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Establishment of an *in vitro* culture and regeneration protocol for the native grass *Polypogon australis* Brong

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Abstract

Polypogon australis Brong. is a native Chilean grass frequently found colonizing metal-rich tailings, yet it lacks an established *in vitro* regeneration system to support controlled experimentation. Here, we report an efficient and reproducible protocol for seed germination, callus induction, and plant regeneration using coleoptile–mesocotyl explants. Seeds were surface-sterilized and germinated on MS medium supplemented with sucrose, achieving 44–51% germination. The coleoptile–mesocotyl junction was identified as the most responsive explant type, showing a callus induction rate of 30.55% after 3–5 weeks. Embryogenic calli predominated under Dicamba treatment and regenerated multiple plantlets without the need for exogenous organogenesis-promoting hormones. Regeneration frequency reached 45.5%, producing uniform and totipotent plantlets suitable for downstream analyses. This protocol overcomes a major bottleneck in *P. australis* research and provides a reliable framework for future physiological and molecular studies involving this ecologically relevant native species.

Introduction

Native plant species that colonize metal-enriched soils, including abandoned mine tailings, often display specialized physiological and biochemical adaptations that enable survival under high concentrations of potentially toxic elements (Ghosh & Singh, 2005; Ghazaryan et al., 2019). These facultative metallophytes hold considerable promise for phytotechnologies such as phytostabilization, phytoextraction, and phytomining, owing to their ability to accumulate, tolerate, or immobilize metals in a cost-effective and environmentally sustainable manner (Futughe et al., 2020). In addition to their applied relevance, these species serve as informative models for studying metal homeostasis and stress tolerance under extreme environmental conditions.

The development of efficient and reproducible *in vitro* propagation systems is fundamental for advancing both basic and applied research in metallophytes. Tissue culture methodologies enable large-scale clonal propagation, provide homogeneous plant material for physiological and biochemical studies, and serve as platforms for downstream molecular and functional analyses (Wijerathna-Yapa & Hiti-Bandaralage, 2023). However, many native grasses exhibit strong recalcitrance to *in vitro* culture, making the establishment of regeneration systems particularly challenging (Kovács et al., 2016; Hinojosa et al., 2014). Despite these limitations, recent advances demonstrate that somatic embryogenesis can be achieved in several non-model Poaceae species when explant selection, auxin balance, and micronutrient availability are optimized. For instance, *Cenchrus ciliaris* now possesses robust embryogenic callus and regeneration systems derived from juvenile tissues (Goyal et al., 2023), while *Chloris gayana* has recently been regenerated and transformed using optimized *Agrobacterium*-mediated protocols (Maybery-Reupert et al., 2025). In addition, long-term embryogenic competence has been achieved in *Miscanthus × giganteus* through fine-tuning of micronutrients such as $CuSO_4$ (Kopeć et al., 2025; Wojtkowiak et al., 2025). These studies collectively highlight the feasibility of overcoming recalcitrance in diverse Poaceae lineages.

Among native grasses adapted to extreme environments, *Polypogon australis* Brong. is a native, non-endemic species widely distributed across Chile and other regions of South America (Ulloa et al., 2011). The species frequently colonizes disturbed environments, including copper-rich mine tailings, riverbanks, and marginal soils, where it demonstrates tolerance to multiple abiotic stressors (Ortiz-Calderón et al., 2008; Lam et al., 2017). Under controlled conditions, *P. australis* has been shown to accumulate metals such as copper and zinc and to exhibit enhanced antioxidant responses when exposed to metal-enriched substrates (Jara-Hermosilla et al., 2017). These characteristics highlight its role as a facultative metallophyte and support its potential for phytoremediation applications.

Despite this ecological relevance and its potential for phytotechnological applications, *P. australis* remains absent from biotechnological pipelines due to the lack of a validated *in vitro* regeneration system, which limits its use in controlled physiological studies, propagation platforms, and genetic transformation approaches.

Importantly, regeneration and transformation capabilities have recently been demonstrated within the *Polypogon* genus. Zhou et al. (2023) established callus induction and *Agrobacterium*-mediated transformation in *Polypogon fugax*, illustrating that members of this genus possess embryogenic competence suitable for tissue culture and transformation. Moreover, successful regeneration has been reported in other native South American grasses, such as *Spartina argentinensis* (Bueno et al., 2012), reinforcing the broader potential for developing *in vitro* culture systems in underrepresented Poaceae adapted to extreme environments.

