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In vitro somatic embryogenesis through pollen and ovule culture in Niger [*Guizotia abbyssinica* (L.f.) Cass.]

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Abstract
Niger [*Guizotia abyssinica* (L.f.) Cass.] is a self-incompatible minor oilseed crop, where conventional inbreeding is slow, making haploid production attractive for rapid line development. Pollen and ovule culture were standardized in genotype RCR-18 in the Department of Genetics and Plant Breeding, College of Agriculture, Raichur during the year 2025-26. Flower buds harvested before anthesis received three durations of cold pre-treatment (24, 48 and 72 hours at 4°C) and were cultured on 14 Murashige and Skoog based media varying in 2,4-D, kinetin and BAP concentration, in a factorial completely randomized design with three replications. Pollen, isolated by three methods, failed to divide or form callus under any treatment, indicating recalcitrance of the male pathway in this genotype. Ovule culture produced callus and gynogenic embryos on six media (M_9-M_{14}), all combining 2,4-D with BAP, among the 14 media combinations evaluated. Fourty eight hours of cold pre-treatment with MS media containing 2.0 mg/l of 2,4-D and 2.5 mg/l of BAP (T_2M_{11}) gave the highest callus formation (63.33 %) and somatic embryogenic response (48.89 %) in the shortest time (28 days). Ovule culture is thus the effective pathway in Niger, with T_2M_{11} identified as the reference treatment for future regeneration studies.

Keywords: *Guizotia abyssinica*, Niger, *In vitro*, somatic embryogenesis, pollen culture, ovule culture, cold pre-treatment

Introduction
Niger [*Guizotia abyssinica* (L.f.) Cass.] is an oilseed crop grown across India, Ethiopia and parts of East Africa, valued for its edible oil, its use as bird feed and a range of traditional medicinal applications. In India, the crop covers close to 83,400 hectares, concentrated in Odisha, Chhattisgarh and other states where it is grown mainly by resource-poor farmers on marginal land (Anon., 2024). Productivity remains low, held back by seed shattering, lodging and most importantly for breeders, a strong self-incompatibility system that makes conventional inbreeding slow and unreliable.

Hybrid and pure-line breeding in a self-incompatible crop depends on homozygous parental lines and forced selfing in Niger rarely delivers these within a practical number of generations. Doubled haploid technology offers a faster route: a haploid plant, once regenerated and chromosome-doubled, is fully homozygous in a single step, without the repeated cycles of selfing that conventional breeding requires. Anther culture has already been standardized for Niger in this laboratory (Anusha 2024) [2], but regenerants recovered from cultured anthers carry a persistent complication, since the anther wall contributes diploid tissue alongside the haploid microspores inside it, so every plantlet obtained must be checked for ploidy before it can be used with confidence.

Pollen culture and ovule culture avoid this ambiguity by excluding the surrounding diploid tissue at the point of inoculation. Pollen culture works through the male gametophyte, in which individual pollen grains, freed from the anther wall, are redirected from their normal maturation path toward division. Ovule culture works through the female side, in which an unfertilized ovule is induced to form callus and embryos without fertilization. Both routes have succeeded in other crops, most notably wheat (Wei 1982) [15] and barley for pollen culture (Wei *et al*. 1986) [16] and sugar beet (Lux *et al*. 1990) [10] and gentian for ovule culture (Doi *et al*. 2011) [5], but neither had previously been attempted in Niger.

The response of both explant types to *in vitro* culture depends heavily on the developmental stage at which they are harvested, the duration and severity of any stress pre-treatment applied beforehand and the concentration and identity of the auxin and cytokinin supplied in the medium (Touraev *et al*. 1997; Ferrie and Caswell 2011) [6, 14]. Cold pre-treatment of flower buds before culture is among the more consistently reported stress cues for triggering androgenic and gynogenic switches in other species, but the duration required is specific to each species and cannot be assumed to carry over from one genus to another.

