juvenile tissue (Harvengt et al, 2001; Klimaszewska et al, 2007; Lelu-Walter et al, 2006; Park et al, 2006). It has proven extremely challenging to achieve SE from mature conifers by *in vitro* manipulation, and many attempts have been made to overcome the inherent phase that is associated with aging (Gupta and Durzan, 1985; Monteuuis, 1991; Bonga, 1997; Merkle et al, 1998; Andersone and Ievinsh, 2002). Traditional methods of *in vitro* manipulation, including complex arrangements of growth regulators and media composition have proven ineffective, and therefore many studies have concluded that the choice

of explants is important when attempting to establish SE from mature trees (McCown, 2000; von Aderkas and Bonga, 2000), (Bonga and von Aderkas, 1992). Ultimately, if success is reached, SE is a powerful tool that can be used for clonal propagation of superior genotypes for out planting (Nehra et al. 2005; Klimaszewska et al. 2007; Klimaszerska et al. 2009) or alternatively as a tool to preserve species at risk.

Several tree species in British Columbia (BC) are becoming vulnerable. Warming trends related to anthropogenic climate change (IPCC 2007) are predicted to decrease the quality and quantity of unique ecosystems. Slow growing, high-elevation species, such as subalpine larch (*Larix lyallii* Parl.) and whitebark pine (*Pinus albicaulis* Engelm), are among the most sensitive to a warming climate (Aitken *et al.* 2008). Optimistically, Aitken *et al*. (2008) have shown that lodgepole pine (*Pinus contorta* Dougl. ex Loud. Engelm.) is one of the species least affected directly by climate change. Unfortunately, it is being affected indirectly; warmer winters have allowed the mountain pine beetle (*Dendroctonus ponderosae)* population to reach astonishing numbers (Aitken *et al*. 2008). This epidemic as a result from warming temperatures, has caused catastrophic changes to BC's forests and as such has caused lodgepole pine to be a potential vulnerable species for the future. A few genotypes have shown resistance to their attack however, if BC's reforestation practises used seedlings which retained these resistant traits then future attacks could be less devastating or even mitigated. The process of breeding a desired species, in this case trees, and then waiting to see what their offspring possess (such as the resistant genotype to the mountain pine beetle) requires far too much time. Although SE can take many months to complete, it is substantially faster than natural regeneration of the desired genotype tree and offers a chance to produce clones with the trait and preserve them.

Subalpine larch is another vulnerable species in BC. Their alpine ecosystem is undergoing drastic change due to increasing temperatures which is resulting in species such as subalpine fir (*Abies lasiocarpa*) to encroach on its native habitat range and intolerable warmer soil temperatures. Although subalpine larch has little commercial value compared to other gymnosperms, it is an ecologically interesting (deciduous gymnosperm with extreme winter weather tolerance) and aesthetically attractive species (Klinka et al. 2000). Subalpine larch exists only in North America including BC and Alberta, Canada and Idaho, Montana and Washington in the United States of America. Their range is limited to high altitudes >1800m and the International Union for Conservation of Nature (IUCN) Red List of Threatened Species includes subalpine larch. Since climate change is causing many unpredictable occurrences, it may be wise for conservationists to way on the side of caution to avoid extirpation, thus avoiding extinction for this unique alpine species. Somatic embryogenesis could ensure future existence of this species as cryopreservation is possible.

Somatic embryogenesis is not necessarily the solution but could serve as an option for the future existence of lodgepole pine and subalpine larch.

Objectives

The purpose of this study was to attempt to obtain embryogenic tissue from lodgepole pine and subalpine larch via tissue culture of megagametophytes. Concurrently, with the preceding objectives the time (stage) of cone retrieval was assessed.

Methods

Subalpine Larch- Experiment #1

Origin of Seeds

Cones were collected, three times bi-weekly during the summer of 2008 between July-August (Table 1) from a small alpine ecosystem in Manning Provincial Park located in British Columbia Canada (Figure 1). This ecosystem is found at the top of Mount Frosty (N49 01.865 W120 49.806) which is the highest peak in the park reaching a height of 2408m. Cones were collected along the trail between the 2000m to 2200m area. Three trees were chosen based on fecundity to ensure subsequent collections could occur weeks to follow. Cones that displayed a purplish coloration, thus open-pollinated female strobili, were chosen, while cones that were green (thus not fertilized or not mature as of yet) were left (Fig 2a). The cones were kept in a refrigerator maintained at 4°C until cultured, and kept separated in bags by parent tree.