Given the ecological importance of *P. australis* and its emerging relevance for phytoremediation and functional genomics, establishing a reproducible regeneration protocol represents a critical first step. The objective of this study was to develop a complete *in vitro* culture and regeneration system for *P. australis* by optimizing seed germination, identifying a responsive explant for embryogenic callus induction, and regenerating plantlets capable of normal root and shoot development. This regeneration platform provides the foundation for future copper physiology studies and positions *P. australis* as a tractable native Poaceae species for integrating phytotechnology with advanced genome engineering approaches.

Results

Seed germination and early seedling development

Surface-sterilized seeds of *Polypogon australis* displayed rapid and uniform germination on MS medium (Fig. 1). Germination began 8 days after sowing (DAS) and increased steadily through 11 and 15 DAS, reaching high cumulative germination percentages under controlled conditions. Seedlings exhibited normal early development, characterized by elongation of the coleoptile and emergence of the first true leaf.

When transferred to 1-L jars containing MS+10S medium, seedlings continued to grow vigorously over a 50-day period (Fig. 2). Shoot and root development progressed consistently across replicates, confirming the reliability of MS-based germination and early-growth conditions for producing uniform explants suitable for subsequent tissue culture experiments.

Callus induction from coleoptile–mesocotyl explants

Explants excised from the coleoptile–mesocotyl junction of 7–10-day-old seedlings produced callus efficiently when cultured on CIM supplemented with 2.5 mg L^{-1} dicamba. Callus initiation was first visible after 12 days of incubation in darkness, and induction frequencies increased with subculture at 3-, 4-, and 5-week intervals (Fig. 2).

Callus quality varied among explants, but a substantial proportion developed into friable, cream-colored tissues characteristic of embryogenic calli. Callus induction percentage remained consistent across experimental replicates, demonstrating that the coleoptile–mesocotyl junction is a responsive explant for *P. australis* callogenesis.

Embryogenic callus formation and shoot regeneration

Calli were categorized based on morphology, color, and tissue organization. Embryogenic calli (ECs) displayed compact structures with distinct nodular regions indicative of somatic embryo development. These ECs were isolated and subcultured for an additional week before regeneration.

Upon transfer to regeneration conditions, ECs produced multiple shoot primordia, followed by elongation of shoots and roots (Fig. 3). Regeneration efficiency, calculated as the proportion of ECs forming shoots, was reproducible across replicates. Regenerated plantlets exhibited normal leaf and root development, confirming the totipotency of *P. australis* embryogenic tissues and the suitability of the regeneration protocol.

Discussion

This study establishes the first complete *in vitro* regeneration system for *Polypogon australis*, a native metallophyte with natural tolerance to copper-enriched environments. By optimizing seed germination, explant selection, and auxin-driven callus induction, we demonstrate that *P. australis* possesses reliable totipotent capacity under controlled culture conditions. These results provide an important methodological foundation for future biotechnological applications in a species that has historically lacked tissue culture resources.

The strong response of the coleoptile–mesocotyl junction confirms the importance of explant choice in overcoming recalcitrance in non-model Poaceae. Similar patterns have been observed in other wild and agronomic grasses, where juvenile tissues often exhibit the highest embryogenic potential. The high

frequency of friable, embryogenic calli obtained under dicamba supplementation reflects the capacity of *P. australis* to undergo somatic embryogenesis when auxin balance and culture environment are optimized.

Regeneration from embryogenic calli was consistent across biological replicates, supporting the reproducibility of the system. Regenerated plantlets developed normal shoots and roots, indicating that the hormonal and nutritional conditions provided were adequate to support both organogenesis and somatic embryo maturation. The stability of this response also suggests that *P. australis* may be amenable to downstream transformation systems, as regeneration efficiency represents one of the most significant constraints in grasses targeted for genetic engineering.

The development of a reliable regeneration platform for *P. australis* is particularly relevant given its ecological distribution in copper-rich mine tailings and its emerging role as a model for metal tolerance. *In vitro* systems enable controlled studies of copper uptake, oxidative stress responses, and metal sequestration mechanisms using uniform plant material, which is difficult to obtain from field-grown tissues. Furthermore, the establishment of embryogenic callus cultures provides the tissue framework needed for *Agrobacterium*-mediated transformation and CRISPR-based transcriptional activation strategies aimed at modulating metal homeostasis pathways.

