Summary

In this report, development of *in vitro* propagation protocols for *Mesomelaena pseudostygia* - a difficult-to-propagate dryland sedge species (Cyperaceae) endemic to Western Australia are described. Various methods for *in vitro* propagation were explored including: shoot culture, organogenesis and somatic embryogenesis, using zygotic embryos as initiation material. *In vitro* multiplication rates for shoots of up to 3.4 ± 1.0 were achieved after six weeks on ½ strength Murashige and Skoog (MS) medium containing both 2.5µM kinetin and 0.5µM 6-benzylaminopurine. Following pulse treatment on ½ MS medium containing 10µM indolebutyric acid and 2µM α-naphthaleneacetic acid for seven weeks plus transfer to medium (without growth regulators) for a further seven weeks shoots showed a peak rooting of 83%. As an alternative, *in vitro* grown shoots could be pulse treated on ½ MS medium with both 100µM indolebutyric acid and 20µM α-naphthaleneacetic acid for one week then placed in Rockwool plugs (under propagation house conditions) for a further seven weeks which resulted in 63% root induction. Rooted plantlets in Rockwool plugs or bare rooted were transferred to potting mixture and maintained in propagation house conditions with very high survival (95% shoots surviving after seven weeks), indicating that micropropagation of *M. pseudostygia* for small to medium scale restoration purposes is achievable. Experiments on inducing embryogenic callus from zygotic embryos indicated that somatic embryos developed in some treatments (containing 2,4-dichlorophenoxyacetic acid) but at a very low frequency, indicating that further optimisation is necessary before mass propagation of *M. pseudostygia* via somatic embryos is feasible.

Development of an *in vitro* protocol for a difficult-to-propagate endemic Australian dryland sedge species *Mesomelaena pseudostygia* (Cyperaceae)

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Abridged title: *In vitro* propagation of *Mesomelaena pseudostygia* (Cyperaceae)

Abstract. *In vitro* propagation for *Mesomelaena pseudostygia* a difficult-to-propagate dryland sedge species (Cyperaceae) endemic to Western Australia is described. Multiple avenues to *in vitro* propagation were investigated: shoot culture, organogenesis and somatic embryogenesis, with zygotic embryos as initiation material. The highest multiplication rate for shoots was 3.4 ± 1.0 after six weeks on basal medium (½ strength Murashige and Skoog) with 2.5µM kinetin and 0.5µM 6-benzylaminopurine. Shoots achieved peak rooting (83%) following a pulse treatment on basal medium containing 10 µM indolebutyric acid and 2 µM α-naphthaleneacetic acid for seven weeks, followed by transfer to medium (without growth regulators) for a further seven weeks. Alternatively, *in vitro* grown shoots were pulse treated on basal medium with both 100 µM indolebutyric acid and 20µM α-naphthaleneacetic acid for one week then placed in Rockwool plugs (under propagation house conditions) for another seven weeks resulting in 63% root induction. Rooted plantlets were also successfully transferred to potting mixture either in Rockwool plugs or bare rooted and maintained in propagation house conditions with ≥95% survival after seven weeks. These results indicate that micropropagation of *M. pseudostygia* is feasible for small to medium scale restoration purposes. The highest frequency of callus induction was from cultured zygotic embryos on basal medium with 5µM α-naphthaleneacetic acid, whereas 2,4-dichlorophenoxacetic acid (2 or 5µM) produced the largest callus sizes. A low frequency of shoot regeneration occurred in zygotic callus tissues in basal medium treatments containing cytokinin (kinetin or thidiazuron at 1µM). A small proportion (<20%) of zygotic embryo callus explants from 2,4-dichlorophenoxyacetic acid treatments were found to be embryogenic, firstly developing embryo-like structures after two weeks on basal medium (minus plant growth hormones), that continued to develop with approximately one in twenty germinating after a further four weeks on basal medium to form small plantlets. Further optimisation is needed to improve somatic embryogenesis efficiency for mass propagation.

Keywords Restoration, embryo culture, organogenesis, somatic embryogenesis, *meta*-Topolin, picloram

Introduction

The Cyperaceae of the Southwest Australian Floristic Region (SWAFR) comprise 22 genera and 164 species (Western Australian Herbarium, 1998-). These 'dryland' Cyperaceae are important understorey components widespread in several Western Australian (WA) ecosystems such as the Kwongan, *Banksia* woodland (Marchant et al. 1987; Meney and Dixon 1988) and northern Jarrah (*Eucalyptus marginata*) forest – vegetation communities concurrent with mining and other human impacts (Koch 2007). Native sedge species typically propagate vegetatively via rhizomes, forming large clonal assemblages (Meney and Dixon 1988) with little apparent genetic variation (von Perger et al. 1994) and are capable of thriving in bare or degraded landscapes (e.g. post-mined sites) where other species struggle to establish, thus colonizing and stabilizing habitats, creating opportunities for other species (Willyams 2005). Consequently the return of native sedge taxa is currently viewed as a high priority for restoration activities in Western Australia (Koch 2007). One important group required for urban bushland restoration is the endemic genus, *Mesomelaena* which consists of only seven species all restricted to the south-west corner of Western Australia. As for other dryland Cyperaceae (e.g. *Lepidosperma* and *Gahnia*) reliable propagation via division, transplantation or seed has proven exceptionally difficult for *Mesomelaena*. This is variously due to the fact that plants are unusually sensitive to root and rhizome disturbance (therefore limiting conventional vegetative propagation approaches) and are prone to erratic seed production, often produce poor quality seed and deep, intractable seed dormancy which may take several years to alleviate under natural soil conditions (Turner unpublished results). Viable seed is consequently valuable and maximising propagation from available seeds is therefore highly desirable (Willyams 2005).

Extraction of seed embryos has been the method of choice for native rush and sedge species as vegetative shoots (from underground rhizomes) have proven too difficult to establish in culture (Meney and Dixon 1995a). Micropropagation has been previously developed for some Cyperaceae species such as *Caustis dioica*, *Tetraria capillaris* and *Lepidosperma* species (Rossetto et al. 1992; Panaia et al. 2009; Kodym et al. 2010) and has historically been an effective avenue for plant production for restoration purposes (Meney and Dixon 1995b). Nevertheless, while reasonably effective for restoration of native sedges in a few cases, *in vitro* shoot multiplication can vary substantially between species and even between different provenance sources and be very slow

therefore increasing the cost per plant to levels that are not commercially viable (Willyams 2005). In contrast, much higher multiplication rates have been achieved for several different Cyperaceae using alternative *in vitro* techniques such as plant organogenesis or somatic embryogenesis (Panaia et al. 2011). For example, an *in vitro* organogenesis protocol was developed for the native Australian sedge *Caustis blakei*, through organogenesis from friable callus with the original explant source being zygotic embryos (Webber et al. 2003). Studies on high output *in vitro* propagation systems such as somatic embryogenesis with native rush and sedge species are few but have been done with some success and estimates exceeding 20,000 somatic embryos per gram of callus tissue with high conversion rates have been quoted for *Baloskion tetraphyllum* (Panaia et al. 2004).