TABLE 1 CONE COLLECTION DATE AND TOTAL NUMBER OF SEEDS CULTURED

Collection date	Total number of cultured megagametophytes	Media
July 21	340	MLV-L MLV-S DCR
August 15	300	
August 22	260	

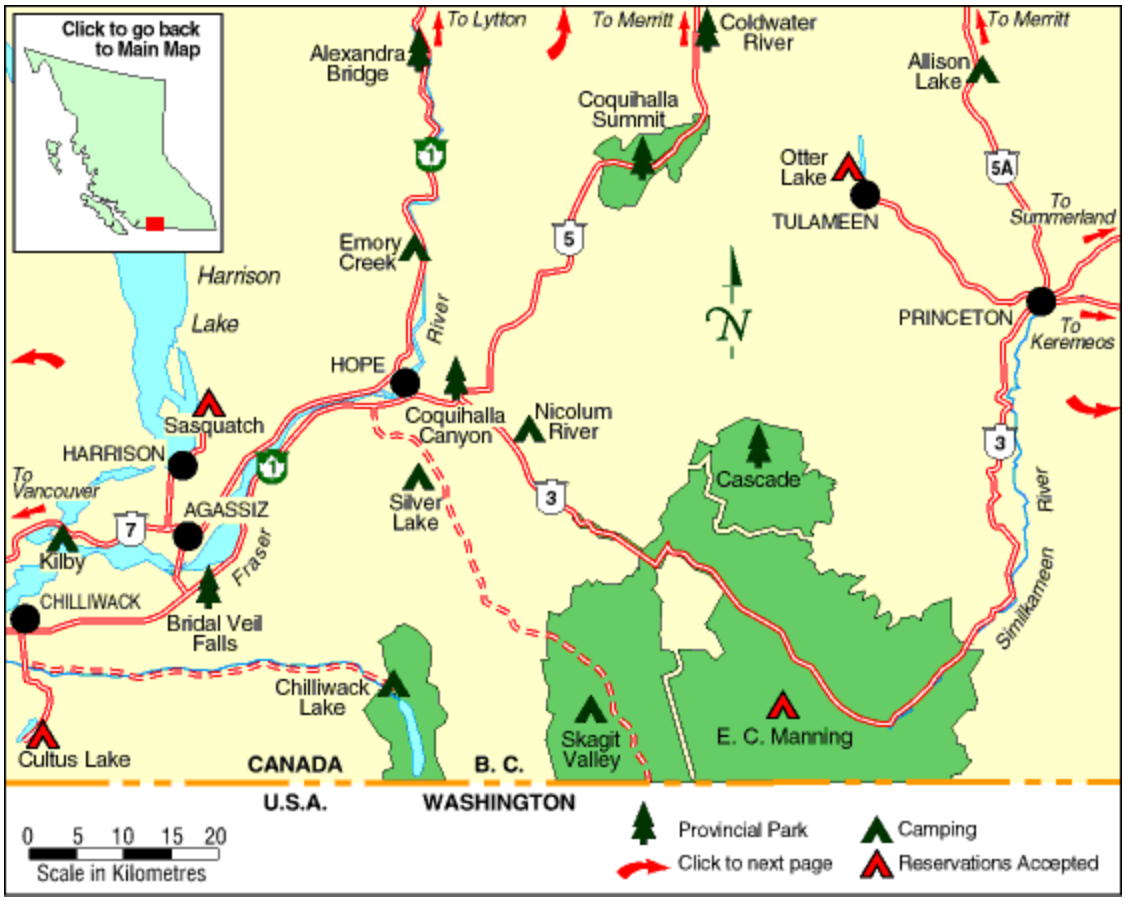

FIGURE 1. MAP OF BRITISH COLUMBIA AND LOCATION OF MANNING PARK PROVINCIAL PARK.

Culturing: Initiation of zygotic embryogenesis

Cones were longitudinally cut with an electric hand saw (Fig 2b). All culturing was preformed in a sterile environment via a laminar flow hood. After cutting cones were sterilized in 95% alcohol for 30-60 seconds. Scales were easily removed from cone stem with sterile forceps and placed on a sterile surface. Subsequently, seeds were emerged in 6% hydrogen peroxide for 20 minutes followed by several rinses with sterile water. Seed sterilization is not necessary for the Lodgepole pine because their cones are serotinous. Gametophytes were extracted from seed coat and placed on 3 different types of media: DCR, MLV standard and MLV low (Table 2). Petri dishes were kept in the dark at room temperature (24°C). The third collection on August

22nd had very poor seed set due to a dipteral outbreak on the trees and therefore only 8 petri dishes per medium type from each tree were made.

FIGURE 2 SUBALPINE LARCH CONES A.) RANGE OF CONE SIZES AND PURPLE COLORING PRESENT. B.) CONE CUT LONGITUDINAL AND HORIZONTALLY TO VIEW INSIDE.

TABLE 2 MEDIA COMPOSITION MODIFIED MLV (LITVAY ET AL. 1985)

	2,4-D	BA	CH	SUCROSE	GELRITE	EXTRA
MLV low	2.2 µM	2.2 µM	1 g/L	2% (w/v)	4 g/L	
MLV standard	9.0 µM	4.4 µM	1 g/L	2% (w/v)	4 g/L	
DCR	9.0 µM	4.4 µM	1 g/L	Maltose	1.5 g/L	Maltose 90mM

Before autoclaving media was adjusted to 5.8. After autoclaving Glutamine (L-glutamine 1g/L) was added.