Overall, this study establishes the first complete *in vitro* regeneration system for *Polypogon australis*, a native metallophyte with strong tolerance to copper-enriched environments. By optimizing seed germination, explant selection, and auxin-driven callus induction, we demonstrate that *P. australis* possesses reliable totipotent capacity under controlled culture conditions. The ability to generate embryogenic tissues and regenerate whole plants positions this species as a tractable non-model Poaceae for physiological studies, *Agrobacterium*-mediated transformation, and CRISPR-based approaches targeting metal homeostasis. This work fills a critical methodological gap and provides a foundation for advancing phytoremediation research and genome engineering in native metallophytes.

Methods

Plant material and seed sterilization

The plants used in this study come from seeds obtained from our experimental nursery. These nursery seeds were originally sourced from wild populations, naturally growing on a copper mine tailings storage facility in northern Chile (Planta Manuel Antonio Matta, Atacama; 27.3° S, 70.3° W) (Lam et al., 2017). These seeds were used as the starting material for all *in vitro* experiments.

Surface sterilization of the *Polypogon australis* seeds followed the protocol of Acemi and Özen (2019), with slight modifications (Fig. 1A–D). Seeds were immersed in 70% (v/v) ethanol for 1 min, followed by 1% (w/v) sodium hypochlorite supplemented with a drop of Tween™ 20 for 10 min, and rinsed three times with sterile distilled water. Sterilized seeds were cultured on Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) supplemented with 1% (w/v) sucrose and 0.43% (w/v) Phytagel®

(pH 5.75–5.8), and incubated at 22°C under a 16/8 h photoperiod with a light intensity of 136 µmol m^{-2} s^{-1}. Germination dynamics were assessed using MS+10S medium following the approach of Noni-Morales et al. (2019). Germination percentage was calculated as:

$$\text{Germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total seeds sown}} \times 100$$

Cumulative germination was recorded at 8, 11, and 15 days after sowing (DAS). Four independent biological replicates were analyzed.

Early growth of P. australis seedlings

Early seedling growth was assessed by germinating 400–500 mg of surface-sterilized seeds on MS medium. After 14 days, groups of five seedlings were transferred into 1-L jars containing MS+10S medium, and growth was monitored for 50 days. Each treatment consisted of three replicates, with five plants per replicate.

Callus induction and regeneration efficiency

Coleoptile–mesocotyl junction explants (~ 0.5 cm segments excised from 7–10-day-old seedlings after emergence of the first true leaf), were cultured on callus induction medium (CIM; MS basal salts supplemented with 2.5 mg L^{-1} dicamba, 11.3 µM) (Fig. 1E-H). Roots were removed prior to culture. Explants were incubated at 24°C in complete darkness, and the most responsive explant type was identified after 12 days. Explants were subcultured onto fresh CIM at 3-, 4-, or 5-week intervals to evaluate callus induction and regeneration efficiency. Callus induction percentage (CI) was calculated as:

$$\text{CI (\%)} = \frac{\text{Number of calli formed}}{\text{Total explants}} \times 100$$

For regeneration, calli were incubated in darkness at 24°C for 2 weeks. Calli were then classified based on size, morphology, and color. Embryogenic calli (ECs) were excised and subcultured on CIM for 1 week. Regeneration percentage was calculated as:

$$\text{Regeneration (\%)} = \frac{\text{Number of calli forming shoots}}{\text{Total ECs}} \times 100$$

All regeneration assays were performed in triplicate using 12 explants per replicate.

Statistical analysis

Germination data were normalized to percentages and modeled using a cumulative Gaussian (inverse normal) distribution to characterize germination dynamics. Statistical analyses were conducted using GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA). For germination assays, four independent biological replicates were evaluated. Early growth experiments included three replicates of five seedlings each. Callus induction and regeneration assays were performed in triplicate with 12 explants per replicate.

A one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, was used to identify significant differences among treatments at $p < 0.05$. Distinct letters above bars indicate statistically significant differences (mean ± SD).