The aim of the project was to develop an *in vitro* propagation system for *Mesomelaena pseudostygia* via shoot culture. Alternatively, organogenesis and/or somatic embryogenesis were also investigated to determine their potential as a more time-efficient and cost-effective avenue for semi-commercial *in vitro* propagation systems for the study species. In the process we also gathered valuable information on *M. pseudostygia* seed characteristics and germination of zygotic embryos that could be useful for future *in vitro* studies of other *Mesomelaena* spp. and related taxa.

Materials and Methods

Culture conditions. Basal medium (BM) consisted of 1/2 strength MS basal salts (Murashige and Skoog, 1962) with 100µM NaFeEDTA, 60mM sucrose, 500µM myo-Inositol, 500µM 4-morpholineethanesulfonic acid (MES), 1µM thiamine hydrochloride, 2.5µM pyridoxine, 4µM nicotinic acid, 0.6% *w/v* agar and pH set at 6.0 prior to autoclaving at 121°C for 20 min. BM was used throughout this study unless otherwise specified. Heat labile plant growth regulator (PGR) compounds gibberellic acid (GA_3), indole-3-acetic acid (IAA), thidiazuron (TDZ) and 6-(4-Hydroxy-3-methylbut-2-enylamino)purine (zeatin, Z) were filter sterilised using 0.2µm microfilters (Acrodisc, Pall Life Science, UK) and aseptically added to media after autoclaving. Standard culture room conditions were 16 hr-photoperiod provided by high output 30W cool white fluorescent lamps with average photosynthetic photon flux density (PPFD) of ~50µmol$m^{-2}s^{-1}$ at culture level (as measured with a Li-cor® quantum radiometer/photometer) with temperature maintained at 23±1°C.

Seed collection, seed characteristics, storage and preliminary viability assessment of wild-collected seed: Seeds of *M. pseudostygia* were collected from mature inflorescences in December 2004 and 2009 from two locations. The older (2004) seed was collected from Carabooda on the Swan Coastal Plain near Perth, Western Australia. Upon collection, seeds were manually extracted from flower heads by gentle rubbing on a coarse metal sieve to dislodge seeds, air dried (~ five weeks @ 24°C), sealed (laminated foil bags) and stored at 5°C until use in this study. In addition fresh seeds were collected in 2009 (14th – 21st December) from four bushland populations in Kings Park in Perth Western Australia. Ten random flower heads were harvested from each individual, with ten flowering plants sampled in each population to determine average seed set per flower. All seeds (mature and fresh) were x-rayed at 18 kV for ten seconds (MX-20 digital x-ray, Faxitron, USA) to examine for seed fill.

In vitro germination (to determine optimal germination and growth of zygotic embryos): seeds were sealed in porous nylon mesh (~100 µm) sachets and surface sterilised in 2% (*w/v*) calcium hypochlorite ($Ca(ClO)_2$) solution with two drops of Tween-80 for 30 min with intermittent vacuum assistance (ten min on, ten min off, ten min on), rinsed three times with sterile distilled water prior to imbibing overnight (> 12 hours) in sterile water. Zygotic embryos were aseptically extracted using a dissecting microscope and placed on the following treatments: BM only (control), BM with 3µM GA_3 +1µM Z; BM with 1, 5, 10 or 20µM 6-benzylaminopurine (BAP); BM with 1, 2, or 5µM N6-(*meta*-hydroxybenzyl)adenine (*meta*-Topolin or *m*T); BM with 0.1, 0.5, 1, 2, 5 or 10µM TDZ. Each treatment was replicated with ten embryos/treatment (five embryos/Petri dish) except for BM and BM with 3µM GA_3 +1µM Z with 30 embryos each. Cultures were incubated in the dark for seven days then moved to standard light and temperature conditions as described above until germination was completed.

In vitro *multiplication of shoots and effects of cytokinins on shoot multiplication.* Seedlings germinated on BM with 3µM GA_3 + 1µM Z with a minimum length of 20 mm (after about 21 days) were placed in tubes with different media: PGR-free BM (control) or BM with 2.5µM 6-furfurylaminopurine (kinetin or kin), 5.0µM kin, 0.25µM BAP, 0.5µM BAP, 2.5µM kin + 0.25µM BAP, 2.5µM kin + 0.5µM BAP, 5.0µM kin + 0.25µM BAP, or 5.0µM kin + 0.5µM BAP. Seven replicates (30 ml tubes) per treatment were prepared, with one shoot/tube. Plantlets were assessed for leaf and shoot number, and shoot height after six weeks. Treatments were repeated twice and results pooled for analysis.

Rooting. Experiment 1 – determination of basic *in vitro* rooting response. Shoots were placed into 30 ml tubes (one plantlet/tube, 50 tubes/treatment) containing the following four media treatments: PGR-free BM, BM with 10µM indolebutyric acid (IBA), 2µM ∝-naphthaleneacetic acid (NAA) or 10µM IBA + 2µM NAA. After seven weeks approximately half (20 to 25) of the living shoots per treatment showing no signs of root initiation were placed into jars (six to nine shoots/jar, three jars/treatment) with PGR-free BM media. 250 ml glass jars each with polypropylene vented closures (10 mm diam. holes sealed with 0.2µm microfilters) were used. The other half of the shoots remained on the original medium. All treatments were evaluated at seven weeks, then again after a further seven weeks (14 weeks total) to determine final rooting percentage.

