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ORIGINAL ARTICLE

Synergistic effects of L-glutamine and inorganic nitrogen molar ratios enhance the induction of somatic embryogenesis of *Pinus maximinoi* H.E. Moore

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Abstract

Clonal breeding programs of *Pinus maximinoi* require the establishment of a robust somatic embryogenesis (SE) protocol to produce enough cell lines to accelerate the effective continuous deployment of elite planting stocks to research and commercial compartments. Somatic embryogenesis was induced from immature zygotic embryo explants enclosed in megagametophytes of *P. maximinoi* collected from two plantations located in different climatic conditions. Cones were collected during the winter months from July to August and the influence of seed family, cone collection date and culture medium formulation, with emphasis on the organic and inorganic nitrogen supply, were studied. Ammonium to nitrate molar ratios of 1:1 and 1:2 in modified Litvay's medium (mLV) produced the highest numbers of extrusions, while a 1:4 ratio mostly produced unhealthy, non-embryogenic extrusions. The formation of a tissue showing a rapidly-proliferating, spiky morphotype was produced in a medium supplemented with 1.5 g/L of L-glutamine. Morphologically advanced cultures with nodular structures were produced in megagametophytes from both plantations in a 1:2 NH_4^+:NO_3^- medium regardless of L-glutamine supplementation levels. The optimal medium for *P. maximinoi* SE induction contained a 1:2 NH_4^+:NO_3^- molar ratio with 1.5 g/L L-glutamine. The synergy between the molar ratio of NH_4^+:NO_3^- and L-glutamine resulted in the highest numbers of extrusions. The overall inductive competence window for somatic embryogenic response in *P. maximinoi* was determined to be from the second week of July to the first week of August for both plantations. The "peak" period was in the fourth week of July 2022. The success of the SE technology in *P. maximinoi* seed families is determined by the optimal inductive competence window of the immature megagametophytes enclosing zygotic embryos and the chemical composition of the induction medium in terms of the ammonium to nitrate molar ratio and the concentration of the L-glutamine used.

Key Message

Interaction between the collection date, genotype of the seed family and nitrogen content of the medium determine the somatic embryogenesis capacity of immature zygotic embryos at the optimal developmental stage.

Keywords Embryogenic cell line · L-glutamine · *Pinus maximinoi*

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Introduction

Pinus maximinoi H.E. Moore is a tropical evergreen 5-needle pine native to Mexico and the Central American countries-Guatemala, Honduras, Nicaragua, and El Salvador (Camcore 2002a). It occurs over a wide range of microclimates and is the most prevalent pine species after *P. oocarpa* in Central America. It grows from 700 to 2400 m above sea level in frost-free areas with well-drained soils receiving 900 to 2200 mm of annual precipitation (Camcore 2002a; 2002b). *Pinus maximinoi* was imported into South Africa in

the early 1990s by an international tree breeding and conservation cooperative, CAMCORE (The Central American and Mexican Coniferous Resources). Provenance trials to study the adaptability and performance of genotypes within families were undertaken to determine its commercial feasibility in southern Africa (Nyoka 1994; Kietza 1988). In South Africa, *P. maximinoi* H.E. Moore shares its species niche with *P. tecunumanii* in moist subtropical areas where frost events seldom occur (Dvorak et al. 2002; Gapare et al. 2001).

The commercial production of *Pinus* species was subsequently threatened by the introduction of the fungal pathogen *Fusarium circinatum*, which saw most forestry companies devising strategies to curb nursery and field losses due to the devastating effects of this pathogen (Wingfield et al. 2008). *Pinus patula*, the most widely planted species in South Africa due to its excellent growth and adaptability, is highly susceptible to the pathogen and forest breeders employed species hybridization through controlled pollination to produce resistant hybrids that ensured a consistent seedling supply and wood production (Mitchell et al. 2011). Infected seedlings in the nurseries show tip die-back, root and collar deformation, and damping off (Wingfield et al. 2008). Commercial forestry experienced high levels of seedling mortality resulting in reduced establishment of plants in the field, poor wood quality and subsequently, reduced wood production.

As knowledge about the biology and distribution of the pathogen increased, numerous studies were undertaken to identify different isolates while also trying to develop potential measures to rid research and commercial nurseries of the pathogen. Application of chemicals and consistently adhering to nursery hygiene measures were the first strategies recommended as these reduced fungal spores of the pathogen to reasonable levels (Amaral et al. 2019; Zamora-Ballesteros et al. 2019; Mitchell et al. 2012). However, the most practical strategies came from a breeding point of view. Breeding populations were screened for resistant genotypes and only these were deployed. Additionally, hybridization with species that showed resistance yielded tolerant hybrid genotypes (Mitchell et al. 2012; 2011). *Pinus patula* x *tecunumanii* and *Pinus patula* x *oocarpa* are examples of such hybrids created to overcome issues with this fungal pathogen, due to the high degree of resistance shown by both pure *P. tecunumanii* and *P. oocarpa* to *F. circinatum* (Mitchell et al. 2012; 2011).

Breeders carefully screened populations of various species by comparing growth and wood traits of *P. patula* as the then industry leader, they found that *P. maximinoi* matched the traits of *P. patula* very well in all respects, while also exhibiting high resistance to *F. circinatum* infestation (Mitchell et al. 2012; 2004). As with most exotics, *P. maximinoi* has its own challenges. It is a shy seed producer; producing only around 15 filled seeds per cone at 8 years in provenance trials (Dvorak et al. 2000; Camcore 2002a). *P. maximinoi* is also known to be difficult to graft as it exhibits grafting incompatibility after a few years (Camcore 2002a). It tends to outgrow the rootstock, such that the grafted tree dies due to scion-rootstock incompatibility (Hodge and Dvorak 2012; Dvorak et al. 2000). Propagation of *P. maximinoi* is currently conducted through seeds and rooted cuttings. Both approaches come with the associated challenges of low seed yields and physiological aging of hedges (Mitchell et al. 2004); whereby cuttings show low rooting capacity once the hedge reaches 4 to 6 years of age.

With vegetative propagation being difficult to implement in *P. maximinoi*, other methods such as somatic embryogenesis (SE) are being explored to produce enough propagules for research trials, commercial compartments, and in vitro gene banks for conservation of germplasm. During SE, plant cells are artificially induced to form somatic embryos, without the involvement of gamete fusion, through manipulation of medium components such as nitrogen ratios and levels, carbon source, gelling agents, and plant growth regulator concentrations (Garcia et al. 2019). Somatic embryos undergo similar phases of morphological development as their zygotic counterparts.

Induction of SE is influenced by several factors, the most critical are the developmental stage of immature zygotic embryos, the genotype of the parent tree selected to provide explants (Yan et al. 2021; Aronen et al. 2009; Lelu-Walter et al. 2006), chemical composition of tissue culture media, and plant growth regulator (PGR) types and concentration (Tretyakova et al. 2020; Becwar et al. 1990). Immature zygotic embryos are explants of choice for induction of SE in *Pinus* species (Nunes et al. 2017) and their responsive period in culture is species-dependent, usually lasting for around 3–4 weeks (Yildirim et al. 2006). This represents a very narrow competence window, which requires careful determination of the collection period of immature green cones, which corresponds to the embryo development during cone development. Immature cones responsive to SE induction are almost one year old in their development and consist of actively dividing embryos forming numerous embryo initials by a process of polyembryony (Bonga et al. 2010; Klimaszewska et al. 2007). Previously, SE attempts for various *Pinus* species have achieved zero to very low initiation frequencies (Pullman et al. 2003). Most of these attempts achieved some improvements when PGRs, media type and nutrients were optimized (Lelu et al. 1999). Plant growth regulators (Gao et al. 2023), carbon sources (Ren et al. 2022; Pullman et al. 2003), solidifying agents and culture temperature (Pereira et al. 2017, 2020), inorganic nitrogen molar ratios (Li et al. 2022), and organic nitrogen in the form of casein hydrolysate and the amino acid L-glutamine are amongst constituents of the medium most often tested for

optimization (Khajuria et al. 2021; Kim and Moon 2007; Smith 1996). Optimal proportions of ammonium (NH_4^+) and nitrate (NO^-_3) for plant development varies from species to species (Duarte-Ake et al. 2022), as do the in vitro environmental conditions, developmental stage of the explant being induced, and total nitrogen concentration of the culture induction medium (Li et al. 2022; Ramage and Williams 2002). A proper balance between these two ionic nitrogen forms plays a critical role in the induction of somatic embryogenesis and proliferation of resultant somatic embryos (Duarte-Ake et al. 2022; Carlsson et al. 2019; Dahrendorf et al. 2018; Niedz and Evens 2007). This balance has been reported to bring about high embryogenic cell differentiation and increased embryo frequency during somatic embryogenesis (Wu et al. 2015).