Declarations

Author contributions statement

J.V.R. conceived and designed the experiments, conducted the experiments, analyzed the data, prepared the figures, and drafted the manuscript. C.O.-C. supervised tissue culture experiments and contributed to methodological optimization. R.G. provided ecological and field expertise. G.H. supervised and contributed to tissue culture methodology and manuscript editing. All authors reviewed and approved the final manuscript.

Competing interests:

The authors declare no competing interests.

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Author Contribution

J.V.R. conceived and designed the experiments, conducted the experiments, analyzed the data, prepared the figures, and drafted the manuscript. C.O.-C. supervised tissue culture experiments and contributed to methodological optimization. R.G. provided ecological and field expertise. G.H. supervised and contributed to tissue culture methodology and manuscript editing. All authors reviewed and approved the final manuscript.

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Data Availability

Data availability: All data generated or analyzed during this study are included in this published article and its supplementary information files. Additional raw data are available from the corresponding author upon reasonable request.Competing interests: The authors declare no competing interests.

References

1. Acemi, A. & Özen, H. Efficient surface sterilization method for seeds used in plant tissue culture studies. *Planr Cell Tissue Organ Cult.* **137**, 1–9 (2019).
2. Bueno, M. S., Sorrequieta, J. I., Feldman, S. R. & Ortiz, J. P. A. *In vitro* plant regeneration of *Spartina argentinensis* Parodi. *Revista Colombiana de Biotecnología*. **14**, 61–69 (2012).
3. Futughe, O., Eze, E., Nnamchi, H. & Onwughalu, J. Prospects of phytomining and hyperaccumulators in sustainable metal recovery. *Environ. Sustain.* **3**, 321–330 (2020).
4. Ghazaryan, K., Movsesyan, H. & Ghazaryan, N. Mechanisms of plant adaptation to heavy metal–contaminated soils. *Environ. Sci. Pollut. Res.* **26**, 1122–1139 (2019).
5. Ghosh, M. & Singh, S. P. A review on phytoremediation of heavy metals and utilization of its byproducts. *Appl. Ecol. Environ. Res.* **3**, 1–18 (2005).
6. Goyal, V. et al. Embryogenic callus and suspension culture development in *Cenchrus ciliaris*: implications for transformation. *Planr Cell Tissue Organ Cult.* **155**, 321–334 (2023).
7. Hinojosa, L., Sanhueza, C. & Carrasco, B. *vitro* regeneration of native Chilean grasses. *Chil. J. Agricultural Res.* **74**, 324–332 (2014).
8. Jara-Hermosilla, R., Cisternas, M. & Sánchez, P. Antioxidant responses of native Poaceae species from copper-contaminated sites. *Plant Physiol. Biochem.* **118**, 612–620 (2017).

9. Kopeć, P. et al. Optimizing micronutrient conditions to improve callus regeneration in *Miscanthus × giganteus*. *Biocatal. Agric. Biotechnol.* **60**, 102883 (2025).
10. Kovács, G., Sági, L. & Kiss, J. Overcoming recalcitrance in grass tissue cultures: current challenges and future directions. *Plant Cell Rep.* **35**, 1501–1512 (2016).
11. Lam, Q. D. et al. Physiological responses of *Polypogon australis* to copper stress in mine tailings environments. *Environ. Monit. Assess.* **189**, 456 (2017).
12. Maybery-Reupert, E. et al. Efficient regeneration and *Agrobacterium*-mediated transformation in Rhodes grass (*Chloris gayana*). *Planr Cell Tissue Organ Cult.* **169**, 435–448 (2025).
13. Murashige, T. & Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497 (1962).
14. Noni-Morales, L., González, H. & Palta, J. A. Germination dynamics of native Poaceae species under controlled conditions. *Seed Sci. Res.* **29**, 223–232 (2019).
15. Ortiz-Calderón, C., Ginocchio, R. & Sauvé, S. Agromining potential of *Polypogon australis* in copper-rich environments. *Environ. Sci. Technol.* **42**, 329–334 (2008).
16. Wijerathna-Yapa, H. & Hiti-Bandaralage, J. Advances in *in vitro* propagation of metallophytes and their applications in environmental biotechnology. *Plant Biotechnol. J.* **21**, 65–82 (2023).
17. Wojtkowiak, A. et al. Long-term retention of embryogenic potential in *Miscanthus × giganteus* callus cultures. *Plant Cell Rep.* **44**, 87–99 (2025).
18. Zhou, F., Meng, Z., Han, Y. & Zhang, Y. Callus induction and *Agrobacterium*-mediated transformation of *Polypogon fugax*. *J. Plant Biochem. Biotechnol.* **32**, 622–631 (2023).