Lodgepole Pine - Experiment #2

Origin of Seeds

Seeds were collected from the Ministry of Forestry seed orchard (307) in Vernon BC Canada (Figure 3) from 20-year-old lodgepole pine trees. Twenty cones of each genotype (10 total) were collected 4 times with 1 week-interval starting June 19, 2008 (Table 3). The cones were kept in a 4°C refrigerator until cultured and kept separated in bags by genotype.

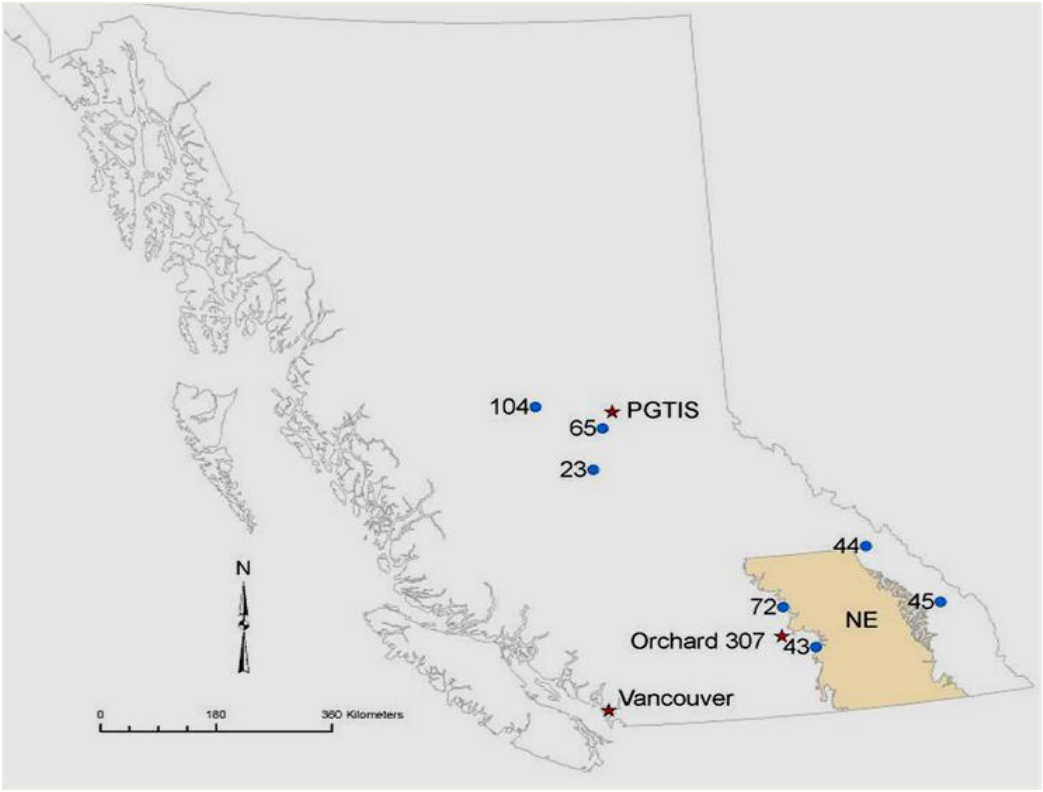

FIGURE 3. SEED ORCHARD #307 LOCATION IN BC

TABLE 3 LODGEPOLE PINE COLLECTION DATES WITH TOTAL RESULTS OF CULTURED SEEDS

Seed family*	Collection date / developmental stage	No of cultured megagametophytes	Medium
	Jun 19 / early dominance	1,177	MLV-L
	Jun 26 / late dominance	1,310	MLV-S
	Jul 3 / pre-cotyledonary	1,224	DCR
	Jul 10 / cotyledonary	947	

***** Genotype 1503, 1504, 1514, 1518, 1520, 1528, 1532, 1533, 1537, 1539

Culturing: Initiation of zygotic embryogenesis

Cones were longitudinally cut with an electric hand saw and then submerged in 70 % ethanol for 10 min. Cut cones were then briefly dried in the laminar flow unit. All culturing was preformed in a sterile environment via a laminar flow hood. Immature seeds were detached from

the cone, and seed coat and nucellus were removed with sterile forceps and the megagametophytes placed on 3 different types of media: DCR, MLV standard and MLV low (Table 2). Petri dishes were kept in the dark at room temperature (24°C).

Data Collection

Petri dishes were monitored daily, for the first week (at short intervals to ensure little light was exposed to cultured seeds). After the first week the chance of unexpected contamination decreased and the petri dishes were only checked weekly for signs of embryogenic tissue forming. All dishes were scored for: survival, callus present, amount loss to contamination, and any seeds showing signs of possible embryogenic tissue forming. Only after scoring was any interesting tissue transferred to fresh media, subsequently biweekly sub-culturing to ensure available nutrients and purification of tissue.