The ability to capture numerous embryonic cell lines for many families within a breeding program is limited by the low initiation frequencies of embryogenic tissue (Salajova and Salaj 2005). Families within a breeding program vary greatly in their ease of propagation with a particular clonal technology due to interaction of genetic factors (Garin et al. 1998). An assessment of the SE success rate relies on the ability to distinguish between the initial outgrowth, an extrusion where the immature embryo or group of embryos push out of the micropylar end of the megagametophyte, and the continuous growth of the extruded tissue (Montalban et al. 2010; Klimaszewska et al. 2007). Under optimal in vitro culture conditions, the continuous tissue growth, often called embryogenic suspensor mass (ESM) or simply embryogenic tissue (ET), leads to numerous established cell lines. These lines are then maintained using a tissue-preserving technology called cryopreservation, which indefinitely stores cell lines at ultra-low temperatures of between -80 to -196 °C (Cao et al. 2022; Martinez et al. 2022; Salaj et al. 2012). Cryopreservation enables the implementation of multi-varietal forestry, the use of tested tree genotypes in forest operations, as the precise genetic material is readily available whenever needed for plant regeneration (Salaj et al. 2022; Lineros et al. 2018). SE technology benefits several other applications such as artificial seed technology (Rihan et al. 2017; Aquea et al. 2008), genetic engineering and transformation, where foreign genes are inserted into genomes of species to improve their adaptability, quality, performance, and yield (Fernandes et al. 2008).

The overall aim of this study was to establish the optimal inductive competence window and factors that enhance both induction and proliferation of embryogenic suspensor masses of *Pinus maximinoi* immature seeds. The main objectives of this study were to investigate: (1) the effect of the NH_4^+:NO_3^- molar ratio and (2) the effect of L-glutamine on the induction of somatic embryogenic tissue of *Pinus maximinoi* families and the proliferation of somatic embryos.

Materials and methods

Climatic data of the plantation sites for cone development and collection

Cone collection for *P. maximinoi* was conducted from July to August for both plantations (Table 1). The two plantations located in different climates received virtually no rain during the cone collection period (Supplementary Fig. 1) as this period falls within the winter season in the Lowveld of Mpumalanga province, which lies within the summer rainfall area of South Africa. The absence of rain allowed for climbing operations for collection of immature seeds; however, some days were occasionally windy and climbing operations had to be delayed or stopped.

Plant material

One-year-old green female cones (Fig. 1a), enclosing immature zygotic embryos of *P. maximinoi,* were collected weekly from open-pollinated (OP) trees of six families. Immature cones were collected from two plantation sites, Tweefontein and Wilgeboom, for which the climatic zones, altitude, mean annual precipitation (MAP), mean annual temperature (MAT) and planting date are shown in Table 1. A total of 15 to 20 immature cones were harvested from each family in the respective plantations and stored for a maximum of two months in a paper bag wrapped in a sealable plastic bag at 4 °C until excision of an explant. The intact cones were washed in distilled water with a few drops of a household antibacterial dish washing liquid soap manufactured by Massmart Holdings Limited. Immature seeds were removed from the cones and surface-disinfected in a laminar flow

Table 1 Climatic characteristics of two plantations in Mpumalanga province of South Africa, planted with open pollinated *Pinus maximinoi* seed families used in the somatic embryogenesis studies

Plantation	Tweefontein	Wilgeboom
Plant date	January 2008	March 2008
Location	Mpumalanga province	Mpumalanga province
Latitude	25°03′50.67'' S	24°57′04.07'' S
Longitude	30°48′51.25'' E	30°56′25.98'' E
Altitude (m.a.s.l.)	1 251	965
Replications	6	6
Plot size (trees)	1 × 6	1 × 6
MAT (°C)	16 – 18	18—19
MAP (mm)	> 1300	1050—1300
Climatic zone	Warm-temperate	Warm-temperate subtropical

Source: SAFCOL planning division, m.a.s.l. = meters above sea level, MAT = mean annual temperature, MAP = mean annual precipitation

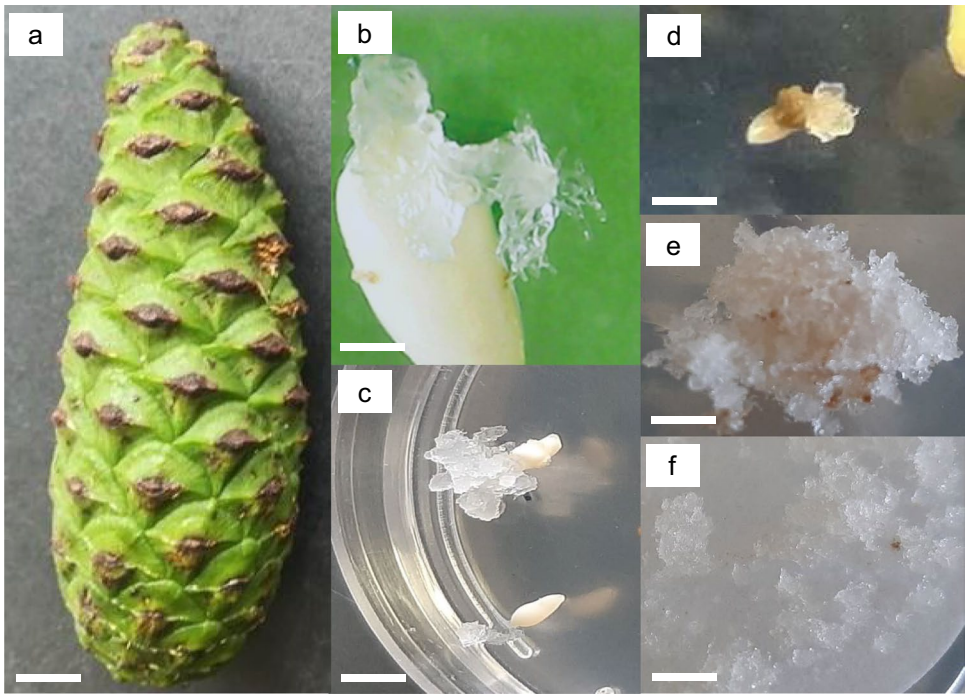

Fig. 1 Induction of somatic embryogenesis in one-year old immature *Pinus maximinoi* megagametophytes. **(a)** one-year old immature *P. maximinoi* cone, *bar* = 80 mm **(b)** spiky extrusion of somatic embryos undergoing proliferation on IM2 medium, *bar* = 3 mm **(c)** smooth extrusion of somatic embryos slowly proliferating on IM1 medium, *bar* = 2 mm **(d)** slimy, necrotized, non-embryogenic extrusion on a solid IM3 medium, *bar* = 1 mm **(e)** embryogenic tissue clump proliferating on a solid mDCR medium, *bar* = 4 mm **(e)** proliferation of embryogenic tissue suspension on a filter paper disc cultured on mDCR medium, *bar* = 5 mm

bench. Extracted seeds were placed in a steel-mesh infuser and dipped 10 times each into of a sequence of disinfectant solutions, starting with distilled water containing three to five drops of surfactant, 10% (v/v) hydrogen peroxide (H_2O_2), followed by 70% (v/v) ethanol and finally 10% (v/v) bleach (0.35% [m/v] NaOCl). After disinfection, the seeds were thoroughly rinsed with sterile water. Megagametophytes containing immature embryos were extracted aseptically using a blade and scalpel that were constantly heat-sterilized at 250 °C in a glass bead sterilizer. Culturing of megagametophytes was carried out as described below.