Figures

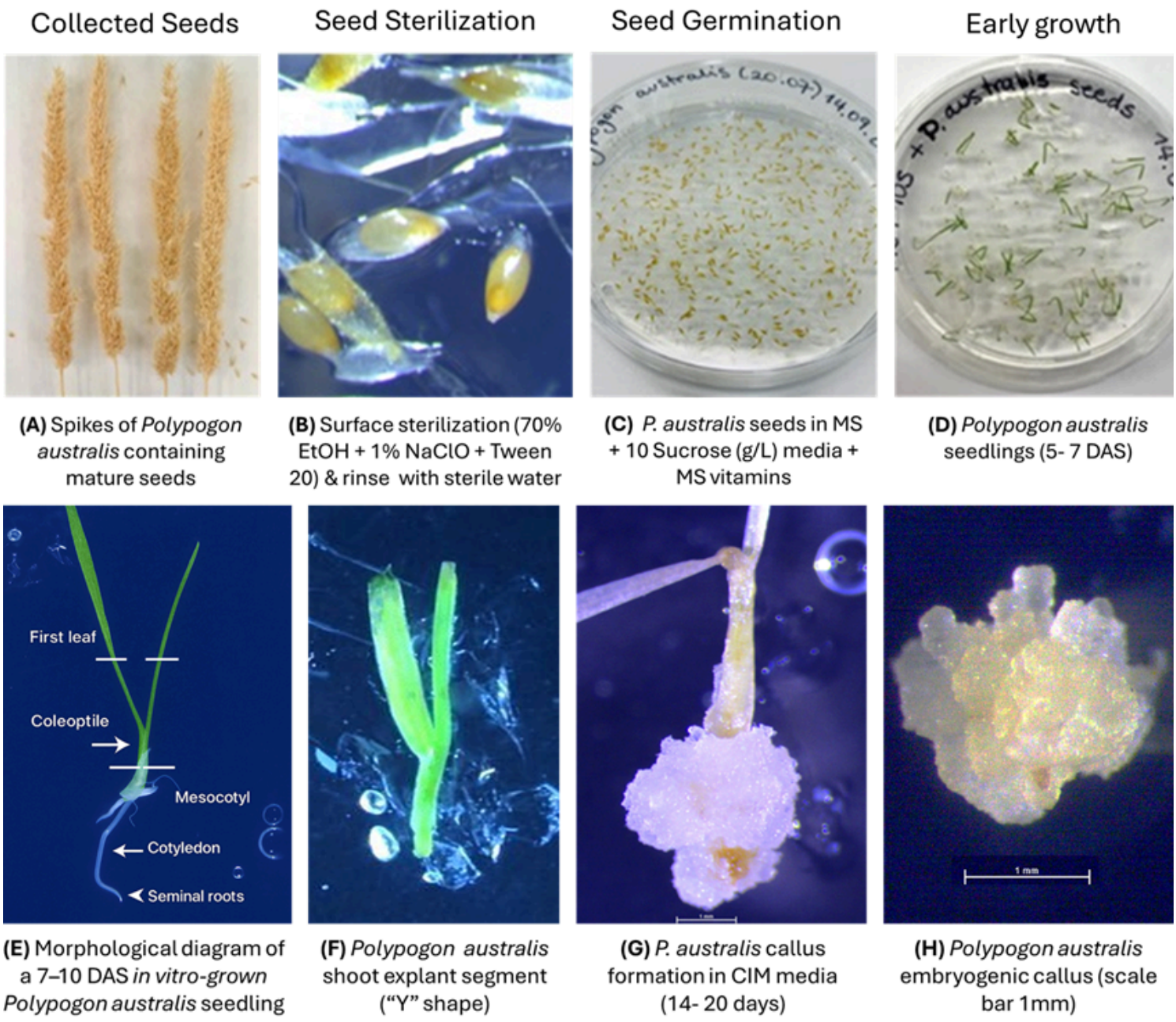

Figure 1

Workflow for *in vitro* seed germination and callus induction in *Polypogon australis*. (A) Spikes collected from field-grown plants; (B) seeds surface sterilization with 70% (v/v) ethanol followed by 1% (w/v) sodium hypochlorite + Tween® 20; (C) seeds cultured on MS medium supplemented with 10 g L^{-1} sucrose and MS vitamins; (D) germination observed within 5–7 days; (E) morphological diagram of a 7–10 DAS *in vitro*-grown seedling; (F) coleoptile–mesocotyl explants excised after emergence of the first true leaf; (G) callus induction observed after 12 days in the dark; (H) embryogenic calli after 3–5 weeks of culture on CIM. Scale bars: 1 cm.