Results

Experiment #1: Subalpine larch

Extrusion, the first evidence of SE initiation, on all medium used; DCR, standard (MLV-S) and low plant growth regulator (PGR) media (MLV-L) was visible for some explants after 4 weeks of culture. However, whole megagametophytes in the majority of explants produced callus after 8–9 weeks of culture, and resulted in mix culture that looked like pro-embryonal mass (PEM) and non-PEM. After 3 months of culture, approximately 10% of callus turned to brown, and vigorously growing calli were observed with a squash sample to identify whether it was PEM or not, no cells were present. After 6 months (Fig 6) most tissue had turned to calli and observations under a microscope confirmed that all samples were not embryogenic (Fig 7).

Although it is almost affirmative that there is no possibility at this time that embryogenic mass (EM) will form, cultures were kept and continued to be transferred and one final observation was taken 18 months after commencement of this experiment, confirming once again that there was no EM present (Fig 8).

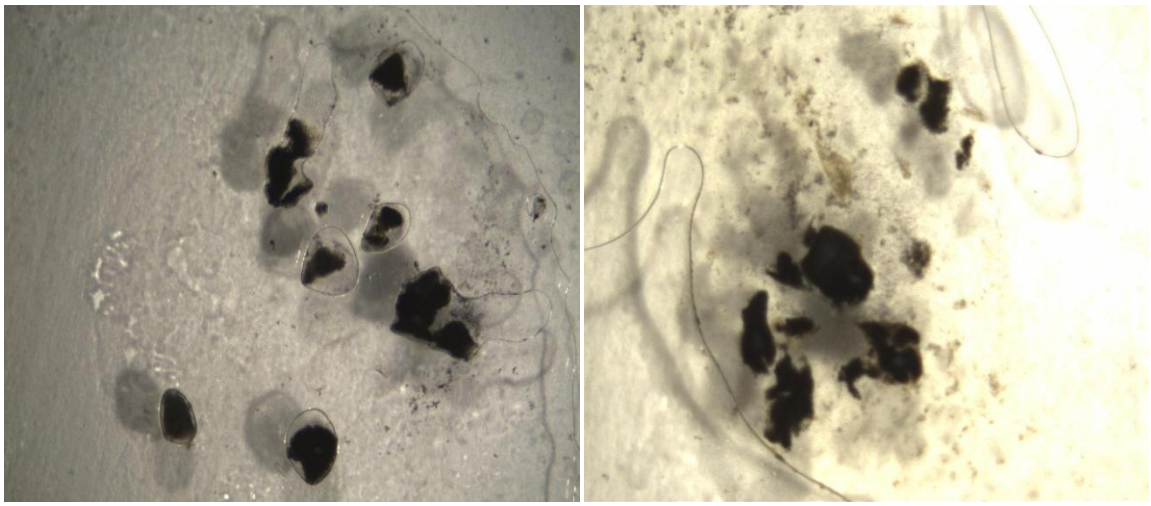

FIGURE 4 DEVELOPMENTAL STAGE OF COLLECTION 3 OF EMBRYO UPON CULTURING