Induction and initiation of embryogenic suspensor masses

Modified Litvay's medium (mLV), as modified by Hargreaves et al. (2009) was used to test the effects of NH_4^+:NO_3^- molar concentration ratios on extrusion and initiation of tissue. Three induction medium (IM) variants that differed in NH_4^+:NO_3^- molar ratios were prepared as follows: IM1 with 1:1, IM2 with 1:2 and IM3 with 1:4 ratio of NH_4^+:NO_3^-. The molar ratios of ammonium nitrate to potassium nitrate were manipulated in the recipe for the basal medium to achieve the ratios of IM1, IM2 and IM3. These variants were supplemented with 0.9 mg/L of 6-benzylaminopurine (BAP), 1.8 mg/L 2.4-dichlorophenoxyacetic acid (2.4-D), 0.9 g/L of casein acid hydrolysate, 40 g/L of sucrose and 4 g/L of Gelrite. The pH was adjusted to 5.8 before sterilization in an autoclave at 121 °C for 20 min. After sterilization, the medium variants were allowed to cool to 50 °C in a laminar flow bench before filter-sterilized L-glutamine, at the different concentrations as described below, was added. Filter sterilization was carried out using a 0.22 µm syringe filter.

Effect of molar ratios of NH_4^+:NO_3^- on the induction of extrusions of the embryogenic tissue of *Pinus maximinoi* families during somatic embryo development

Megagametophytes from both Tweefontein and Wilgeboom plantations were arranged in five replications of culture dishes with five explants each and cultured on IM1, IM2 and IM3 with 1:1, 1:2 and 1:4 molar ratios of NH_4^+:NO_3^- respectively, supplemented with 0, 1.5 or 4.4 g/L L-glutamine in 65 mm × 15 mm sterile disposable plastic petri dishes (Labocare™). IM1, IM2 and IM3 replications with 0 g/L L-glutamine served as negative controls. Petri-dishes were sealed with Parafilm® M and cultures were maintained in the dark at 23 °C. These cultures were visually examined weekly for tissue extrusion and contamination. The megagametophytes were not subcultured during the experiment, which ran for 8 to 16 weeks. Extrusions were visually recorded when zygotic embryos were pushed out of the micropylar end of the megagametophyte and formed an initial embryonal growth in IM1, IM2 and IM3 media. In a few lines, embryonal growth was very slow and eventually ceased to proliferate on media IM1, IM2, and IM3.

Effect of L-glutamine concentrations on induction of the extrusion of somatic embryos

Megagametophytes of OP seed families from Tweefontein and Wilgeboom plantations were arranged in five replications with five explants each on IM2 medium petri-dishes supplemented with respective L-glutamine concentrations of 0, 0.5, 1.5, 3.0, and 4.4 g/L. IM2 medium replications supplemented with 0 g/L L-glutamine served as control petri-dishes for the experiment. Cultures were again maintained in the dark at 23 °C for 8–16 weeks.

Embryogenic tissue maintenance for captured cell lines

Captured cell lines from both experimental trials described above were maintained on modified DCR medium (mDCR, Gupta and Durzan 1985) with 400 mg/L of ammonium nitrate replaced by 400 mg/L of ammonium sulfate. The BAP and 2.4-D were reduced to 0.5 mg/L respectively, while sucrose was reduced to 20 g/L and casein hydrolysate to 0.5 g/L. Phytagel (4 g/L) was added to solidify the medium and pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Filter-sterilized L-glutamine was added at 0.25 g/L to the cooled medium in a laminar flow bench before decanting to sterile plastic disposable petri dishes.

Clumps of each captured cell line were suspended in distilled water supplemented with 0.25 g/L L-glutamine and poured over a filter paper disk in a Büchner funnel with low-pressure suction from a peristaltic pump. This suction took a few seconds to drain the excess liquid from the tissue; and cells attached to the filter paper were placed onto the mDCR medium. Sterile petri dishes with cell layers on top of filter papers were sealed with Parafilm® M and maintained in the dark at 23 °C for 7 to 10 days.

Data collection and statistical analysis

To screen seed families for their ability to induce SE in culture, we collected immature cones from six seed families starting from July to August of 2022 for both Tweefontein and Wilgeboom plantations. Five replications were prepared for each seed family per experiment of each plantation. Each culture dish with IM1, IM2, or IM3 medium contained five replications, each with five megagametophytes of tested OP seed families, and was visually assessed, and scored individually for extrusion zygotic embryo after 8 to 16 weeks in culture. The proliferating captured cell line was considered stabilized after reaching between 5 to 10 mm of the clumping mucilaginous tissue diameter. The main effects of seed family, collection dates, plantation, L-glutamine, NH_4^+: NO_3^- molar ratio, replication and relevant interactions were evaluated by ANOVA, using R statistical software (R. 2015). Percentage data on frequency of extrusion and initiation of embryogenic cell lines were arcsine transformed. Combined ANOVA analysis was performed to compare the two plantations for extrusion and initiation frequencies. A linear model was used that considered seed families, plantations, L-glutamine concentration, and ammonium to nitrate molar ratios to be fixed effects. Collection dates and all possible interactions were considered as random effects. Significant differences between means were determined using the Fisher's Least Significant Differences (LSD) post hoc test at a significance level of 5%.

Results

Effect of seed families and the geographical location on the extrusion rate of the putative embryogenic tissue

We intended to check whether there were any immature zygotic embryo development disparities across two *P. maximinoi* plantations due to geographic location. Megagametophytes that were collected from Tweefontein and Wilgeboom; and cultured onto respective induction media after 6 to 12 weeks, started exhibiting extrusions of translucent to white mucilaginous cell masses from the micropylar ends (Fig. 1b). Extrusions from IM1 and IM3 media progressed into non-proliferating tissue clumps, while those from IM2 readily formed proliferating tissue clumps around the micropyle. The proliferating clumps of the putative embryogenic tissue formed a soft, whitish translucent tissue mass which could be easily multiplied by repetitive subcultures. Two phenotypes were seen from the proliferating tissue masses, a spiky phenotype characterized by elongated suspensor cells on the periphery of the clumps (Fig. 1b), and a smooth phenotype mostly characterized by spherical cells on the periphery of the clumps (Fig. 1c). We observed that the spiky tissue masses undergo proliferation quickly when compared to the smooth tissue masses, which require prolonged subculturing before producing substantial amounts of tissue mass.

There was considerable variation between seed families in terms of positive SE responses. We achieved an overall initiation frequency of 3.84% in Tweefontein and captured 12 stably proliferating embryogenic cell lines. Most of these cell lines were achieved in the megagametophytes of the seed family M094 collected from 18 July to 3 August 2022 (Table 2).

Conversely, a total of 1700 megagametophytes was extracted from cones of 5 out of 6 seed families collected in Wilgeboom and produced an overall extrusion frequency of 21.12% (Table 3). An overall initiation frequency of 9.54% was achieved and 36 stably proliferating embryogenic cells lines were successfully captured in

Table 2 Overall comparison of SE responses of immature megagametophytes of two open pollinated *Pinus maximinoi* seed families in Tweefontein plantation based on cone collection dates

Family/date	Total plated	Total extruded	Extrusion %	Total initiated	Initiation %	ECL
M664	**330**	**101**	**30.61**	**14**	**4.24**	**2**
22-Jul	330	101	30.61	14	4.24	2
M094	**1259**	**333**	**26.45**	**47**	**3.73**	**8**
18-Jul	315	80	25.40	13	4.13	4
20-Jul	10	2	20.00	2	20.00	1
22-Jul	305	105	34.43	18	5.90	3
26-Jul	320	116	36.25	2	0.63	1
28-Jul	5	3	60.00	0	0.00	0
3-Aug	60	7	11.67	5	8.33	1
8-Aug	244	20	8.20	7	2.87	0
Total	1589	434	27.31	61	**3.84**	**12**

ECL, embryogenic cell lines

Table 3 Overall comparison of SE responses of immature megagametophytes of five open pollinated *Pinus maximinoi* seed families in Wilgeboom plantation based on cone collection dates