The present study was undertaken to standardize a protocol for callus and embryo induction through pollen and ovule culture in Niger genotype RCR-18. Three durations of cold pre-treatment were combined factorially with fourteen MS-based media differing in 2,4-D concentration and in the cytokinin. The callus and embryogenic responses obtained were compared between the two explant types while analysis were performed separately.

Material and Methods
1. Plant material and growing conditions
Niger genotype RCR-18, developed and maintained at the University of Agricultural Sciences, Raichur, was raised in a greenhouse on a raised beds measuring 1.5 m × 10 m at a spacing of 30 cm × 10 cm. Sowing was staggered at ten-day intervals through the growing season so that flower buds of the required stage were available continuously for culture. Irrigation, weeding, fertilizer application and plant protection followed standard practice for the crop.

The crop was also grown in pots placed in the glasshouse to ensure continuous and uninterrupted supply of flower buds for culture. The biotic and abiotic factors adversely affecting the plant growth were avoided in this growing condition.

2. Explant collection and cold pre-treatment

The flower buds were harvested before anthesis, a stage at which the disc florets had not yet opened and the anthers had not dehisced. Harvested buds were subjected to cold pre-treatment at 4℃ for one of three durations before surface sterilization and culture: 24 hours (T_1), 48 hours (T_2) or 72 hours (T_3).

3. Culture medium

Murashige and Skoog (MS) basal medium was prepared from macronutrient, micronutrient, iron and vitamin stock solutions, with sucrose at 30 g/l and agar at 8 g/l and the pH adjusted to 5.8 before autoclaving at 121℃ for 15 to 20 minutes. Fourteen media (M_1 to M_{14}) were prepared by supplementing this basal medium with 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations ranging from 0.5–3.0 mg/l in factorial combination with kinetin at concentrations of 1.0 mg/l to 2.0 mg/l (M_1 to M_6) and 6-benzylaminopurine (BAP) at concentrations of 1.0 mg/l to 3.0 mg/l (M_7 to M_{14}) as summarized in Table 1.

Table 1: Media combinations evaluated for pollen and ovule culture

Sl. No.	Code	Medium composition
1	M_1	MS + 2,4-D (0.5 mg/l) + Kinetin (1.0 mg/l)
2	M_2	MS + 2,4-D (1.0 mg/l) + Kinetin (1.5 mg/l)
3	M_3	MS + 2,4-D (1.5 mg/l) + Kinetin (2.0 mg/l)
4	M_4	MS + 2,4-D (2.0 mg/l) + Kinetin (2.0 mg/l)
5	M_5	MS + 2,4-D (2.5 mg/l) + Kinetin (2.0 mg/l)
6	M_6	MS + 2,4-D (3.0 mg/l) + Kinetin (2.0 mg/l)
7	M_7	MS + 2,4-D (0.5 mg/l) + BAP (1.0 mg/l)
8	M_8	MS + 2,4-D (1.0 mg/l) + BAP (1.5 mg/l)
9	M_9	MS + 2,4-D (1.5 mg/l) + BAP (1.5 mg/l)
10	M_{10}	MS + 2,4-D (2.0 mg/l) + BAP (2.0 mg/l)
11	M_{11}	MS + 2,4-D (2.0 mg/l) + BAP (2.5 mg/l)
12	M_{12}	MS + 2,4-D (2.5 mg/l) + BAP (2.5 mg/l)
13	M_{13}	MS + 2,4-D (3.0 mg/l) + BAP (2.5 mg/l)
14	M_{14}	MS + 2,4-D (3.0 mg/l) + BAP (3.0 mg/l)