Rooting. Experiment 2. This experiment examined the interaction of auxin pulse treatment (applied as BM + auxins or Clonex rooting gel), substrate (BM or Rockwool plugs) and incubation conditions (culture room or propagation house) on rooting response, root length and shoot survival after seven weeks (not including one week for initial *in vitro* pulse auxin treatment where applied). Root induction treatments in detail were: (a) BM alone; (b) BM with 100µM IBA + 20µM NAA continuously for seven weeks; (c) BM with 100µM IBA + 20µM NAA for one week then transferred to PGR-free BM, culture room (d) BM with 100µM IBA + 20µM NAA for one week then moved to Rockwool plugs (one plant/plug, ten plugs/container) in 2L polypropylene containers, culture room; (e) BM with 100µM IBA + 20µM NAA for one week then Rockwool plugs, propagation house (f) Rockwool plugs with no auxin treatment, inside a container, culture room (g) Rockwool plugs with no auxin treatment, propagation house; (h) shoot bases dipped in Clonex rooting gel (8,000 ppm IBA) then shoots placed in Rockwool plugs, culture room; and (i) shoot bases dipped in Clonex rooting gel then shoots placed in Rockwool plugs, propagation house. Fifty shoots were used per treatment and rooting percentage and average root length per treatment recorded after seven weeks. Rockwool plugs were not sterilized or modified and wetted with sterile distilled water prior to use. Standard propagation house conditions consisted of 70% shading, approximately 14 hours daylight, temperature maintained at about 21°C via evaporative cooling (range approximately 18 to 25°C), automatic misting of plants and root zone heating of 25±1 °C was provided by a bench top heating mat.

Rooting. Experiment 3. This experiment examined effects of possible root disturbance on transfer of bare-rooted shoots to glasshouse conditions to assess whether or not using Rockwool plugs (or similar) is a necessity for plantlet survival. Pre-rooted plantlets (160) were used to assess four

different potting mixture transferral approaches to determine the sensitivity of bare-rooted shoots (compared to rooted shoots in Rockwool) to disturbance on transfer to nursery potting mixture. Eighty plantlets were derived from tissue culture with the agar washed off roots prior to potting up. Another 80 plants were derived from plantlets rooted in Rockwool plugs under propagation house conditions and plantlets were placed directly into potting mixture without removal of the Rockwool plugs. All plants were potted into forestry tubes (120 mm x 50 mm x 50 mm) containing pasteurised native plant potting mixture comprising four parts composted jarrah (*Eucalyptus marginata*) sawdust: two parts nursery sand: one part coarse river sand, 3 kg Osmocote® slow-release fertilizer (nine month) and 0.5 kg ferrous sulphate per 1 m^3 of mixture and the pH adjusted to 6 by adding lime and dolomite. Once potted, 80 plants (40 bare rooted and 40 in rock wool plugs) were placed onto bottom heat and another 80 plants were placed onto a cold frame under standard propagation house conditions (ambient temperature). Plants were compared for survival and growth after seven weeks.

Callus initiation and regeneration. Two types of explants were used: (i) zygotic embryos and (ii) coleoptile segments derived from *in vitro* germinated embryos. Treatments above were replicated with at least ten explants (five/Petri dish). Callus appearance, relative growth measure by increase in diameter (mm) and evidence of regeneration (i.e. organogenesis and/or somatic embryogenesis) recorded at the conclusion of the experiments (six weeks). Data from the most successful of these treatments is indicated in Table 6. All callus induction experiments were incubated at 25±1°C in total darkness unless otherwise specified.

(i) callus initiation from zygotic embryos (effects of various auxins on callus induction, growth and regeneration). Excised embryos were placed on BM with different concentrations of auxins only: zero (control), 1, 2, 5 or 10µM 2,4-D; 1, 2, 5 or 10µM NAA and 1, 10 or 40µM of 4-Amino-3,5,6-trichloropicolinic acid (picloram); or auxin (2,4-D) combined with various cytokinins: 2µM 2,4-D + 0.5 or 1µM BAP, 2µM 2,4-D + 1, 2 or 5µM *m*T, 2µM 2,4-D + 1, 5 or 10µM TDZ and 5µM 2,4-D + 0.5 or 1µM BAP. As the volume of callus formed was generally low, callus with a minimum of 5 mm diameter was consolidated from all previous 2,4-D experiments and placed on PGR-free BM and BM with 1µM kin or 1µM TDZ to encourage conversion into somatic embryos and/or plantlets. At least ten callus explants were set up for each treatment. Cultures were incubated in the dark (at 25°C) for one week before transferring to standard culture conditions. Evidence of organogenesis, somatic embryogenesis and plantlet development were recorded at weekly intervals up to six weeks.

(ii) callus initiation from coleoptiles (effects of various auxins on callus induction, growth and regeneration). To obtain coleoptile segments, dissected embryos were initially grown in darkness on BM with 3µM GA_3 +1µM Z, before moving to light after seven days culture. Seedlings ~20 mm in length were removed and basal 5 to 10 mm coleoptile segments excised. The explants were placed on BM with different concentrations of auxins and cytokinins alone, or in combinations thereof: zero (control), 1, 2, 5 or 10µM 2,4-D; 1, 5 or 20µM BAP; 1, 2 or 5µM *m*T; 1µM NAA; 0.1, 0.5, 1, 2 or 10µM TDZ; 2µM 2,4-D + 0.5 or 1µM BAP; 5µM 2,4-D + 0.5 or 1µM BAP; 2µM 2,4-D + 1, 2 or 5µM *m*T; and 2µM 2,4-D + 1µM TDZ.

Data analysis. Differences in data were analysed for statistical significance using either the Analysis of Variance (ANOVA) (Tables 1, 3 and 5) when data were expressed as a count, health rating or measurement or with a binomial generalised linear model (GLM) with a logistic link function (Tables 1, 2, 4, 5 and 6) where data were expressed as a proportion or percentage (McCullagh and Nelder 1989). For the Analysis of Variance, data were transformed to conform to ANOVA assumptions i.e. normal distribution and homogeneity of variance. Differences between replicate treatments were separated with Fisher's protected least significant difference (PLSD) with a 95% confidence interval (Ott 1993). All analyses were carried out using GenStat statistical software package (GenStat Ninth edition version 9.2.0.153). Depending on the data presented tables show either standard errors (SE), standard deviations (SD) or 95% confidence intervals (95% CI).

Results

Seed collection, seed characteristics, storage and viability. The 2009 Kings Park collection from 40 plants resulted in a total of approximately 1500 seeds with a mean of 37.8±3.3 seeds/plant (Table 1). Seed colouration was highly variable ranging from dark brown to white. X-ray analysis showed white seeds to be empty, whereas approximately one third of brown seeds were filled. Seed fill varied between the four populations. One population was found to have a significantly lower seed fill ($P < .05$) at 24.1% compared to the other three populations that ranged from 29 to 36% (Table 1). This equated to about 10 viable seeds per plant on average (or one viable seed per inflorescence sampled) hence approximately 400 seeds were potentially suitable for embryo extraction for this study.