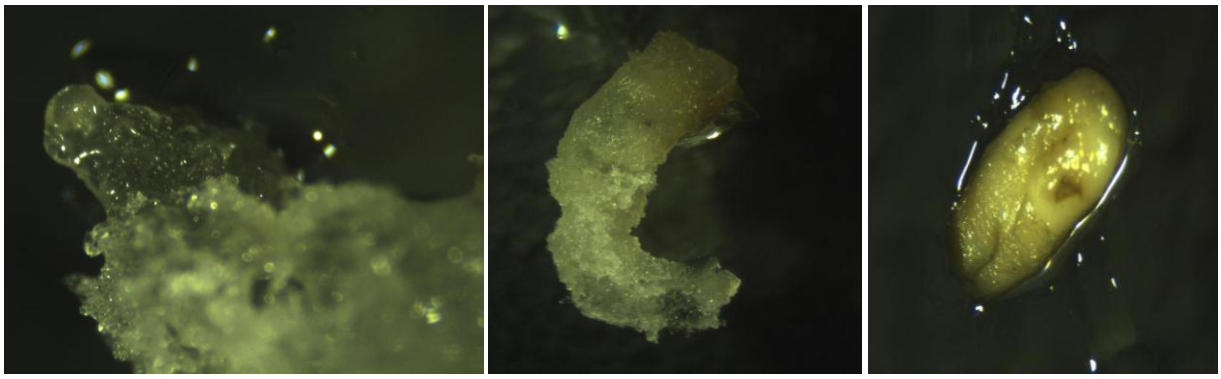

FIGURE 5. AFTER 3 MONTHS POTENTICAL EMBRYOGENIC TISSUE AMONGST CALLUS TISSUE

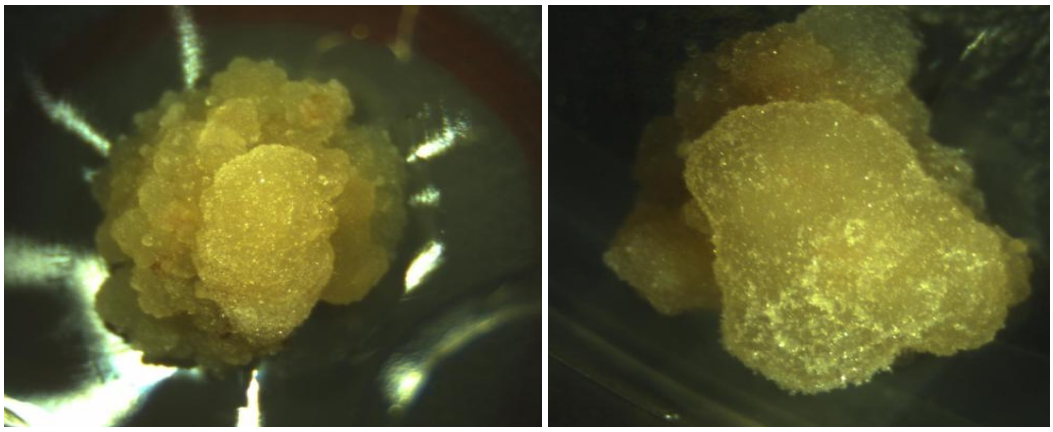

FIGURE 6. SUBALPINE LARCH AFTER 6 MONTHS CALLUS TISSUE ONLY PRESENT

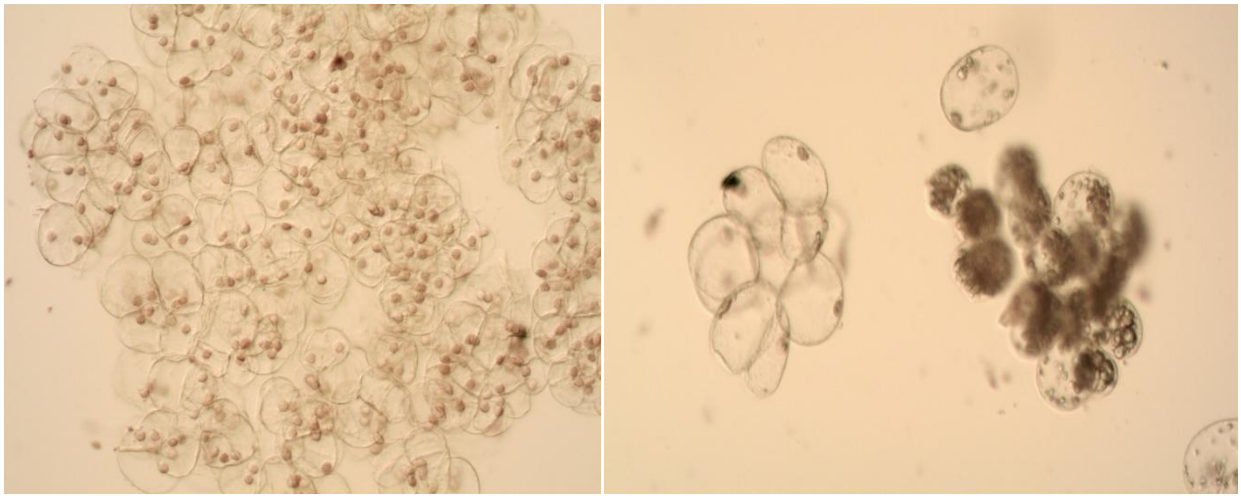

FIGURE 7. SUBALPINE LARCH AFTER 6 MONTHS SQUASH OF CALLUS NO SIGNS OF CALLUS TISSUE

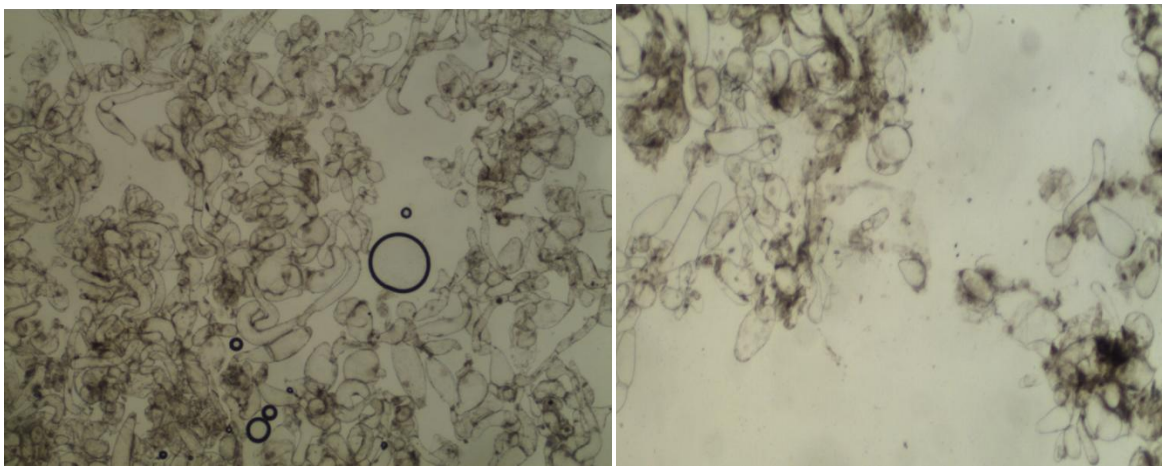

FIGURE 8. SUBALPINE LARCH AFTER 18 MONTHS SQUASH OF CALLUS TISSUE WITH NO SIGNS OF EMBRYOGENIC TISSUE

Experiment #2: Lodgepole Pine

Extrusion, the first evidence of SE initiation, on both standard (MLV-S) and low PGR media (MLV-L) was visible for some explants after 2 weeks of culture (Fig. 9, C). However, whole megagametophytes in the majority of explants produced callus after 4–5 weeks of culture (Fig. 9, D), and resulted in mixture of PEM and non-PEM. After 8 weeks of culture, approximately 50% of callus turned to brown. Vigorously growing calli were used to make squash to establish embryogenic tissue presence. Some calli, derivable from the 3rd and 4th collection, showed PEM cell division but not in 1st and 2nd collections (Fig. 9, E).