4. Pollen culture

Harvested flower buds, after the respective cold pre-treatments, were surface sterilized to ensure the contamination does not hinder the cultures. Sterilized flower buds were dissected under aseptic conditions and healthy anthers excised from the disc florets. Pollen was released by three methods, each run in parallel on the same set of media: (i) Release of pollen from anthers into sucrose solution followed by culture wherein healthy anthers were excised and the pollen was released into sterile sucrose solution by gently teasing the anthers followed by extraction and culture of the solution containing pollen (ii) Direct isolation of pollen grains followed by culture wherein the pollen were isolated from the anthers using needle and cultured onto the respective media and (iii) Crushing of anthers in sucrose solution followed by centrifugation at 800 rpm for five minutes, followed by culture of pollen bearing supernatant separately from the pelleted debris. Pollen obtained by each method, subjected to each cold pre-treatment, was cultured on all fourteen media in three replications, initially subjected to darkness until visible response was observed and further placed in a growth chamber with a 16 hours light and 8 hours dark photoperiod maintained at the temperature regime of 24 ℃ to 28℃.

5. Ovule culture

Surface-sterilized flower buds were opened aseptically and unfertilized ovules were excised from the disc florets with careful handling to avoid mechanical injury. Ovules from each cold pre-treatment were plated on all fourteen media in three replications, kept in darkness until callus initiation was observed and then moved to the 16 hours light and 8 hours dark under 24℃ to 28℃ temperature regime.

6. Maintenance of callus

Callus showing continued growth was sub-cultured at 21 days intervals, onto fresh medium of the respective composition to avoid the hinderance of the explant to undergo division and subsequent embryogenesis due to the exhaustion of nutrients and the plant growth regulators *viz*., 2,4-D, kinetin and BAP. The cultures were monitored for the appearance of embryogenic structures.

7. Observations recorded

The parameters explaining the effect of cold pre-treatment and media were recorded as the number of days from callus formation, the percentage of callus formation, the number of days for embryogenesis and the percentage of embryogenesis. The number of days for callus formation and number of days for embryogenesis were considered from the day of inoculation, while the percentage of callus formation and percentage of embryogenesis were calculated among the total number of explants cultured.

8. Statistical analysis

Data for pollen culture and ovule culture were analyzed separately, each as a two factorial completely randomized design with cold pre-treatment (three levels) and medium (fourteen levels) as factors, in three replications. Treatment means were compared by Duncan's Multiple Range Test following analysis of variance, standard errors and critical difference values were computed for each comparison.

Results

1. Pollen culture

None of the pollen cultures, across any of the three isolation methods, the three durations of cold pre-treatment or the fourteen media combinations, progressed beyond the culture phase to produce callus. Pollen grains degenerated within a few days of plating rather than dividing and no callus or embryo-like structure were recovered from any replicate. Ovules taken from the same flower buds, under the same sterilization, thermal and hormonal regime, gave a reproducible response (Section 3.2), so the absence of a pollen response is attributable to the pollen system itself rather than to a general fault in aseptic technique or ambient culture conditions.

2. Ovule culture: callus formation

The analysis of variance indicated that cold pre-treatment, medium and their interaction had highly significant effects on both days to callus formation and percentage of callus

formation (Table 2, P<0.01). Ovules produced callus only on the six media combinations with 2,4-D and BAP at the higher concentration range tested (M_9 to M_{14}) (Table 4), the six 2,4-D + kinetin media (M_1 to M_6) and the two lowest 2,4-D + BAP combinations (M_7 and M_8) produced no callus under any pre-treatment.

Of the three cold pre-treatment durations, forty-eight hours (T_2) gave the best result, with 29.81 per cent callus formation in a mean of 15.00 days compared to 24 hours (28.61 % in 15.80 days) and 72 hours (23.06 % in 16.25 days) as indicated in Table 3.

Among the six responsive media, callus formation was highest on the media combination M_{11} (2,4-D 2.0 mg/l + BAP 2.5 mg/l) at 47.41 per cent in the shortest mean time of 14.0 days and declined on either side of this combination, falling to 3.33 per cent on the media containing lowest concentration of BAP *i.e.*, M_9, and back down to 3.33 per cent on the highest concentration medium M_{14} (Table 4).