In vitro *germination* (optimal germination and growth conditions for zygotic embryos). PGR-free BM resulted in 80% germination of *M. pseudostygia* embryos after four weeks, while BM with 3µM GA_3 + 1µM Z resulted in 90% of embryos growing. Treatments incorporating only BAP demonstrated a significant decline in germination as the concentration increased ranging from 100% with 1µM BAP to only 20% in response to the incorporation of 20µM BAP to BM and many of the growing embryos appeared distorted or malformed (Table 2). BM with 1, 2 or 5µM *m*T in comparison resulted in 100 to 70% of embryos germinating, while BM incorporating TDZ varied from 50 to 80%, though there were no significant differences between the concentrations assessed (Table 2). In light of these results BM with 3µM GA_3 + 1µM Z was used thereafter for embryo germination and growth as this treatment provided fast (three weeks) and consistent embryo germination (≥90%), and resulted in healthy growing seedlings for use in other experiments as required.

In vitro *multiplication of shoots* (effects of cytokinins). The combined effects of kin and BAP significantly increased multiple shoot production in *M. pseudostygia*. The highest number of shoots was produced by incorporating 2.5µM kin + 0.25 or 0.5µM BAP, or 5µM kin + 0.25µM BAP, with a mean increase of at least three shoots per individual per six week cycle (Table 3). Cultures maintained on GR-free BM had the lowest productivity (0.7±0.4) with most shoots failing to multiply at all. Approximately 14% of shoots on media containing 0.5µM BAP appeared hyperhydric and stunted. Shoot death was sporadic for all treatments with approximately 5% of shoots becoming necrotic after six weeks culture. However, new emergent shoots remained healthy and continued to divide. Nearly half of the shoots produced on medium incorporating 2.5µM kin + 0.25 or 0.5µM BAP were between 25 and 44 mm tall; while more than half of the shoots cultured on 5µM kin + 0.25µM BAP were between 5 - 24 mm in height.

TABLES 1, 2, 3

Rooting. Experiment 1 - determination of basic *in vitro* rooting response: Shoots on PGR-free BM or on BM with auxins displayed very low levels of root induction (0 – 7%) following seven weeks incubation (Table 4). However, when transferred to PGR-free BM for an additional seven weeks root production improved for all treatments (Table 4). Root induction was significantly higher ($P < .05$) for those treatments originally incorporating IBA, either in combination with NAA or by itself (more than 80% rooting success). The two treatments with IBA retained on the original medium for the additional seven weeks also showed some level of root production though this was

much lower (14 and 32%). Shoots on PGR-free or on medium containing only 2µM NAA produced roots when moved across to fresh BM (30 and 47% respectively) (Table 4). Survival of shoots in all treatments was very high (>90%).

TABLES 4, 5

Rooting. Experiment 2 (interaction of auxin pulse treatment, substrate and incubation conditions on rooting)**:** Survival of shoots in this rooting experiment ranged from 86-100% (Table 5). Shoots kept on BM with 100µM IBA + 20µM NAA for the entire experiment under culture room conditions had the lowest level of root production (0%) (Table 5). All treatments incorporating Rockwool plugs displayed a higher level of root induction (>20%) regardless of whether auxin was incorporated into the treatment or not. The highest levels of root induction (≥60%) were observed for the NAA + IBA *in vitro* pulse treatment prior to placement in Rockwool plugs and the Clonex rooting gel treatment followed by transfer to Rockwool plugs in the propagation house (*Fig. 1a - c*). Shoots subjected to the same two treatments but incubated under culture room conditions (rather than the propagation house) displayed lower levels of root production (23–30%). Roots were the longest on treatments incorporating Clonex rooting gel (Table 5).

FIGURE 1

Rooting. Experiment 3 (effect of root disturbance on plantlet transfer to the glasshouse): Transfer of rooted plantlets to potting substrate resulted in >90 % survival whether bare-rooted or retained in Rockwool plugs, indicating little or no disturbance or transplant shock on bare-rooted plantlets during the transfer to potting mixture phase (data not shown). All plantlets displayed a similar level of health after seven weeks in potting mixture (*Fig. 1d*). However, plants that had been maintained with bottom heat during this time were slightly larger than those maintained without bottom heat though this was not significant (data not shown).

Callus initiation and regeneration (auxin effects on initiation, growth and regeneration). (i) Zygotic embryos: BM with 10µM 2,4-D elicited significantly ($P < .05$) higher callusing response in zygotic embryos (average 40% callus production after six weeks) when compared to GR-free medium (0%), 1µM 2,4-D (0%) or 2 and 5µM 2,4-D (10% each) (Table 6). BM with 5µM NAA resulted in callus production in 35% of zygotic embryos within four to six weeks with an average diameter of 4.8±1.6 mm, while the 2µM NAA treatment resulted in 20% callusing; both these treatments were

significantly higher than callusing from 1µM (10%), or 10µM (15%) NAA treatments. BM with picloram resulted in significantly more zygotic embryos callusing at 40µM (30 %) and 10µM (20%) concentrations compared to 1µM or control treatments (zero response) (Table 6). Most calli appeared to be compact while friable calli was rarely formed but when present appeared white or yellow. Amongst all treatments, the largest individual calli pieces formed on 2,4-D media (up to 14 mm diam.).

TABLE 6

Among other treatments for which data is not presented in Table 6, BM with IAA showed zero callusing response at all concentrations, while BM with 0.5 or 1µM BAP combined with 2µM 2,4-D resulted in a maximum of 10% zygotic embryos developing callus. Raising 2,4-D to 5µM combined with 0.5µM BAP resulted in 20% callusing response but increasing BAP to 1µM (with 5µM 2,4-D) resulted in no net increase in callus production. BM with *m*T combined with 2µM 2,4-D resulted in 10% callusing (2 or 5µM *m*T) but zero response with 1µM *m*T alone. BM with TDZ combined with 2µM 2,4-D resulted in 20% callus response (1 or 10µM TDZ) but zero response with 5µM TDZ alone.

As available callus material was very limited at this stage of the study all calli from previous 2,4-D treatments (2, 5 and 10µM) were pooled and placed on BM (minus PGRs), BM + 1µM kin or BM + 1µM TDZ (data not presented). On BM (minus PGR) most calli developed multiple roots within four weeks. A small proportion (<20%) of these callus explants were found to be embryogenic, firstly developing embryo-like structures (*Fig. 1e*) after two weeks that continued to develop with approximately one in twenty germinating after a further four weeks on BM to form small plantlets (*Fig. 1f*). No somatic embryos were observed in calli transferred onto BM + 1µM kin or 1µM TDZ; however, 60% of explants cultured on 1µM kin developed multiple roots and 10% of callus explants formed multiple shoots within two weeks of subculture. For calli placed on BM + 1µM TDZ, multiple roots were observed in 30% of explants after two weeks, whereas multiple shoots were formed in 20% of callus explants after three weeks.