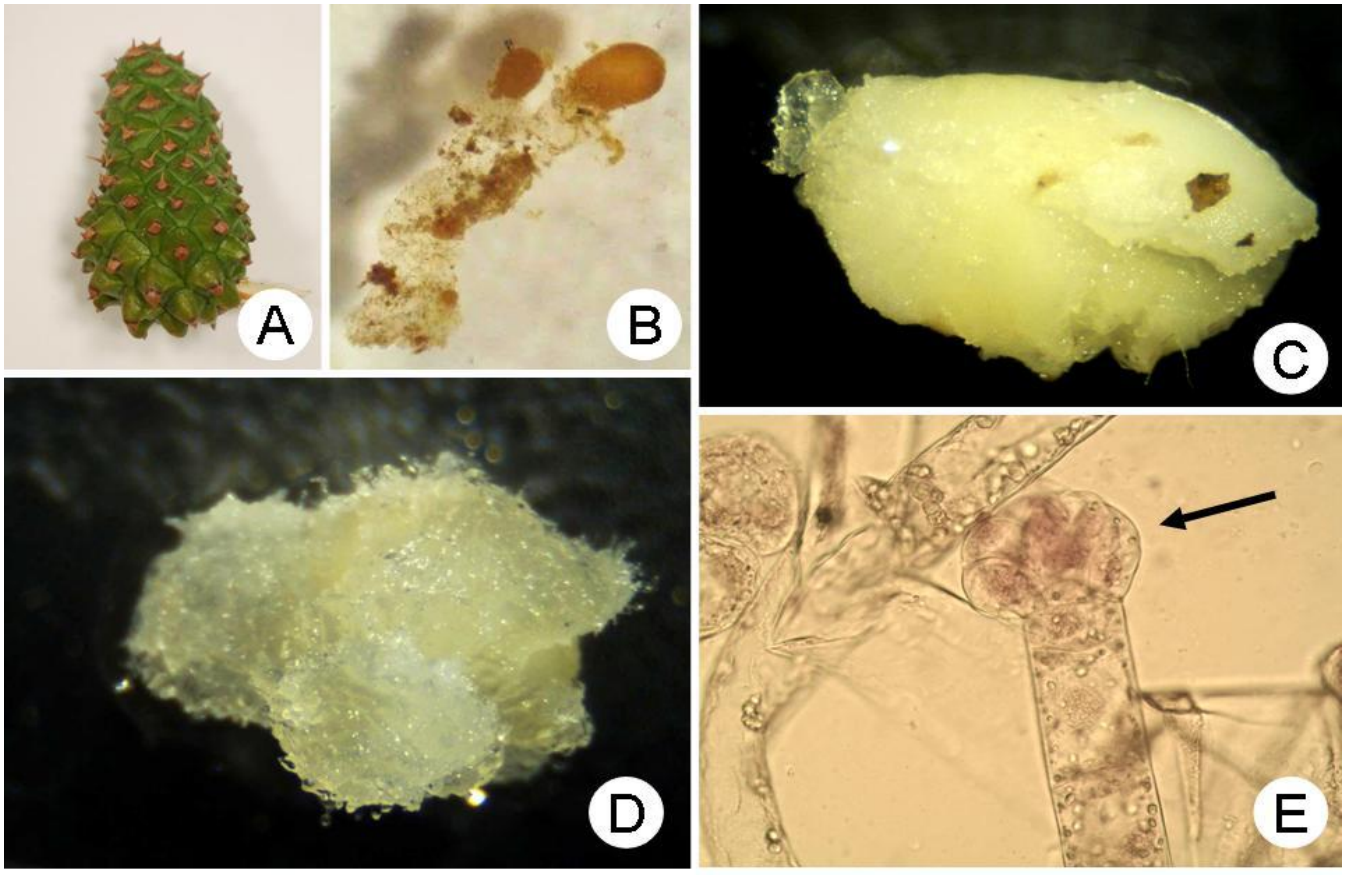

FIGURE 9. IMMATURE ZYGOTIC EMBRYO (ZE) CULTURE OF LODGEPOLE PINE. A. CONES USED AS MATERIAL, B. IMMATURE ZE IN LATE DOMINANT STAGE, C. EXTRUSION THROUGH THE MICROPYLE AFTER 2 WEEKS OF CULTURE ON PEM INDUCTION MEDIUM, D. CALLUS FORMATION AFTER 4 WEEKS OF CULTURE, E.PEM CELL DIVISION (ARROW).

Discussion

The first step in SE establishment, initiation, is known to be influenced by strong genetic control (Park et al. 1993; Lelu et al. 1999; Klimaszewska et al. 2001; Miguel et al. 2004; Niskanen et al. 2004). If this step is not achieved evidently the following steps (proliferation (maintenance), maturation, post-maturation, germination and conversion to plants, early growth ex vitrum and cryopreservation) will not occur. Given that both subalpine larch and lodgepole pine were unsuccessful at producing the desired formation of embryogenic tissue leads one to suspect either the media or timing of fertilization in seeds to be at fault, although neither could be exclusively the cause.

Within the genus *Pinus* it is not unusual to date for these results; its current success of initiation is very low thus limiting the ability to capture cell lines for varietal forestry and commercial application (Hargreaves et al. 2009; Park et al. 2006). This is an issue since many pines species are economically important, in particular for BC's lodgepole pine. Unfortunately for subalpine larch, it holds no economic value for BC.

Spruce was the first conifer to achieve success in SE establishment in which mature embryos were used. Subsequently, scientists believed that this timing in embryo development would be identical for all conifers; however this protocol did not achieve the same results for other genus's such as *Pinus*. For example, when pine embryos (from different species) were dissected from mature seeds SE was initiated at a very low frequency, or not at all (Klimaszewska and Cyr 2002). As of today, SE initiation typically comes from immature embryos for most conifers except for spruce (Klimaszewska and Cyr 2002). In this study, both lodgepole pine and subalpine larch, immature seeds were cultured from immature cones, thus a possible explanation for unsuccessful initiation is from the possible chance that these suspected

immature seeds contained nothing in the megagametophyte or a degenerated embryo. Also, it could be possible for the lodgepole pine, even though their embryos showed the correct stage for collections 3 and 4, that for this species this is the incorrect time for cone collection. This is highly unlikely given the collections were broken up into weekly intervals to prevent this window from being missed.