The interaction between cold pre-treatment and medium was significant for both parameters. The combination of 48 hours of cold pre-treatment with M_{11} (T_2M_{11}) produced 63.33 per cent of callus formation in 14.00 days, when compared to the same medium under 24 hours of cold pretreatment (46.67 %) or 72 hours of cold pre-treatment (32.22 %) and better results than every other treatment combination in the experiment (Table 5).

3. Ovule culture: Gynogenic somatic embryogenesis

Organization of callus into embryo-like structures followed a pattern similar to callus formation. Cold pre-treatment, medium and their interaction again had highly significant effects on the number of days to embryogenesis and percentage of embryogenesis (Table 2, P<0.01). The 48 hours of cold pre-treatment gave the highest embryogenic response with 22.44 per cent in a mean of 30.00 days, against 16.67 per cent (31.60 days) under 24 hours and 13.61 per cent (32.5 days) under 72 hours of cold pre-treatment (Table 3).

Media combination M_{11} outperformed the other responsive media with 35.56 per cent of embryogenesis in a mean of 14.00 days, compared with a maximum of 22.22 per cent on M_{12} and a negligible response on M_9 and M_{14} (Table 4). The residual, non-embryogenic fraction of the callus, tissue that proliferated without organizing into embryo-like structures, was highest on M_{10} (14.44 %) and lowest on M_9 and M_{14}.

The strongest interaction and the best response recorded in the study for callus formation and embryogenesis was 48 hours of cold pre-treatment combined with the media combination M_{11} (T_2M_{11}) that led 48.89 per cent of cultured ovules to produce embryo-like structures (Fig. 2a-c) within 28 days (Table 5), over the same medium under 24 hours (30.00 %) or 72 hours (27.78 %) of cold pre-treatment as depicted in Fig. 1a-i.

Table 2: Analysis of variance for callus formation and gynogenic embryogenesis from cultured Niger ovules

Source of variation	Degree of freedom	Days to callus formation (MSS)	Percentage of Callus formation (MSS)	Days to embryogenesis (MSS)	Percentage of Embryogenesis (MSS)
Cold pre-treatment (T)	2	33.64**	434.22**	134.57**	198.68**
Medium (M)	13	461.14**	2228.90**	1844.57**	1046.15**
T × M	26	41.49**	84.64**	165.96**	44.83**
Error	84	0.02	0.44	0.10	0.18
Total	125	–	–	–	–

**Significant at 1% level of significance.

Table 3: Effect of duration of cold pre-treatment on callus formation and gynogenic embryogenesis from cultured Niger ovules

Cold pre-treatment	Days to callus formation (d)	Percentage of callus formation (%)	Days to embryogenesis (d)	Percentage of embryogenesis (%)
T_1 (24 h)	15.80 ^{b}	28.61 ^{b}	31.60 ^{b}	16.67 ^{b}
T_2 (48 h)	15.00 ^{a}	29.81 ^{a}	30.00 ^{a}	22.44 ^{a}
T_3 (72 h)	16.25 ^{c}	23.06 ^{c}	32.50 ^{c}	13.61 ^{c}
SE(m)±	0.02	0.10	0.05	0.06
CD (1%)	0.09	0.38	0.18	0.24

Means followed by the same letter within a column do not differ significantly.

Table 4: Effect of medium composition on callus formation and gynogenic embryogenesis from cultured Niger ovules

Medium	Days to callus formation (d)	Percentage of callus formation (%)	Days to embryogenesis (d)	Percentage of embryogenesis (%)
M_9	18.00 ^{e}	3.33 ^{e}	18.00 ^{e}	0.00 ^{f}
M_{10}	16.33 ^{d}	22.22 ^{d}	16.33 ^{d}	7.78 ^{d}
M_{11}	14.00 ^{a}	47.41 ^{a}	14.00 ^{a}	35.56 ^{a}
M_{12}	15.33 ^{c}	33.33 ^{b}	15.33 ^{bc}	22.22 ^{b}
M_{13}	16.33 ^{d}	23.33 ^{c}	16.33 ^{d}	11.11 ^{c}
M_{14}	15.00 ^{b}	3.33 ^{e}	15.00 ^{b}	3.33 ^{e}
SE(m)±	0.05	0.22	0.10	0.06
CD (1%)	0.19	0.83	0.38	0.24

Note: The media combinations M_1 to M_8 gave no response under any level of cold pre-treatment.