Family/date	Total plated	Total extruded	Extrusion %	Total initiated	Initiation %	ECL
M244	**75**	**26**	**34.67**	**21**	**28.00**	**3**
27-Jul	75	26	34.67	21	28.00	3
M544	**340**	**85**	**25.00**	**37**	**10.88**	**8**
12-Jul	220	72	32.73	21	9.55	1
3-Aug	120	13	10.83	16	13.33	7
M784	**415**	**114**	**27.47**	**38**	**9.16**	**17**
12-Jul	135	42	31.11	13	9.63	1
20-Jul	250	69	27.60	24	9.60	16
3-Aug	30	3	10.00	1	3.33	0
M094	**810**	**121**	**14.94**	**61**	**7.53**	**7**
20-Jul	330	58	17.58	22	6.67	1
27-Jul	225	49	21.78	29	12.89	4
3-Aug	135	10	7.41	0	0.00	0
12-Aug	55	24	43.64	8	14.55	2
25-Aug	115	4	3.48	4	3.48	0
M815	**60**	**13**	**21.67**	**5**	**8.33**	**1**
20-Jul	60	13	21.67	5	8.33	1
Total	1700	359	21.12	61	**9.53**	**36**

ECL, embryogenic cell lines

Wilgeboom. Megagametophytes that came from cones of seed family M784 collected between 12 July and 2 August 2022 produced the most embryogenic cell lines compared to the other four seed families. Developmental differences between megagametophytes from seed families harvested between two plantations were evident, most megagametophytes of seed families from Wilgeboom showed opaqueness in the second week of July 2022 while those from Tweefontein were still translucent and soft at the same period, and only started to take on an opaque appearance in the third week of July 2022.

Effects of the collection date on extrusion of the putative embryogenic tissue from Tweefontein and Wilgeboom plantation sites

To determine the optimal cone collection window for *P. maximinoi* SE induction, we compared SE responses of megagametophytes from different collection dates and plantations. Megagametophytes sampled in the first week of July 2022 were immature, soft and translucent in both collection sites. During the second week of July 2022, a proportion of some megagametophytes started to show opaqueness,

although there were differences in opaqueness between different families of collection sites. Cultured opaque megagametophytes started to show extrusions on the micropyle after 2 to 4 weeks, whilst translucent megagametophytes became brown and died. Two out of five open-pollinated seed families, M094 and M664, from Tweefontein plantation exhibited opaqueness in the second week of July 2022 (Fig. 2a), while most megagametophytes collected from Wilgeboom were opaque during the same period (Fig. 2b). For these two families at the Tweefontein site, extrusion frequencies began with low levels on 18 July 2022 and peaked on 26 July 2022 (Fig. 2c), with the inductive competence window from 18 July to 8 August 2022 (Fig. 2c). The megagametophytes from the other 4 seed families collected in Tweefontein remained translucent and nonresponsive from 18 July to 8 August 2022. Winter rains interfered with cone sampling in Tweefontein during mid-August 2022 and cone harvesting was stopped as climbing activities could not be undertaken when trees were wet and slippery. We could have achieved capture of the other four seed families if the sampling period was extended and winter rains had not interfered with climbing activities.

Extrusions from megagametophytes collected in Tweefontein plantation were achieved over a short period of six weeks, from the third week of July 2022 to the second week of August 2022 with several extrusions in the fourth week (Fig. 2c); while for Wilgeboom plantation they were achieved over a broader period of seven weeks, from the second week of July 2022 to the end of August 2022 (Fig. 2d). Five out of six tested seed families responded positively, and the highest extrusion rate was achieved in the fourth week of July 2022 (Fig. 2d). The only seed family that did not produce extrusions in Wilgeboom was M664, even though most megagametophytes from this family were opaque. The inductive competence window for extrusion of putative embryogenic tissue in Wilgeboom was between 12 July and 25 August 2022. Based on these results, the consistent sampling of immature cones through the developmental period of zygotic embryos across plantations can be used to determine the optimal inductive window for effective SE in *P. maximinoi* seed families. Opaqueness is associated with readiness of zygotic embryo for induction of SE, as most responsive seeds that produced initiations were opaque.

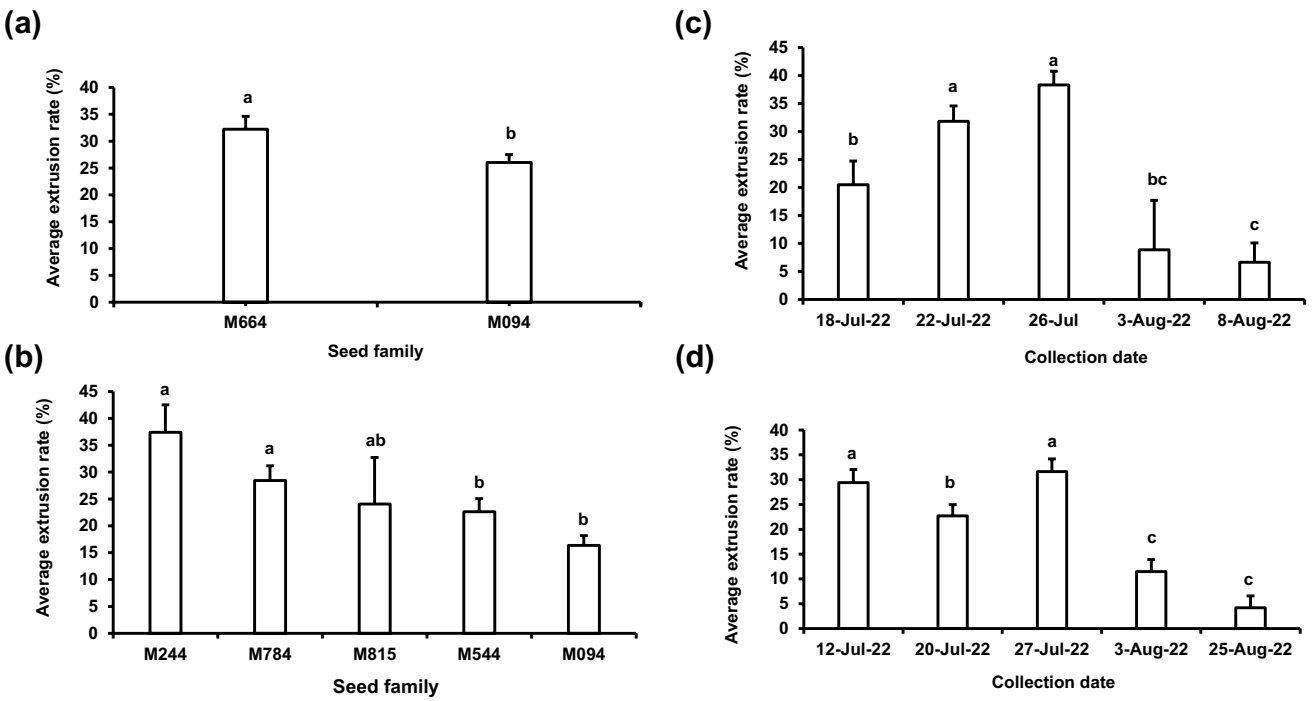

Fig. 2 The inductive competence window of *Pinus maximinoi* seed families harvested in Tweefontein and Wilgeboom plantations in the year 2022. **(a)** Average induction of extrusions of putative embryogenic tissue from two open pollinated families of *P. maximinoi* on mLV medium with varying L-glutamine concentrations over seven collection dates in Tweefontein plantation. **(b)** Effects of seed family on average induction of extrusions of putative embryogenic tissue of *P. maximinoi* plated on mLV medium over seven collection dates in Tweefontein plantation. **(c)** Effects of collection dates on the average induction of extrusions of putative embryogenic tissue from five open pollinated seed families of *P. maximinoi* on mLV medium with varying NH_4^+:NO_3^- ratio in Wilgeboom plantation. **(d)** Effects of seed family on average induction of extrusions of putative embryogenic tissue of *P. maximinoi* plated on mLV medium with varying NH_4^+:NO_3^- ratio over seven collection dates in Wilgeboom plantation. Different letters above mean error bars indicate a significant difference at the 5% significance level according to Fisher's LSD post hoc test

Effect of nitrogen supplements on SE induction in *P. maximinoi*

Effects of the ammonium to nitrate molar ratio on SE induction in *P. maximinoi*

Firstly, we wanted to assess the effect of ammonium to nitrate molar ratio on extrusion of embryogenic putative tissue; various molar ratios of the inorganic nitrogen in an mLV medium variant were tested. The 1:1,1:2 and 1:4 ammonium to nitrate molar ratios were tested in the mLV basal medium. In Tweefontein there were no significant differences in SE responses due to NH_4^+:NO_3^- molar ratios. The analysis of variance (ANOVA) for average extrusion rate (%) in Tweefontein indicated that replication, seed family and cone collection date were the only sources of variation that were statistically different at $P < 0.05$ (Table 4).