In past experiments, it has been easy to produce EM for species within the genus *Larix.* Consequently, scientists are confident that we know the optimal zygote developmental stage and fairly confident with standard media composition. One may argue this point due to the fact that most of the *Larix* species used have been hybrids for past SE success (Klimaszewska & Cyr 2002). There could be several reasons why no EM was formed from subalpine larch. If one was to compare this experiment with previous *Larix* experiments, the KI staining to observe zygotic embryo's present failed to show a developed embryo from immature seed in all three collections. A possible explanation for this failure could be due to a pollination problem and this could be from multiple reasons however the following two are the most suspected. First hypothesis, given their natural habitat, high isolated altitudes, there were not enough trees for sound cross pollination. This increases poor genetic diversity with the possible increase for self pollination. Overall, a low population of trees decreases chances for pollination.

Second hypothesis is perhaps the year that this study was done happened to be a year of low cone crop availability. While larch cones were being collected it was hard at first to find trees with cones present. Then when cones were appearing on trees they were scarce, some trees holding only a few cones with an overall average of 6 to 7 cones per tree and many trees with zero cones present. It is common in conifer trees to have variations in reproduction rates from year to year also referred to as mast seeding. In some species for example, such as Douglas fir

(*Pseudotsuga menziesii*), this cycle usually occurs every two years and is very consistent. *Larix* has an inconsistent reproduction cycle, good cone crops are produced infrequently at irregular intervals (U.S. Department of Agriculture 1948). Therefore, this species decreases its odds on its own self. To decrease successful reproduction probability even more so, Owens *et al*. (1998) investigate several methods for pollination in conifers and show that *Larix*, has one of the more complex and timely methods. For many conifers after pollen enters fertilization occurs within days (Owens *et al*. 1998). Within the *Larix* genus, the pollen remains at the distal end of the micropylar canal for 5–6 weeks; then it is carried inwards and after several steps and stages fertilization occurs (Owens *et al*. 1994). In addition, germination rates of subalpine larch seed are apparently very poor (Arno and Habeck 1972). Stratified seed produced no seedlings in two studies (U.S. Department of Agriculture 1948), thus regeneration as a whole seems to be a limiting factor for subalpine larch's existence.