Means followed by the same letter within a column do not differ significantly.

Table 5: Interaction effect of cold pre-treatment × medium on callus formation and gynogenic embryogenesis from cultured Niger ovule

Treatment	Days to callus formation (d)	Percentage of callus formation (%)	Days to embryogenesis (d)	Percentage of embryogenesis (%)
T_1M_9	–	0.00 [l]	–	0.00 [j]
T_1M_{10}	16 [c]	16.67 [i]	32 [bc]	3.33 [i]
T_1M_{11}	14 [a]	46.67 [b]	28 [a]	30.00 [b]
T_1M_{12}	15 [b]	27.78 [e]	30 [ab]	23.33 [d]
T_1M_{13}	18 [e]	23.33 [g]	36 [d]	10.00 [g]
T_1M_{14}	–	0.00 [l]	–	0.00 [j]
T_2M_9	18 [e]	3.33 [k]	–	0.00 [j]
T_2M_{10}	15 [b]	30.00 [d]	30 [ab]	13.33 [f]
T_2M_{11}	14 [a]	63.33 [a]	28 [a]	48.89 [a]
T_2M_{12}	14 [a]	46.67 [b]	28 [a]	30.00 [b]
T_2M_{13}	15 [b]	32.22 [c]	30 [ab]	16.67 [e]
T_2M_{14}	14 [a]	3.33 [k]	28 [a]	3.33 [i]
T_3M_9	–	0.00 [l]	–	0.00 [j]
T_3M_{10}	18 [e]	20.00 [h]	36 [d]	6.67 [h]
T_3M_{11}	14 [a]	32.22 [c]	28 [a]	27.78 [c]
T_3M_{12}	17 [d]	25.56 [f]	34 [cd]	13.33 [f]
T_3M_{13}	16 [c]	14.44 [j]	32 [bc]	6.67 [h]
T_3M_{14}	–	0.00 [l]	–	0.00 [j]
SE(m)±	0.09	0.38	0.18	0.24
CD (1%)	0.33	1.43	2.77	0.90

Note: The media combinations M_1 to M_8 gave no response under any level of cold pre-treatment.
Means followed by the same letter within a column do not differ significantly.