Conversely, we found that NH_4^+:NO_3^- molar ratios produced a greater variation for extrusion of putative embryogenic tissue in seed families harvested in Wilgeboom. Interaction effects that were statistically significant were NH_4^+:NO_3^- molar ratios, collection date x NH_4^+:NO_3^- molar ratios (CD x NR), seed family x collection date x L-glutamine concentration (SF x CD x L-gln) and seed family x NH_4^+:NO_3^- molar ratios x L-glutamine concentration (SF x NR x L-gln) (Table 4). The significant interaction between collection date (CD) and ammonium to nitrate molar ratio (NR) in Wilgeboom plantation is presented in Supplementary Table 1. Both 1:1 and 1:2 media produced on average more extrusions than 1:4 medium i.e., IM1 and IM2 > IM3 on respective collection dates for seed families (Fig. 3a, Supplementary Table 1). Based on these results, we realized that the 1:2 NH_4^+:NO_3^- molar ratio produced morphologically advanced cultures with a spiky phenotype, while cultures resulting from 1:1 and 1:4 NH_4^+:NO_3^- molar ratios failed to produce stably proliferating clumps and eventually formed a slimy mass of dead cells.

Effects of the L-glutamine concentration on SE induction of megagametophytes plated on 1:2 ammonium to nitrate molar ratio medium (IM2)

Secondly, we wanted to assess whether an L-glutamine concentration gradient on the medium with a specific NH_4^+:NO_3^- molar ratio would selectively produce maximum proliferative embryogenic cultures. We tested 0, 0.5, 1.5, 3.0 and 4.4 g/L L-glutamine in 1:2 NH_4^+:NO_3^- molar ratio on mLV medium. The analysis of variance (ANOVA) for average extrusion rate (%) in both plantations revealed that there was no preference of a specific L-glutamine concentration (Table 5).

Conversely, seed family was the only source of variation that was statistically significant at $P < 0.05$ in Wilgeboom plantation, while only collection date was statistically significant in Tweefontein plantation (Table 5). None of the other sources of variation was statistically significant. Based on these results, any L-glutamine concentration tested can successfully be used to achieve extrusions from cultured megagametophytes (Fig. 3b, c).

Table 4 Analysis of variance for average extrusion rate in open pollinated *Pinus maximinoi* seed families harvested in Wilgeboom and Tweefontein plantations for evaluation of the effects of NH_4^+:NO_3^- on extrusion rate of putative embryogenic tissue. Statistical significance at $p < 0.05$ using R software and means comparison performed using the Fisher's LSD post hoc test

Plantation	Wilgeboom				Tweefontein			
Source of variation	df	MS	F-value	Pr(> F)	df	MS	F-value	Pr(> F)
Replication	4	678.3	2.84	0.03*	4	721.6	2.63	0.04*
Seed family (SF)	4	1676.8	7.01	3.57e-05***	1	1320.7	4.82	0.03*
Collection date (CD)	4	2947.1	12.32	1.29e-08***	6	2325.8	4.48	5.31e-08***
NH_4^+:NO_3^-	2	2670.0	11.16	3.18e-05***	2	137.6	0.50	0.61
L-glutamine (L-gln)	3	360.9	1.51	0.22	3	426.5	1.56	0.20
SF x CD	2	422.4	1.77	0.17	2	389.0	1.42	0.25
SF x NR	8	281.3	1.18	0.32	9	376.8	1.37	0.20
CD x NR	8	613.9	2.57	0.01*	2	133.0	0.49	0.62
SF x L-gln	9	264.5	1.11	0.36	7	435.4	1.59	0.14
CD x L-gln	8	432.0	1.81	0.08	4	250.1	0.91	0.46
NR x L-gln	4	175.7	0.73	0.57	4	102.1	0.37	0.83
SF x CD x NR	3	256.4	1.07	0.36	12	256.8	0.94	0.51
SF x CD x L-gln	3	844.9	3.53	0.02*				
SF x NR x L-gln	12	492.9	2.06	0.02*				
CD x NR x L-gln	12	280.3	1.17	0.31				
SF x CD x NR x L-gln	2	24.9	0.10	0.90				
Residuals	141	239.3			160	274.2		

*Significant at $P < 0.05$, ***significant at $P < 0.001$

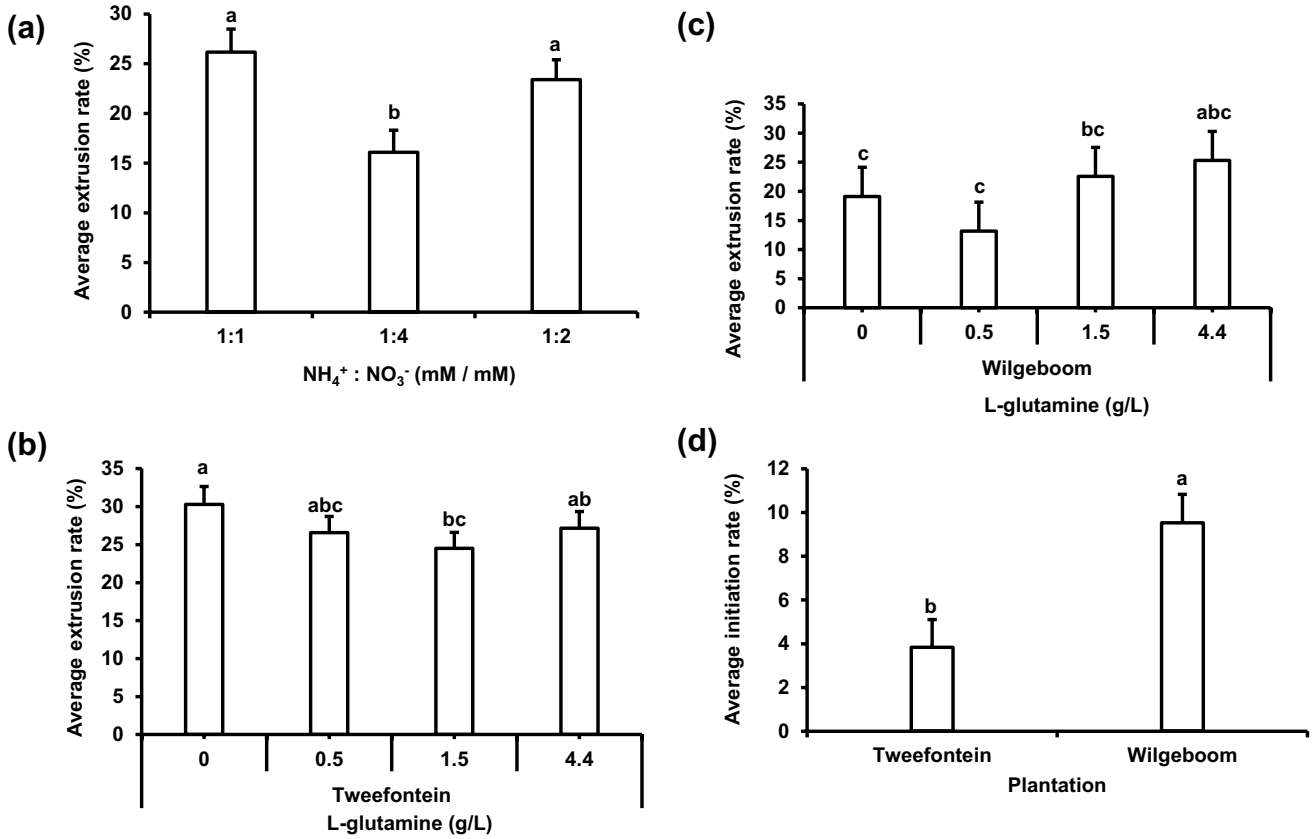

Fig. 3 The influence of the L-glutamine concentration and the molar ratio of NH_4^+: NO_3^- on the embryogenic response of somatic embryos in Tweefontein and Wilgeboom plantations. **(a)** the overall average extrusion rate of somatic embryos per NH_4^+: NO_3^- molar ratios of five open pollinated seed families of *Pinus maximinoi* plated on mLV medium. **(b)** average extrusion rate of embryogenic tissue from megagametophytes harvested from two open pollinated *Pinus maximinoi* seed families cultured in the mLV medium with varying concentrations of L-glutamine for material collected in Tweefontein, **(c)** Average extrusion rate of the embryogenic tissue from megagametophytes harvested from five open pollinated *Pinus maximinoi* seed families cultured on mLV medium with varying concentrations of L-glutamine for material collected in Wilgeboom, **(d)** Overall average initiation of embryogenic tissue from six open pollinated seed families of *Pinus maximinoi* on mLV medium with varying NH_4^+: NO_3^- molar ratios and L-glutamine concentrations over eleven collection dates in Tweefontein (n = 1589 seeds) and Wilgeboom (n = 1700 seeds) plantations. Letters above the error bars indicate a significant difference at the 5% significance level according to Fisher's LSD post hoc test