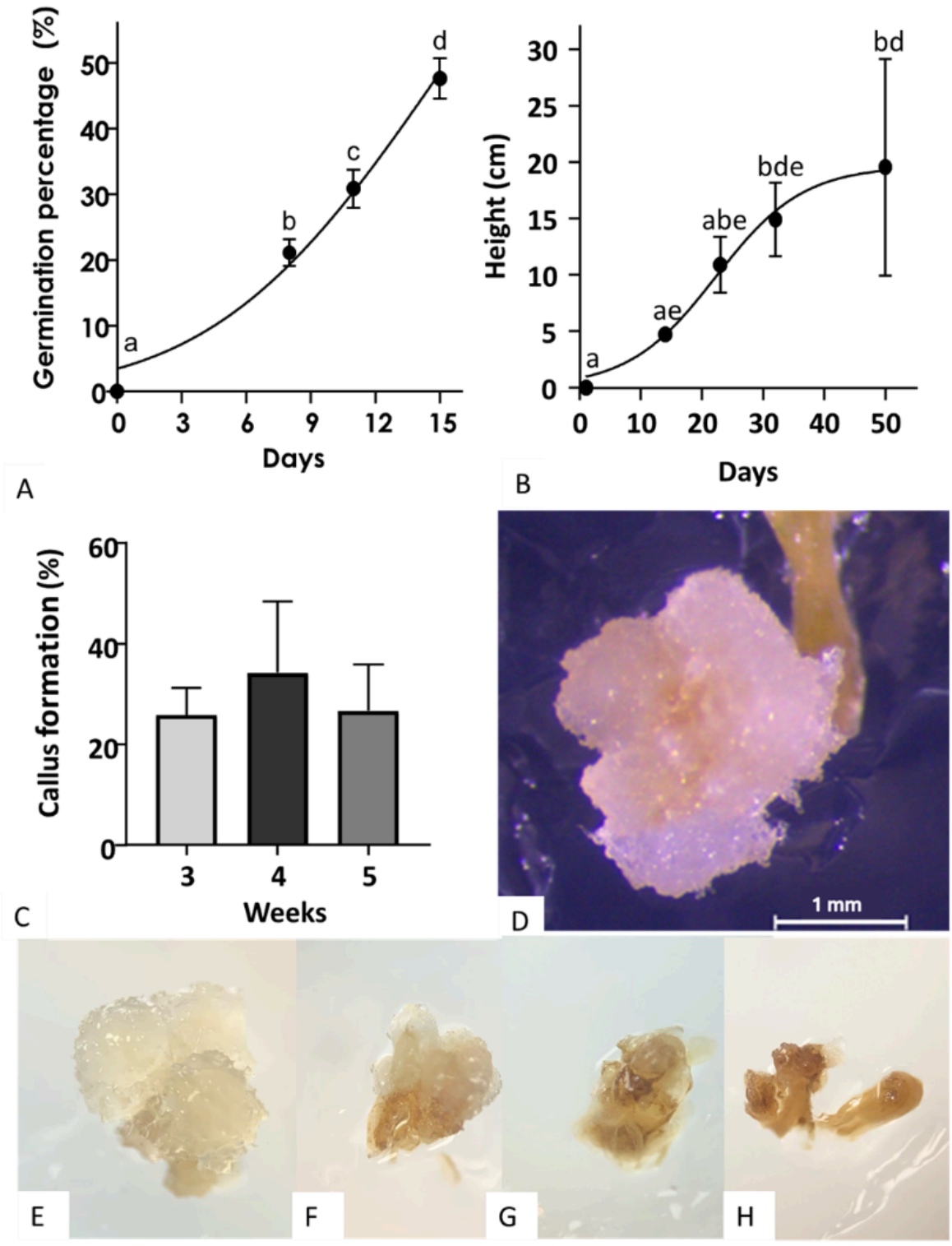

Figure 2

Early growth of *P. australis* seedlings cultured *in vitro*. (A) Fourteen-day-old seedlings on MS medium; (B) transplanted seedlings in 1-L jars containing MS+10S medium; (C) shoot elongation after 25 days; (D) root system development after 50 days; (E–F) representative seedlings showing uniform growth under controlled conditions. Scale bars: 1 cm.