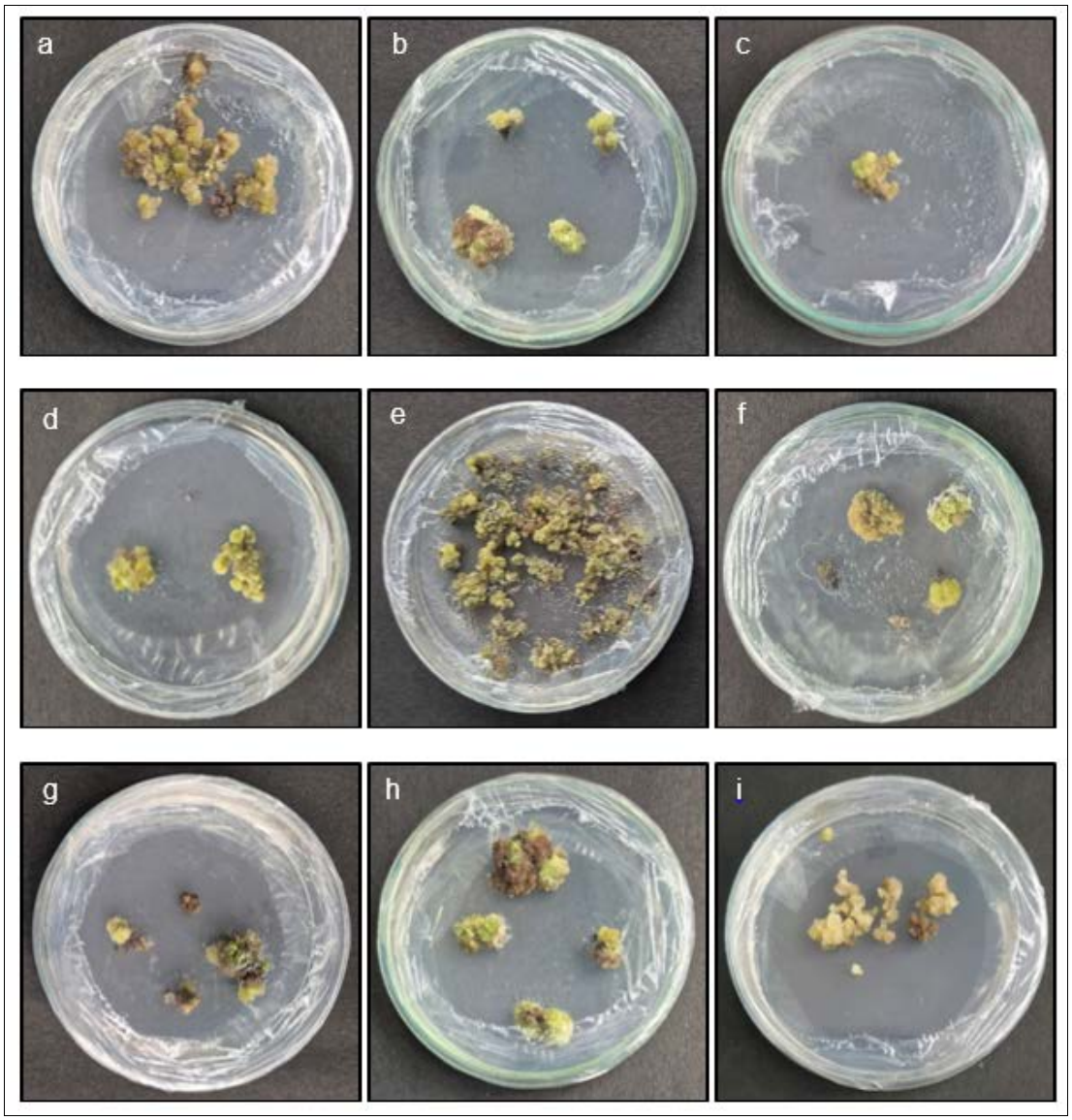


Fig 1: Embryogenesis through ovule culture from flower buds cold pre-treated for the treatment combinations: a T_1M_{11}, b T_1M_{12}, c T_1M_{13}, d T_2M_{10}, e T_2M_{11}, f T_2M_{12}, g T_2M_{13}, h T_3M_{11}, i T_3M_{12}