Table 5 Analysis of variance for average extrusion rate in *Pinus maximinoi* open pollinated seed families harvested in Wilgeboom and Tweefontein plantations for evaluation of the effects of L-glutamine on extrusion rate of putative embryogenic tissue. Statistical significance at $p < 0.05$ using R software and means comparison performed using the Fisher's LSD post hoc test

Plantation	Wilgeboom				Tweefontein			
Source of variation	df	MS	F-value	Pr(> F)	df	MS	F-value	Pr(> F)
Replication	4	227.4	0.70	0.59	4	161.1	0.65	0.63
Seed family (SF)	4	1599.2	4.91	0.00**	1	9.8	0.04	0.84
Collection date (CD)	5	756.2	2.32	0.05	4	11.00	4.48	5.38e-07***
L-glutamine (L-gln)	4	180.3	0.55	0.69	4	402.1	1.62	0.18
SF x L-gln	12	418.4	1.29	0.24	4	560.3	2.25	0.07
CD x L-gln	9	208.8	0.64	0.75	12	328.9	1.32	0.23
SF x CD	2	516.1	1.59	0.21				
SF x CD x L-gln	3	140.3	0.43	0.73				
Residuals	64	325.6			71	248.5		

*Significant at $P < 0.05$, ** significant at $P < 0.01$, ***significant at $P < 0.001$

Combined effects of the ammonium to nitrate molar ratio and the L-glutamine concentration on extrusion rate of putative embryogenic tissue

After the analysis in respective individual collection plantations, data from both plantations were pooled to investigate possible interactions between factors influencing extrusions and initiation of somatic embryogenesis in zygotic embryos (Table 6). We found several interactions to be significantly different for extrusion and initiation frequencies across plantations with respect to NH_4^+:NO_3^- molar ratio composition of the media and L-glutamine supplementation.

The rate of extrusion of putative embryogenic tissue was consistently high for the 1:1 and 1:2 NH_4^+:NO_3^- molar ratio modifications (Fig. 3a). The interaction between seed family, collection date and L-glutamine concentration are presented in Supplementary Table 2. Media consisting of 1.5 and 4.4 g/L L-glutamine combinations produced a statistically similar number of extrusions compared to 0 g/L L-glutamine. The extrusion rate depended on seed family, molar ratio of inorganic nitrogen and supplementation with L-glutamine (Supplementary Table 3). Some seed families yielded the highest extrusion rates at either 1.5 or 4.4 g/L L-glutamine with 1:1 or 1:2 NH_4^+:NO_3^- molar ratio respectively, however, 1.5 g/L L-glutamine and 1:2 NH_4^+:NO_3^- molar ratio predominantly produced spiky proliferative embryogenic extrusions. Both inorganic nitrogen molar ratio and L-glutamine concentration of the induction media played a significant role in influencing the SE response of in vitro cultured megagametophytes. The interaction between the collection date, genotype of the seed family and nitrogen content of the media determines the capacity of immature zygotic embryos collected at optimal developmental stage to undergo induction of somatic embryogenesis when optimal in vitro conditions are established.

Table 6 Analysis of variance for average extrusion rate in *Pinus maximinoi* across Tweefontein and Wilgeboom plantation for evaluation of the effect of NH_4^+:NO_3^- ratio and L-glutamine concentration of six open pollinated seed families collected over eleven collection dates. Statistical significance at $p < 0.05$ using R software and means comparison performed using the Fisher's LSD post hoc test

Source of variation	df	MS	F-value	Pr(> F)
Seed family (SF)	5	1292	4.77	0.00***
Plantation (P)	1	5926	21.86	4.38e-06***
Collection date (CD)	10	3123	11.52	2.48e-14***
NH_4^+:NO_3^- ratio (NR)	2	1318	4.86	0.00**
L-glutamine (L-gln)	3	221	0.81	0.49
SF x CD	2	425	1.57	0.21
P x CD	1	15	0.06	0.82
SF x NR	10	399	1.47	0.15
P x NR	2	565	2.08	0.13
CD x NR	16	490	1.81	0.03*
SF x L-gln	11	177	0.65	0.78
P x L-gln	3	723	2.67	0.05*
CD x L-gln	16	439	1.62	0.06
NR x L-gln	4	317	1.17	0.32
SF x CD x NR	3	239	0.88	0.45
SF x CD x L-gln	3	965	3.56	0.01*
SF x NR x L-gln	16	330	1.22	0.25
P x NR x L-gln	4	281	1.04	0.39
CD x NR x L-gln	24	260	0.96	0.52
SF x CD x NR x L-gln	2	20	0.07	0.93
Residuals	310	271		

* Significant at $P < 0.05$, ** significant at $P < 0.01$, *** significant at $P < 0.001$

Cell line capture and maintenance of the ESMs from Tweefontein and Wilgeboom plantations

To promote proliferation of the extruded putative embryogenic tissue we reduced the concentration of L-glutamine to 0.25 g/L. Based on the combined results from both plantations, some families responded well in a medium devoid of L-glutamine by producing high numbers of extrusions, while the 1.5 g/L of L-glutamine concentration seemed to produce morphologically-advanced cultures. From this data, an optimized medium was developed based on the synergy between the NH_4^+:NO_3^- molar ratio and the L-glutamine concentration, for the promotion of extrusions to initiations, which comprised of a 1:1 NH_4^+:NO_3^- molar ratio in the mLV basal medium enriched with 0.25 g/L L-glutamine (IM4). All extruded tissue for putative cell lines were subcultured onto IM4 to promote the production of proliferative somatic embryos. Tissue clumps of 5 mm to 10 mm diameter were considered captured and ready for further steps of the process. It took about two weeks for IM4 medium to produce "subculturable" ESM clumps. Most initiations of embryogenic tissue came from the material harvested from Wilgeboom plantation (Fig. 3d). All captured cell lines were successfully maintained on a semi-solid mDCR medium. Modified DCR medium comprised of reduced concentration of PGRs, enzymatic casein hydrolysate, L-glutamine, and sucrose. ESM maintained on a semi-solid mDCR medium without the filter paper support took 3 to 4 subcultures before producing enough proliferative clumps (Fig. 1e), while it only took 7 to 10 days for the cultures that were maintained on a filter paper disc to produce enough tissue mass for further steps of the SE process (Fig. 1f). The morphology of cell lines growing on this medium, regardless of culturing method, was mostly morphologically well-organized with distinct

suspensor cells on the edges of the vigorously proliferating tissue mass (Fig. 1e).

Discussion

We have successfully achieved somatic embryogenesis in *P. maximinoi* using immature zygotic embryos from megagametophytes of trees from seed families grown in warm temperate and subtropical plantation sites. We achieved an overall extrusion rate of 27.31% for warm temperate and 21.12% for subtropical plantation (Table 2 and 3). Numerous efforts to induce SE in *Pinus* species have reported very low initiation frequencies using immature zygotic embryos within megagametophytes as explants (Kim and Moon 2014; Hosoi and Maruyama 2012), with initiation frequencies generally in the range of 0–10% (Becwar et al. 1990). The responsive embryos are only available over a short period of time yearly and therefore it is important that the optimal collection time is determined (MacKay et al. 2006). Mature zygotic embryos have been tested for SE induction since they would be readily available throughout the year, however, they also produced very low initiation frequencies compared to immature zygotic embryos (Lara-Chavez et al. 2011). This prevents the incorporation of SE as a breeding tool for forest tree improvement programs due to the very low number of genotypes captured, which would pose a risk of genetic erosion in breeding programs (Attree and Fowke 1993). Breeding programs generally aim to work with many genetically distinct genotypes to ensure sufficient genetic diversity for selective breeding and continued genetic gain, while implementing a multi-varietal forestry (MVF) strategy, which allows propagation of elite genotypes consistently over time (Nunes et al. 2017).