Callus initiation and regeneration (auxin effects on initiation, growth and regeneration). (ii) Coleoptile segments: Coleoptile explants exhibited severe browning with at least 50% dying within six weeks compared to about 30% for zygotic embryos. Callus formation on coleoptiles was only observed on the following media*:* 2,4-D alone at all concentrations with 2,4-D at 5µM eliciting a

significantly greater (*P* < *.05*) callusing response (55%) compared to all other treatments (Table 6). PGR-free BM (control treatment) elicited no response. Overall, callus production from coleoptile segments was very low, certainly much less than achieved with zygotic embryos exposed to similar PGR treatments. Only coleoptiles treated with 5µM 2,4-D produced significant amounts of callus. This yellow callus was only observed at the basal ends of coleoptile segments and was similar in appearance to callus derived from zygotic embryos and observed to be occasionally organogenic (shoot buds in ~ 20% calli) but not embryogenic.

Discussion

Seed collection of *M. pseudostygia* illustrates the poor seed set and viability problems typical of many native Australian sedges reported previously e.g. *Lepidosperma*, *Gahnia* and *Tetraria* (Willyams 2005; Kodym et al. 2010, Panaia et al. 2009; Kodym and Delpratt 2010). Adding to these significant seed quality issues, the reliable germination of viable seeds from most dryland Cyperaceae (including *Mesomelaena* spp.) has proven to be extremely difficult (Meney et al. 1995a; Panaia et al. 2009; Kodym et al. 2010; Turner unpubl. data). *Mesomelaena* spp. are predominantly clonal and their usual means of multiplication is through vegetative division of the rhizome, hence the low viable seed production observed with wild plants and the presence of large numbers of non viable seeds may simply be a lingering genetic trait (as found in many related taxa) and not greatly influenced by environmental conditions (Willyams 2005; Panaia et al. 2009). As viable seeds for restoration are likely to be reasonably scarce and given current germination and dormancy problems with *Mesomelaena* species, the mass propagation of *M. pseudostygia* through *in vitro* techniques is all the more important for the large-scale supply of plants for restoration. The current study emphasises the importance of qualitative assessment of seed viability in species such as *M. pseudostygia* as this aspect is seldom featured in most micropropagation protocols, yet can have far reaching effects on the success and repeatability of *in vitro* propagation (Panaia et al. 2009).

Embryo culture has been reported as the most effective method for *in vitro* propagation of several dryland rush and sedge species (*Caustis dioica*, *Lepidosperma drummondii* and *Tetraria capillaris*) where intractable seed dormancy has required excision of zygotic embryos to initiate germination and growth of plants in culture (Rossetto et al. 1992; Panaia et al. 2009; Kodym et al. 2010). In this study a range of PGR treatments (including BAP, *m*T and TDZ) allowed germination and growth of excised zygotic embryos of *M. pseudostygia* to varying extents. *Meta*-Topolin (*m*T) is an active aromatic cytokinin first isolated from poplar leaves (Strnad et al. 1997); and has been shown to

improve *in vitro* shoot and root production ratio in some ornamental plants (Amoo et al. 2010). In *Harpagophytum procumbens*, *m*T reportedly decreased the incidence of shoot tip necrosis due to its lower toxicity levels (Bairu et al., 2009). We did not observe that *m*T provided superior growth of excised zygotic embryos of *M. pseudostygia* in this study. Thidiazuron (TDZ) has been shown to influence the endogenous ratio of auxins to cytokinins (Visser et al. 1992). At low concentrations ($< 1\mu M$), the compound promotes shoot regeneration in some crop species such as barley and wheat (Shan et al. 2000); and was observed to enhance somatic embryogenesis in *Baloskion tetraphyllum* (Panaia et al., 2004). In *Mesomelaena* however, no significant differences in growth responses of zygotic embryos were found from 0.1 to 10μM TDZ. In comparison, BAP simply suppressed growth of zygotic embryos at concentrations tested above 1μM. BM with 3μM GA_3 and 1μM zeatin appeared to provide the most consistent and quickest germination and seedling growth as experienced with other native sedge species (Meney and Dixon 1995).

Micropropagation via shoot culture of selected Cyperaceae is currently the best way to produce new plants for broad acre restoration under semi-commercial conditions (as currently practiced by Alcoa of Australia's Marrinup nursery in Western Australia) though the number of new shoots produced per cycle is generally relatively low (can be < 1.3 new shoots per four week cycle with some species) (Willyams 2005). In this study *M. pseudostygia* shoot multiplication required a combination of kinetin and BAP to achieve the best response (av. 3 x multiplication rate/six week culture period). This result compares well with published data on micropropagation of other native dryland sedge species such as *Lepidosperma squamatum* (1.4 x), *L. tenue* and *L. drummondii* (1.2 – 1.3 x), and *L. laterale* (1.3 – 2.8 x) (Willyams 2005; Panaia et al. 2011; Kodym et al. 2012).

Initial rooting experiments with shoots of *M. pseudostygia* solely under *in vitro* conditions exhibited a high capacity for rooting (>80%) in response to auxin. However prolonged incubation on auxin medium was inhibitive of root development, with roots only readily growing once plantlets were removed from auxin medium onto a PGR-free BM that resulted in a total incubation time of 14 weeks to achieve maximum root induction. Alternatively *in vitro* propagated shoots of *M. pseudostygia* when pulse treated with auxin treatment *in vitro* (for one week) or Clonex rooting gel applied to shoot bases, were found to be capable of being directly rooted (≥60%) in Rockwool plugs under standard nursery conditions with > 90 % of rooted plantlets established and growing in substrate after seven weeks. While the rooting percentage of shoots was found to be lower than achieved in initial *in vitro* experiments, the time to produce viable rooted plantlets was effectively halved. This result is at least as good or better than micropropagation results published with other

native sedge species (Willyams 2005) and has the potential to reduce some of the costs associated with achieving adequate root induction under tissue culture conditions and the (usually) slow process of acclimating rooted shoots from the *in vitro* environment to *ex vivo* growing conditions.