Perhaps the latter hypothesis on unsuccessful pollination is incorrect, and all cones had been successfully pollinated, which is highly the case for the lodgepole pine given that pollination is monitored and sometimes created artificially in an orchard. This would then lead to the explanation that the developmental stage of the zygotic embryos from seeds was not at an optimal stage to produce EM. Some species have recalcitrant characteristic, so they have a very narrow window to produce EM (Park et al. 2006). This can also mean that they can produce only certain specific developmental stage of the embryo (Park et al. 2006). Usually researchers during the first year of an experiment use this time to screen for the optimal development stage (Park et al. 1993). At this time immature cones are supposed to be collected every week for one to two months. In this case for alpine larch it was extremely hard to get materials from the trees because of their location and the short number of cones. Furthermore, given that subalpine larch have less

than 3 months growing season (Arno & Habeck 1972), perhaps this increases the rate of embryo development. Perhaps cone collections for this species should have been taken several times per week instead to ensure the proper stage of embryo development was obtained. This could potentially explain why the first and second collections had zero images with a microscope for their embryo development and the third showed some sort of tissue cluster, perhaps a post fertilization stage.

The media, although highly unlikely, could be another possible reason why this experiment was unsuccessful. Two different components could be considered as possible impediments: gelling agent concentration used and growth regulator concentrations added. Von Arnold (1987) found no difference when comparing agar to GELRITE for the initiation success of SE from mature zygotic embryos of *Picea abies*. Conversely Klimaszewska (1989) used *Larix* embryogenic cultures and found that it was extremely difficult to maintain high quality cultures on medium containing a low level of GELRITE (1 g/L) and when initiated on medium gelled with 7 g/l of agar proliferated and grew best when transferred to medium gelled with 4 g/l of GELRITE. However, when the effect of gelling agents was investigated in regards to the development of *Picea abies* somatic embryos, thus after initiation phase, Tremblay and Tremblay (1991) found that GELRITE was superior to agar. GELRITE was used in this experiment and has not been found as of today as an inhibitor for either *Pinus* or *Larix's* success for SE therefore, it is highly unlikely that GELRITE had any hindrance on this project.

Another possible reason, although low, why this experiment did not produce EM is that medium composition in regards to growth regulators wasn't suitable for inducing EM from immature zygotic embryos. Previous studies have shown the media preference for conifers such as *Pinus* and *Larix* species is a combination of auxin and cytokinin for growth regulators with a

semi-solid media and nutrients (Klimaszewska and Cyr 2002). An attempt to improve initiation, Hargreaves et al. (2009) used multiple families from *Pinus radiate,* and compared its success with other media; including a modified Litvay medium and the Litvay medium was the most successful with a 70% success rate per family. In addition, for some genotypes within maritime pine (*Pinus pinaster*) loss of early embryogenic ability could be decreased using maltose-based media with no plant growth regulators present (Breton et al. 2005).

Given that larch has a very short growing window, one may suspect its SE response to be quicker, however this was not the case since any growth signs on the media for larch took twice as long when compared to lodgepole pine. In fact, because the subalpine larch was not responding after the third week there were pessimistic assumptions that nothing was going to develop from their megagametophytes. There is no evidence to suggest, but perhaps the room temperature (24°C) where the petri dishes were stored was impeding EM formation for subalpine larch. Naturally, the subalpine larch only inhabit environments with lower temperatures (Worrel 1993), perhaps the storage of megagametophytes, at least for the initial EM formation, should be kept in a room with a lower regulated temperature since this would more closely resemble the actual seed's natural environment. The reason for only concerning the initial EM is because Breton et al. (2005) have evidence to suggest that EM initiation and early somatic development are not connected.

Conclusions

Although the desired results were not obtained this does not conclude that embryogenic tissue is not possible to obtain from lodgepole pine and subalpine larch. Instead, this study should encourage further research for the fundamentals for success such as media composition and correct timing for cone collection for these species. These two factors should not be considered the only possible elements and an unbiased approach in future research would help for the discovery if the existences for other factors does exist. In addition, I would like to challenge scientists to proceed with caution with their generalizations of assuming a species success may apply to its entire genus. For example, within the *Larix* genus there is high variation in growth rates, cold hardiness, form, pest resistance, and other characteristics (Shearer 2008). For such a diverse genus is it wise to assume SE procedures will be similar for all *Larix* species?

This study did not achieve success in producing embryogenic tissue, for lodgepole pine or subalpine larch, but further research may shed light on the parameters necessary to establish SE for these species.

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