Previous studies have established that the ability of immature zygotic explants to initiate extrusions is influenced by the collection date of the cones (Becwar et al. 1990). We collected highly responsive explants within opaque megagametophytes around the third to fourth week of July for both warm temperate and subtropical plantations (Fig. 2c and d). Our results corresponded with those of Humanez et al. (2012) for *P. pinaster* megagametophytes, where the highest initiation response was achieved from the middle to the end of July of 2011 in Spain. The immature embryos within opaque megagametophytes of the same cone were often not developmentally uniform, possibly due to the cone position on the tree crown and weather conditions they were exposed to during pollination, as has been observed previously (Reeves et al. 2018; Lelu-Walter et al. 2006). In other studies, too, the explant's ability to initiate embryogenic tissue was significantly influenced by the collection date of the cones (Miguel et al. 2004) and the geographical location from where cones were collected (Humanez et al. 2012). In our study, megagametophytes from the subtropical plantation showed a substantially higher embryogenic response of 9.53% initiation compared to 3.84% initiation in the megagametophytes from the warm temperate plantation (Fig. 3d).

We found enormous differences in the abilities of tested seed families to produce SE responses (Fig. 2a and b), which suggests that the selection of highly responsive seed families could possibly be used to increase the number of captured cell lines (Montalban et al. 2012). Some studies have reported that cones collected at the optimal developmental stage readily initiated somatic embryos in the media lacking PGRs (Maruyama et al. 2007; Lelu et al. 1999), however, all our treatments contained a uniform PGR concentration and did not include a negative control for PGRs. We observed great variation in seed family effects on the extrusion and initiation rates (Fig. 2a and b). We found that the induction of SE in *P. maximinoi* clearly showed seed family dependence, as there were major differences between the different families tested. Only a small proportion of extrusions yielded initiations that later gave rise to stably proliferating cell lines (Table 2 and 3, Fig. 2c and d). Somatic embryogenesis in *Pinus* species is notorious for being seed parent specific (Humanez et al. 2012).

Although we achieved encouraging results for the SE initiation in *P. maximinoi*, there is still a need for optimization of protocols to enable capture of as many genotypes as possible (Niedz and Evens 2007). In some studies, initiation frequencies were improved by adjusting zygotic explant preparation and optimization of the chemical composition and PGR supplements of the induction media. Hargreaves et al. (2011) raised initiation frequency in *P. radiata* from 44 to 93% in an mLV medium called Glitz medium, using excised zygotic embryos instead of intact megagametophytes. Park et al. (2006) used excised zygotic embryos of *P. pinaster* cultured onto an mLV-based medium with CPPU (N-(2-chloro-4-pyridyl)-N′-phenylurea), nickel, high cobalt, and vitamins to achieve a high initiation rate of 76.3%. Levels of PGRs in the induction media were tested in *P. strobus* intact megagametophytes, and lower than standard levels produced the highest initiation rate of 34.1% on an mLV-based medium (Klimaszewska et al. 2001). We therefore tested the effects of adjusting the levels of the various forms of nitrogen supplements in the induction medium, to improve both extrusion and initiation rates of somatic embryos of immature *P. maximinoi* intact megagametophytes. The synergistic reduction of the inorganic NH_4^+:NO_3^- molar ratio and the levels of organic nitrogen, in the form of L-glutamine, positively influenced SE induction. We found that the extrusions that were able to initiate embryogenic cultures came from 1:1 and 1:2 NH_4^+:NO_3^- media, while 1:4

NH_4^+:NO_3^- medium mostly produced wet extrusions which eventually turned non-embryogenic (Fig. 1d). Extrusions achieved in a 1:2 NH_4^+:NO_3^- molar ratio medium supplemented with 1.5 g/L L-glutamine produced morphologically advanced embryogenic suspensor masses with spiky, elongated suspensor cells on the edges of the clumps (Fig. 1b). Therefore, an optimal medium for *P. maximinoi* somatic embryogenesis induction contained a 1:2 NH_4^+:NO_3^- molar ratio with 1.5 g/L L-glutamine. From this data, an optimized medium was developed based on the synergy between the NH_4^+:NO_3^- molar ratio and the L-glutamine concentration, for the promotion of extrusions to initiations, which comprised of a 1:1 NH_4^+:NO_3^- molar ratio in the mLV basal medium supplemented with 0.25 g/L L-glutamine. We achieved overall initiation frequencies of 3.84% and 9.53% for warm temperate and subtropical plantations respectively (Tables 2 and 3). Overall, the study produced a total of 48 stably proliferating cell lines across both plantations, from cones harvested between the second week of July 2022 and the first week of August 2022 respectively. We successfully maintained cell lines in a DCR medium with 0.5 mg/L of both 2.4-D and BAP plant growth regulators.

The optimization of the type and the concentration of nutrients contained in the induction medium promotes the induction and proliferation of somatic embryos (Niedz and Evens 2007). Although almost all NH_4^+:NO_3^- molar ratios with respective L-glutamine concentrations produced extrusions from cultured megagametophytes, there were noticeable differences in both the frequencies of extrusion between the different seed families and their morphotype, that is, the spiky, smooth, and the wet-slimy morphotypes (Fig. 1b, c and d). The concentration of L-glutamine used to supplement the medium nitrogen content appeared to be critical for induction of SE in *P. maximinoi*. Some seed families were more responsive to induction conditions and produced significantly higher extrusions that later initiated mucilaginous tissue clumps or had to be transferred onto a cell line capture medium (IM4) to promote the proliferation of somatic embryos. This can be attributed to the genotype of the mother plant from which explants were sourced. The inhibitory role of the L-glutamine for ESM proliferation was evident, as several extrusions started to proliferate vigorously as clumps and were readily captured when subcultured onto the IM4 medium with a reduced L-glutamine concentration (Khlifi and Tremblay 1995). A small proportion of extrusions growing on the IM2 medium was able to establish proliferative cell lines, while other extrusions ceased to grow and disintegrated into a wet-slimy non-embryogenic mass (Maruyama and Hosoi 2019; MacKay et al. 2006; Percy et al. 2000). Lara-Chavez et al. (2011) also found similar results with *P. oocarpa* extrusions that were not able to form somatic embryos, and hence failed to establish stable cell lines. Conversely, Maruyama et al. (2007) found that all *P. armandii* genotypes that were contamination-free successfully produced established cell lines that could be readily captured. The ease with which cell lines are captured probably reflects the genetic background and physiological maturity or stage of development of the induced zygotic explant. According to Klimaszewska et al. (2007), it is advisable to distinguish between an initial extrusion and a stable, continuously proliferating extruded tissue when assessing ESM initiation.