The auxin 2,4-D has been documented to promote callus production in several Cyperaceae and Restionaceae species (Panaia et al. 2004; Wang et al. 2004). When applied at low concentrations, 2,4-D greatly enhances callus production in *Baloskion tetraphyllum* (Panaia et al. 2004) and *Lepidosperma drummondii* (Panaia et al. 2011). Kodym et al. (2012) reported callus induction from seedling explants of *L. laterale* and *L. concavum* with a combination of 2,4-D (2, 5 or 10µM) and 1µM zeatin. However, in *M. pseudostygia* callus production was erratic and insignificant in some 2,4-D treatments. Callus response by zygotic embryos cultured on NAA were more consistent, however when a response was induced by 2,4-D, calli produced were at least three times larger than those produced by NAA. It is possible that combinations of auxins such as 2,4-D and NAA would be beneficial in eliciting more consistent callus response and greater volume of callus tissue in *M. pseudostygia*. In addition, auxin to cytokinin ratio has also been found to be a crucial factor in callus culture maintenance for many species, including *Juncus effusus* where the combination of 0.44 µM BAP and 9.06 µM 2,4-D was ideal for callus proliferation (Xu et al. 2009). Picloram was found to increase callus production in *M. pseudostygia* at higher concentrations investigated, with 40µM picloram in particular eliciting the largest volumes of callus. In some wetland monocots such as *Typha latifolia*, picloram induced higher callus proliferation than 2,4-D or NAA (Rogers et al. 1998). As there were no marked differences in morphology of *M. pseudostygia* callus produced by picloram compared to other auxins in this study, picloram could be useful in amplifying *M. pseudostygia* callus volume as a pre-requisite to organogenesis or somatic embryogenesis.

Cytokinins commonly promote regeneration of shoots from callus tissues, a phenomenon observed in many monocotyledonous taxa including *Typha* spp., *Juncus* spp. and *Scirpus robustus* (Rogers 2003; Wang et al. 2004). This was also true for *M. pseudostygia*, where the inclusion of a cytokinin (kin or TDZ) resulted in zygotic embryo callus differentiation to shoots through organogenesis. Root differentiation from *M. pseudostygia* zygotic embryo callus occurred in BM (minus PGR) or BM with cytokinins in this study.

Zygotic embryos appeared to produce calli nearly twice as large (on average) as coleoptile sections with *M. pseudostygia*. By comparison, young coleoptile segments appear to be the best explants for callus production in other related species such as *Baloskion tetraphyllum* and *Lepidosperma* spp.

(Panaia et al. 2004; Kodym et al. 2012). However with *M. pseudostygia* necrosis and browning was severe (for reasons as yet unresolved) in coleoptile explants and in many cases the coleoptiles died before any growth response could occur. With zygotic embryos, browning was also severe in several treatments, however, callus tissues formed by the embryos continued to proliferate even after the onset of browning. This suggests that the presence of phenolics is not necessarily detrimental (at least for zygotic embryos) and could possibly be beneficial to *M. pseudostygia* cell proliferation, as embryo browning was usually observed before embryo growth. Indeed a browning response has been observed to be beneficial to embryogenesis in species such as in *Paulownia* (Radojevic 1979). Overall, zygotic embryos were clearly the best option for reliable callus initiation in *M. pseudostygia* in this study however, if additional research can resolve the early loss of *M. pseudostygia* coleoptile explants due to browning and necrosis better results may be obtained for callus production and regeneration using coleoptile explants in future experiments.

Somatic embryogenesis was observed with *M. pseudostygia* in embryogenic callus from zygotic embryos, but at a very low frequency. At this stage somatic embryogenesis is not a sufficiently developed alternative to standard shoot micropropagation for *M. pseudostygia*, however the few somatic embryos that formed spontaneously in callus treatments and were subsequently transferred to BM for development converted to plantlets readily, indicating the feasibility of somatic embryogenesis as a micropropagation tool for this species. Indeed somatic embryogenesis has already been demonstrated in several *Lepidosperma* spp. and *Baloskion tetraphyllum* with specific auxin type and concentration fundamental in eliciting embryogenic callus from either young coleoptile explants or zygotic embryos (Panaia et al. 2004, 2011; Kodym et al. 2012) though the numbers produced were vastly greater that those observed for *M. pseudostygia* in this study.

Conclusions

The imperative to establish an efficient *in vitro* propagation protocol for *Mesomelaena* spp. is highlighted by the poor seed quality as found in this study and no feasible conventional vegetative propagation options suitable for mass propagation. Germination of *M. pseudostygia* zygotic embryos was enhanced in several experimental treatments and shoots from resulting seedlings were able to be multiplied adequately *in vitro* (three-fold per six week incubation period). Rooting of *M. pseudostygia* shoots could be readily accomplished either *in vitro* or *ex vitrum* and pre-rooted plantlets were found to be relatively straightforward to establish in potting mixture under nursery conditions. These results suggest that micropropagation of *M. pseudostygia* plants to facilitate small

to medium-scale restoration objectives is achievable. Zygotic embryos were found to be superior to coleoptile explants in terms of reliable callus induction and while organogenesis and somatic embryogenesis from this callus both occurred to a small degree, additional research is still required to improve efficiency. It is also anticipated that outcomes from this study will be valuable for *in vitro* propagation research on other closely related species including *M. stygia* subsp. *deflexa* which is currently rated as a priority three species (Western Australian Herbarium, 1998–).

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Figure legends

Figure 1. *M. pseudostygia* (a) *in vitro* shoots in 2 L containers under culture room conditions; (b) untreated shoots placed directly into Rockwool plugs and incubated under propagation house conditions; (c) plantlets following four weeks incubation under propagation house conditions in Rockwool after dipping in Clonex rooting gel; (d) rooted plantlets transferred to 50 mm pots with substrate and growing for seven weeks under propagation house conditions; (e) somatic embryo formation on BM showing a range of somatic embryo development stages (bar = 500 µm) and; (f) growth of a *M. pseudostygia* somatic embryo into a plantlet (bar = 1 mm).

Table Legends

Table 1. Seed collection and seed fill of *M. pseudostygia* from four Kings Park bushland populations sampled in December 2009.

Table 2. Percentage of excised *M. pseudostygia* embryos exhibiting growth (germination and seedling development) after four weeks on BM alone (control), BM + GA_3 and zeatin or BM with various concentrations of BAP, *me*T or TDZ.

Table 3. Average increase (mean ± SE) in the number of shoots per *M. pseudostygia* individual seedling after six weeks incubation on ½ MS basal medium incorporating different Kinetin and BAP concentrations.

Table 4. Root induction for *M. pseudostygia* tissue culture grown shoots incubated on four different media for seven weeks after which time approximately half were transferred across to fresh BM with a second group retained on the original media.