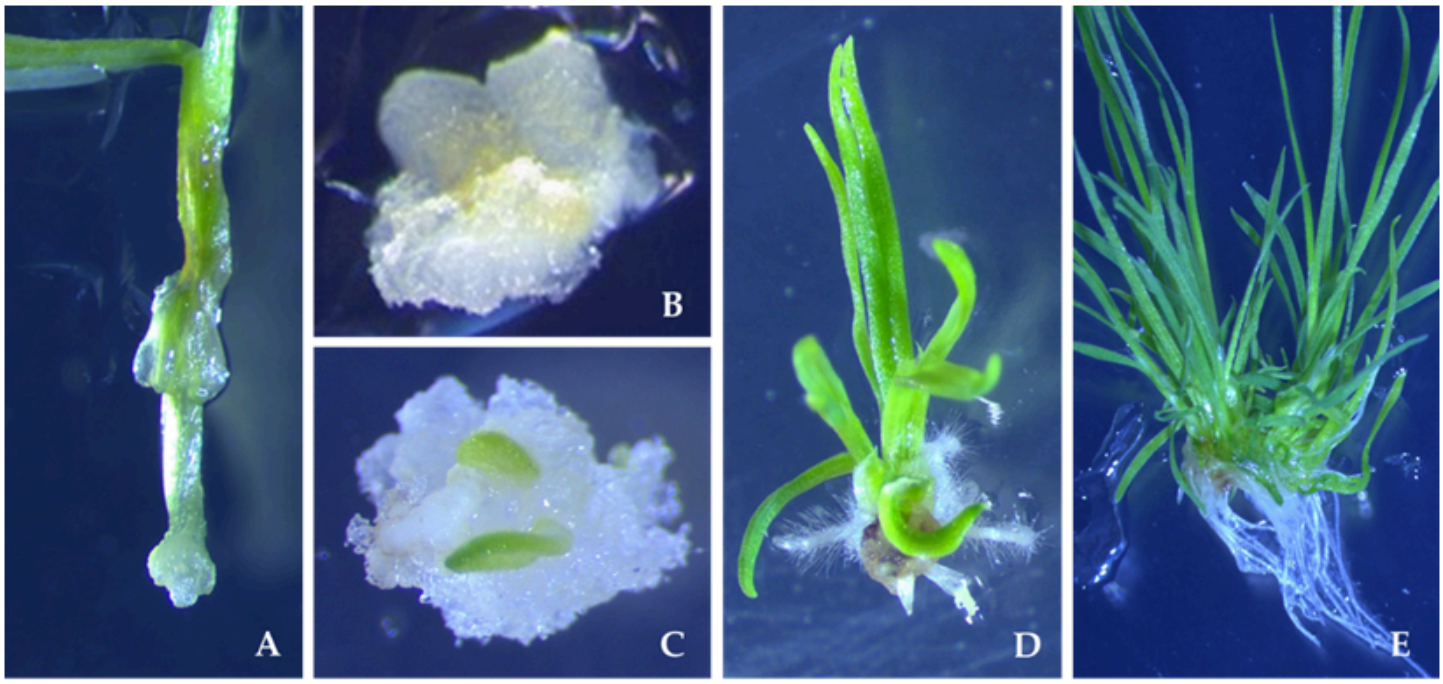

Figure 3

Callus induction and regeneration from coleoptile–mesocotyl explants. A) Explant placement on CIM supplemented with dicamba; (B) callus initiation at 12 days; (C) proliferating callus at 3 weeks; (D) embryogenic callus showing nodular structures; (E) shoot primordia emerging during regeneration; (F–G) regenerated plantlets with developing shoots and roots; (H) fully regenerated plantlet prior to acclimatization. Scale bars: 1 cm.