It has been postulated that there could be stimulatory compounds endogenously contained by megagametophytes that control the viability of the extruded tissue in culture, and their identification and manipulation could improve the initiation frequencies during somatic embryogenesis (Carneros et al. 2009). Most studies have reported a positive role of L-glutamine supplementation in promoting growth and proliferation of extruded tissue (Carlson et al. 2017; Finer et al. 1989); however, in our study a contradictory result was observed for material harvested from the warm temperate plantation, as most induced extrusions were from the media lacking L-glutamine enrichment (Fig. 3b). This suggested that L-glutamine at a particular concentration could be inhibitory for the induction of somatic embryos harvested in trees grown in different plantation sites, depending on the endogenous nitrogen content of respective genotypes (Sotiropoulos et al. 2005). Filner (1966) similarly found that the impact of reduced organic nitrogen in cell cultures undergoing cell division and growth was not always positive. Von Arnold (1987) also noticed a strong negative effect of L-glutamine, which repressed the induction of somatic embryos in *Picea abies*. Conversely, seed collection from the subtropical plantation achieved the highest number of extrusions in a medium supplemented with 4.4 g/L L-glutamine, which was the highest concentration used in this study (Fig. 3c). Again, it is possible that this could be due to the nitrogen composition of the soil where the donor trees grew. A study to determine the endogenous nitrogen content of a growing zygotic embryo within the megagametophytes could help determine the threshold needed by these embryos to undergo dedifferentiation into somatic embryos in culture. The results from this study suggest that L-glutamine must be added as a supplement to the medium. However, additional assimilation of L-glutamine in a nitrogen self-sufficient megagametophyte could lead to repression of induction of somatic embryos from the embryonal tissue, as suggested by material from the warm temperate plantation (Fig. 3b), where 0 g/L L-glutamine yielded the highest number of extrusions, more than both 1.5 and 4.4 g/L L-glutamine. Conversely, some megagametophytes would need an L-glutamine supplementation to produce a high number of extrusions, as evident in the material harvested from the subtropical plantation (Fig. 3c), where a consistent increase from 0.5 to 4.4 g/L L-glutamine concentration progressively

yielded more extrusions when a synergy with a particular nitrogen molar ratio is achieved. These results reflect the indispensability of nitrogen in a plant tissue culture medium to enhance morphogenic responses during differentiation of cells. An interplay between the inorganic nitrogen sources (NH_4^+ and NO_3^-) acts synergistically with organic nitrogen source (L-glutamine) to enable competent cells to undergo differentiation and multiplication, while achieving embryogenic competence to produce somatic embryos.

The empirical results of this study should be considered in the light of some experimental limitations. Experiments on the effect of NH_4^+:NO_3^- molar ratio and the L-glutamine concentration were limited by the low number of genotypes and plantation sites studied. Conclusions about responses of genotypes for extrusion and initiation of ESM could not be drawn with certainty as the number of seed families covered for the purpose of this study were very low, being limited by low seed set in the families present in the plantations. The study, however, clearly indicated the synergistic effect of L-glutamine levels and inorganic nitrogen molar ratios on improving the induction and proliferation of *P. maximinoi* somatic embryos (Zhang et al. 2019). The higher L-glutamine concentration with 1:1 NH_4^+:NO_3^- molar ratio is required for enhancing extrusions in certain genotypes growing in specific locations, such as in the subtropical plantation. Conversely, genotypes from the warm temperate plantation yielded the highest embryogenic response in a medium with 1:1 NH_4^+:NO_3^- molar ratio devoid of L-glutamine. Responses of megagametophytes from warm temperate and subtropical plantations suggest a need for a two-phase extrusion step to enhance extrusion rates per collection plantation site. We achieved extrusions in a 1:1 or 1:2 NH_4^+:NO_3^- molar ratio medium supplemented with a higher L-glutamine concentration of either 1.5 or 4.4 g/L in the subtropical plantation, this was followed by a reduced 0.25 g/L L-glutamine concentration in the 1:1 NH_4^+:NO_3^- molar ratio medium (IM4), which promoted proliferation of somatic embryos into embryogenic suspensor masses. Similarly, extrusions from the warm temperate plantation growing in a 1:1 or 1:2 NH_4^+:NO_3^- molar ratio medium lacking L-glutamine were also subjected to an IM4 medium to promote the capture of cell lines. Robinson et al. (2009), found contrasting results when they subcultured *P. taeda* embryogenic cultures onto a medium with a slightly high L-glutamine concentration to achieve morphologically advanced cultures. This suggests that the assimilation of L-glutamine by cultured megagametophytes is species-specific. Somatic embryogenesis is a stress-controlled morphogenic in vitro process (Walther et al. 2022), hence, the varying ionic ratios of NH_4^+:NO_3^- together with L-glutamine concentration may have resulted in an inductive stress, which caused embryonal cells to undergo dedifferentiation into somatic embryos. Although here we have examined the synergistic effects of nitrogen supplementation in the media, future work should also consider the effects of PGRs, culture temperature, carbon source and levels, carbon to organic nitrogen ratio, levels and types of solidifying agents and enzymatic casein hydrolysate concentrations to develop an optimized medium for maximal initiation of embryogenic callus for *P. maximinoi* (Pereira et al. 2020; Sun et al. 2022).

The use of germplasm conservation technology such as cryopreservation has allowed the implementation of multivarietal forestry, which enables breeders to work with the same group of tested cell lines over time (Varis et al. 2022; Martinez et al. 2022; Nunes et al. 2017). Our future work on *P. maximinoi* will employ cryopreservation technology to preserve juvenility of established cell lines while field tests are planted and kept growing until assessments are concluded (Cao et al. 2022; Varis et al. 2022). It must also be noted that there is very little information about *P. maximinoi* reproduction and vegetative propagation. Its propagation is mostly by rooted cuttings and, to a limited extent, by seeds due to variable seed yields and heterozygosis shown by offspring in the research and commercial compartments (Perek et al. 2022). SE coupled with cryopreservation will serve to support *P. maximinoi* breeding programs with an unlimited supply of elite planting stocks (Martinez et al. 2022). This study adds a tool to the breeder's toolbox to help supplement planting stocks when there is an increasing demand than supply of seedlings for commercial plantations. Forest trees are planted and managed to meet the sawmiller's requirements as the end of the timber process value chain. A recent study by Wessels et al. (2024) revealed that *P. maximinoi* grown in the Southern Cape of South Africa has provided excellent poles and sawn boards with superior bending strength and stiffness properties. A study was undertaken to evaluate 14-year-old *P. maximinoi* trees performance for pulp production in Brazil and performed similarly to reference *P. taeda* for pulping properties as lignin, holocellulose, extractives content, basic density, and lower ash content (Coelho et al. 2021). This makes this species a potential commercial candidate for supplying poles, structural timber, and pulping products such as paper and extractives for global wood and processed wood markets.

Conclusion

Somatic embryogenesis was successfully established in the *P. maximinoi* immature megagametophytes by simultaneous adjustments of molar ratios of NH_4^+:NO_3^-, and L-glutamine concentration of the respective induction medium. The 1:1 NH_4^+:NO_3^- mLV medium supplemented with 4.4 g/L L-glutamine produced the greatest numbers of extrusions for the megagametophytes collected in the subtropical plantation, while 1.5 g/L L-glutamine

supplementation in the same medium composition produced most extrusions in the warm temperate plantation. An induction medium consisting of 1:2 NH_4^+:NO_3^- molar ratio produced morphologically advanced cultures with nodular structures in the collections from both plantations, regardless of L-glutamine supplementation levels. The synergy between the NH_4^+:NO_3^- molar ratio and reduced L-glutamine concentration seem to positively influence the extrusion of putative embryogenic tissue and the initiation of somatic embryos from immature zygotic explants collected at an optimal embryo developmental stage. The optimal medium for *P. maximinoi* somatic embryogenesis induction contained a 1:2 molar ratio with 1.5 g/L L-glutamine. The use of a 1:1 NH_4^+:NO_3^- mLV cell line capture medium supplemented with 0.25 g/L of L-glutamine to subculture all extrusions significantly improved the initiation frequencies of ESMs and the number of cell lines captured. This work provides new insights into *P. maximinoi* clonal breeding through tissue culture by offering an alternative to seed propagation and rooted cuttings production of planting stocks. The resultant plantlets could be encapsulated as artificial seeds to improve their handling and synchronize the production of high-quality seed stocks for nurseries (Rihan et al. 2017; Aquea et al. 2008; Sparg et al. 2002). Furthermore, embryogenic cultures could be cryopreserved to establish gene banks for future purposes (von Arnold et al. 2002; Lineros et al. 2018; Salaj et al. 2022). Another potential advantage of this work could be the facilitation of genetic transformation studies aimed at the incorporation of foreign genes of economic importance for *P. maximinoi* elite breeding material (Fernandes et al. 2008).

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Author contributions PSN, AAM and PNH designed and coordinated the study. PSN carried out somatic embryogenesis induction experiments, analyses and drafted the manuscript with guidance from AAM and PNH. AAM and PNH corrected the English language and coherence of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Conflict of interest The authors declare no conflict of interest. SAFCOL had no role in the design of the study, in collection of data, analyses and interpretation of results, and preparation of the manuscript or in the decision to publish the results.

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