Table 5. Interaction effects of pulse auxin treatment, substrate and incubation conditions on root induction and root length of *in vitro* grown shoots of *M. pseudostygia*.

Table 6. Effects of auxin-only treatments of 2,4-D, IAA, NAA and picloram, on callus production with zygotic embryos and coleoptile segments of *M. pseudostygia* after six weeks in culture.

Table 1.

[1]Population	Mean number of seeds per individual plant sampled (± SE)	Mean seed fill (% + 95 % CI)
1	32.7 ± 5.7[a2]	31.8[a] (27.0 – 37.0)
2	42.3 ± 8.5[a]	24.1[b] (20.3 – 28.4)
3	37.9 ± 3.4[a]	29.8[a] (25.4 – 34.6)
4	38.1 ± 7.9[a]	36.5[a] (31.8 – 41.4)
Mean	37.8 ± 3.3	30.6 (28.1 – 32.7)

[1]For each population 10 inflorescences were randomly collected from 10 individuals and assessed for seed production and seed viability.

[2]Different lower case superscript letters signify significant treatment differences ($P < 0.05$) within columns.

Table 2.

Treatment	% Growth (+ 95 % CI)
BM only (Control)	80.0[a1] (62.3 – 90.9) (n = 30)
BM +	
3.0 µM GA_3 &1.0 µM Z	90.0[a] (73.6 – 97.3) (n = 30)
BM +	
1.0 µM BAP	100.0[a] (75.1– 100.0) (n = 10)
5.0 µM BAP	60.0[a] (31.2 – 83.3) (n = 10)
10.0 µM BAP	30.0[b] (10.3 – 60.8) (n = 10)
20.0 µM BAP	20.0[b] (4.6 – 52.1) (n = 10)
BM +	
1.0 µM *m*T	100.0[a] (75.1– 100.0) (n = 10)
2.0 µM *m*T	90.0[a] (57.4 – 100.0) (n = 10)
5.0 µM *m*T	70.0[a] (39.2 – 89.7) (n = 10)
BM +	
0.1µM TDZ	70.0[a] (39.2 – 89.7) (n = 10)
0.5 µM TDZ	60.0[a] (31.2 – 83.3) (n = 10)
1.0 µM TDZ	70.0[a] (39.2 – 89.7) (n = 10)
2.0 µM TDZ	50.0[a*] (23.7 – 76.3) (n = 10)
5.0 µM TDZ	70.0[a] (39.2 – 89.7) (n = 10)
10.0 µM TDZ	80.0[a] (47.9 – 95.4) (n = 10)

[1] Different lower case superscript letters signify significant treatment differences ($P < 0.05$) within columns when compared to the control treatment (n = embryos per treatment).

Table 3.

Treatment	Mean increase (± SE)
BM (control)	0.7 ± 0.4^{a}[1]
2.5 µM kin	$1.0 \pm 0.4^{a,b}$
5.0 µM kin	$1.6 \pm 0.6^{a,b,c}$
0.25 µM BAP	$1.4 \pm 0.5^{a,b,c}$
0.5 µM BAP	$2.3 \pm 0.6^{a,b,c,d}$
2.5 µM kin + 0.25 µM BAP	$3.3 \pm 1.2^{c,d}$
2.5 µM kin + 0.5 µM BAP	$3.4 \pm 1.0^{c,d}$
5.0 µM kin + 0.25 µM BAP	3.4 ± 0.5^{d}
5.0 µM kin + 0.5 µM BAP	$2.7 \pm 0.9^{b,c,d}$

[1]Different lower case superscript letters signify significant treatment differences ($P < 0.05$).

Table 4:

Treatment	[1]7 weeks only Before movement to BM		14 weeks Maintained on original media		14 weeks Transferred to BM after 7 weeks	
	n	Root induction (% + 95 % CI)	n	Root induction (% + 95 % CI)	n	Root induction (% + 95 % CI)
BM (control)	46	0.0^{aA2} (0.0 – 6.7)	22	0.0^{aA} (0.0 – 13.0)	23	30.4^{aB} (15.4 – 51.1)
10 µM IBA	49	4.1^{aA} (3.7 – 10.4)	21	14.3^{aA} (4.1 – 35.5)	22	81.8^{bB} (60.9 – 93.3)
2 µM NAA	45	6.7^{aA} (5.0 – 11.9)	19	0.0^{aA} (0.0 – 14.8)	19	47.4^{aB} (27.3 – 68.3)
10 µM IBA + 2 µM NAA	50	0.0^{aA} (0.0 – 6.2)	25	32.0^{bB} (17.1 – 51.7)	24	83.3^{bB} (63.5 – 93.5)

[1]Note: Following the first seven week period all shoots with roots were removed from the trial and no longer assessed.

[2]Different lower case superscript letters signify significant differences ($P < 0.05$) within columns when compared to the control (1/2 MS basal medium only) treatment. Different upper case superscript letters signify significant differences ($P < 0.05$) within rows of the same medium type when compared to the control treatment (n = shoots assessed per treatment).

Table 5.

[1]Treatment	Culture environment	Survival (% + 95 % CI)	Root induction -surviving shoots only- (% + 95 % CI)	Average root length (mm ± SD)
(a) BM only	Culture room	86.0[a 2] (73.5 – 93.4) (n[3] = 50)	11.3[b] (4.6 – 24.9) (n = 43)	10.6 ± 6.1[a,b] (n = 5)
(b) BM+ 100 µM IBA+20 µM NAA	Culture room	100.0[a] (93.9 – 100.0) (n = 50)	0.0[a] (0.0 – 6.2) (n = 50)	0.0 ± 0.0[4] (n = 0)
(c) BM+ 100 µM IBA+20 µM NAA for 1 wk then moved to fresh BM	Culture room	100.0[a] (93.9 – 100.0) (n = 50)	14.0[b] (6.6 – 26.5) (n = 50)	9.1 ± 4.0[a] (n = 7)
(d) BM+ 100 µM IBA+20 µM NAA for 1 wk then moved to Rockwool plugs	Culture room	86.0[a] (73.5 – 93.4) (n = 50)	30.2[b,c] (18.5 – 45.2) (n = 43)	15.2 ± 8.5[a,b] (n = 13)
(e) BM+ 100 µM IBA+20 µM NAA for 1 wk then moved to Rockwool plugs	Propagation house	94.0[a] (83.2 – 98.6) (n = 50)	64.1[d] (49.5 – 76.1) (n = 47)	26.2 ± 7.9[c,d] (n = 30)
(f) Untreated shoots in Rockwool plugs	Culture room	95.0[a] (82.6 – 99.5) (n = 40)	23.9[b,c] (12.8 – 39.4) (n = 38)	17.0 ± 10.6[a,b,c] (n = 9)
(g) Untreated shoots in Rockwool plugs	Propagation house	92.0[a] (80.7 – 97.4) (n = 50)	41.1[c,d] (27.6 – 54.7) (n = 47)	22.9 ± 10.0[b,c] (n = 19)
(h) Rockwool plugs after treatment with Clonex rooting gel	Culture room	94.0[a] (83.2 – 98.6) (n = 50)	45.5[c,d] (31.4 – 58.8) (n = 47)	32.3 ± 18.6[d,e] (n = 21)
(i) Rockwool plugs after treatment with Clonex rooting gel	Propagation house	90.0[a] (78.2 – 96.1) (n = 50)	60.0[d] (45.4 – 73.0) (n = 45)	36.4 ± 20.7[e] (n = 27)

[1]Standard incubation time was seven weeks (notwithstanding one week incubation for *in vitro* auxin treatment).
[2]Different lower case superscript letters signify significant differences ($P < 0.05$) within columns when compared to the control treatment within columns.
[3]n = shoots per treatment.

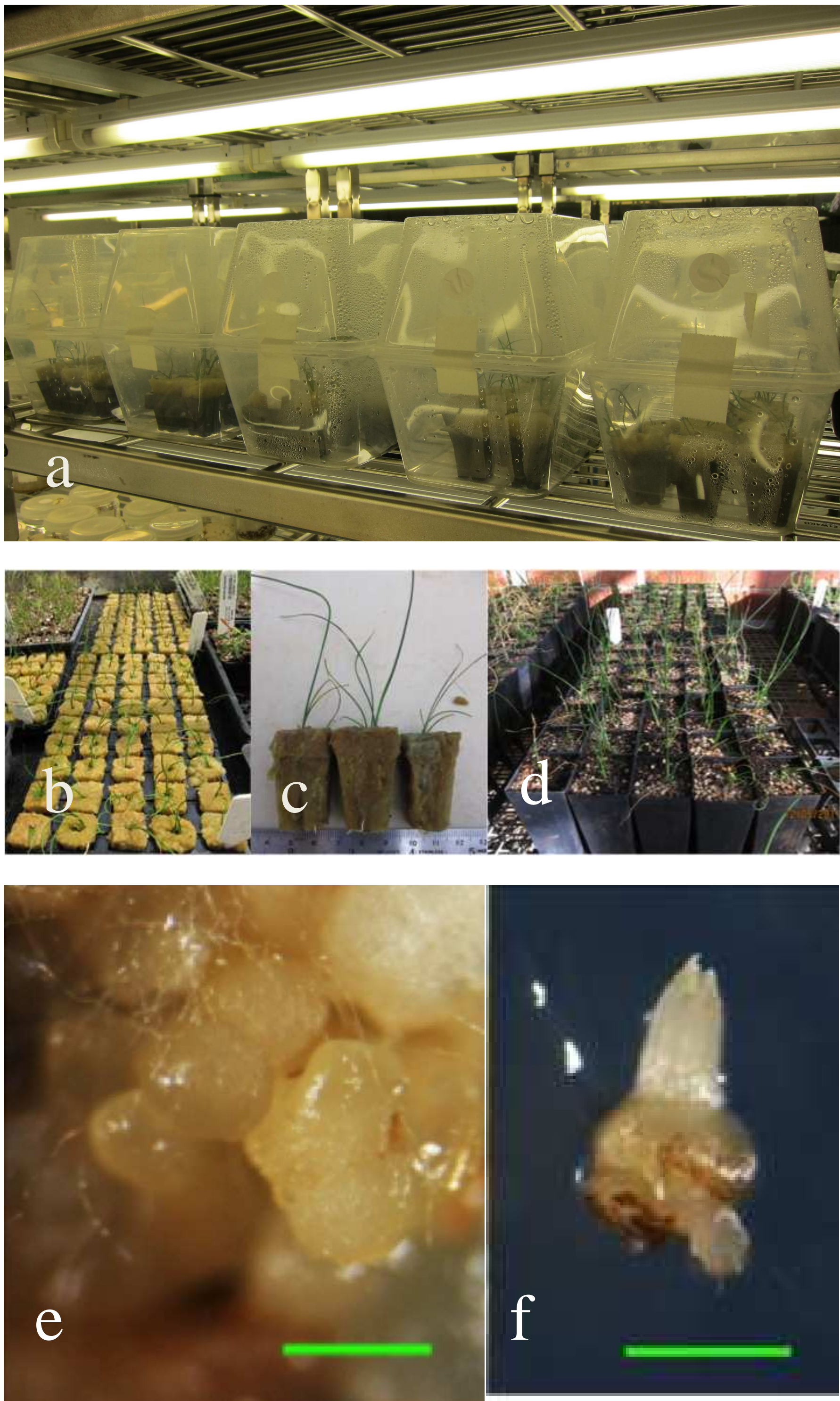
[4]Note: no plants formed roots with this treatment combination hence this treatment was excluded from statistical assessment of root length.

Table 6.

PGR Treatment (μM)	Zygotic embryos n	Zygotic embryos % Explants with callus (+ 95 % CI)	Coleoptile segments n	Coleoptile segments % Explants with callus (+ 95 % CI)
Zero (control)	30	0.0[a1] (0.0 – 9.9)	10	0.0[a] (0.0 – 24.9)
2,4-D				
1	10	0.0[a] (0.0 – 24.9)	20	15.0[a] (4.4 – 36.9)
2	10	10.0[a] (0.0 – 42.6)	20	20.0[a] (7.5 – 42.2)
5	10	10.0[a] (0.0 – 42.6)	20	55.0[b] (34.2 – 74.2)
10	30	40.0[b] (24.6 – 57.7)	10	10.0[a] (0.0 – 42.6)
NAA				
1	20	10.0[a] (15.7 – 31.3)		-[2]
2	20	20.0[b] (7.5 – 42.2)		-
5	20	35.0[b] (18.0 – 56.8)		-
10	20	15.0[a] (4.4 – 36.9)		-
Picloram				
1	20	0.0[a] (0.0 – 14.1)		-
10	20	20.0[b] (7.5 – 42.2)		-
40	20	30.0[b] (14.3 – 52.1)		-

[1]Different lower case superscript letters signify significant differences ($P < 0.05$) within columns when compared to the control treatment (n = embryos per treatment).
[2]Not assessed indicated by '-'..

Figure 1.



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