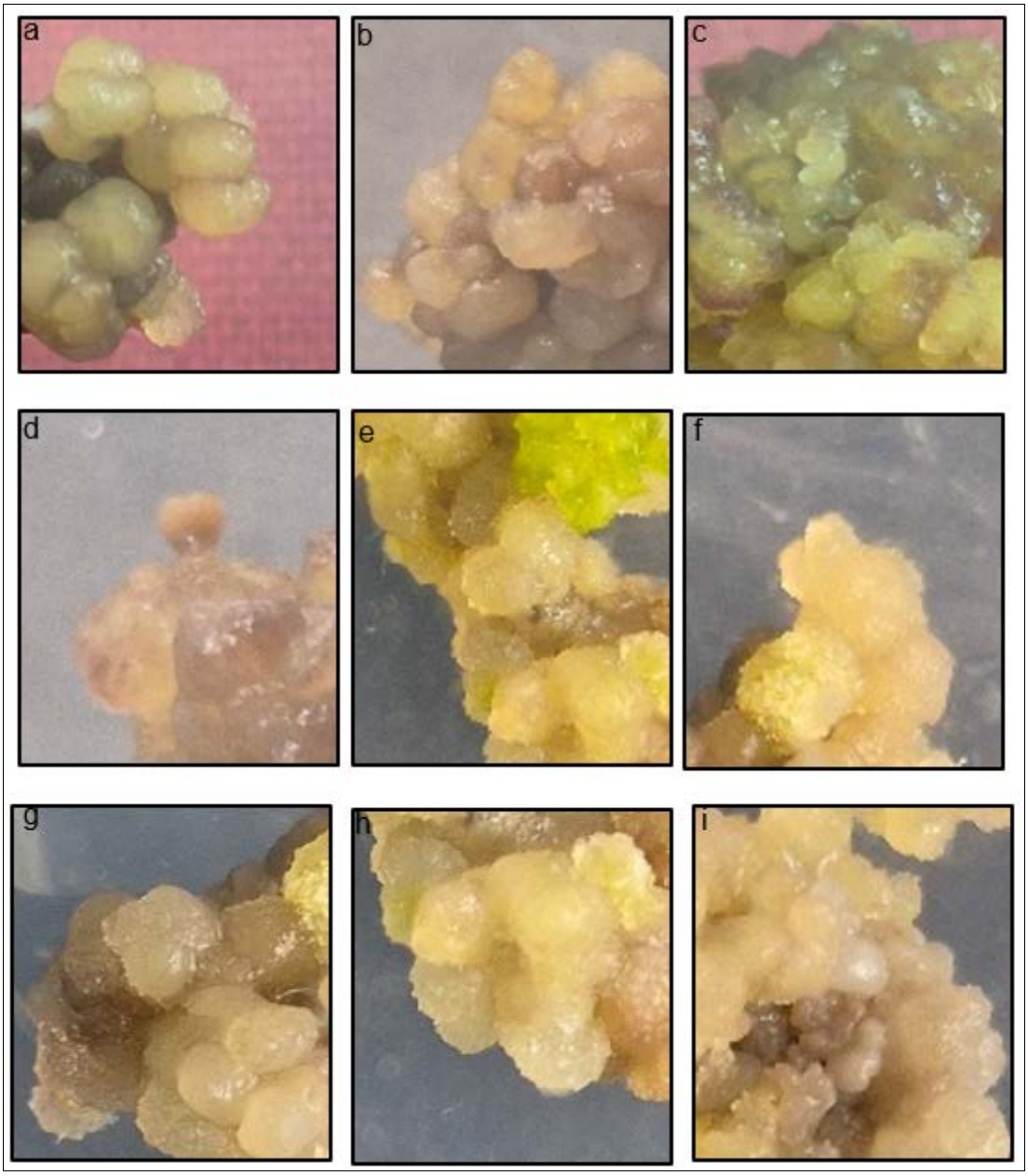


Fig 2: Embryogenic callus derived from ovule culture: a-c globular stage, d-f heart stage, g-i torpedo stage

Discussion

1. Pollen culture

Androgenic competence, the capacity of pollen to switch from its normal maturation path to a dividing phase, is inherently variable across taxa and appears modest within the Asteraceae relative to cereals such as wheat and barley, where most successful androgenesis protocols have been developed (Wei *et al*., 1986; Ferrie and Caswell, 2011) [6, 16]. Flower buds in this study were harvested at a single, chronologically defined stage without cytological confirmation of microspore nuclear stage; because floret maturation on the Niger capitulum proceeds unevenly, this assumption may have placed a meaningful share of the sampled pollen outside the narrow uninucleate to early bicellular window in which reprogramming was possible (Datta and Wenzel 1987) [4].

The cold pre-treatment durations tested were carried over from those validated for ovule culture. Protocols that have successfully induced androgenesis in cereals have generally used substantially longer or more intense stress exposure, running to several weeks in wheat (Datta and Wenzel 1987) and rice (Gao *et al*. 2024) [4, 7], while isolated pollen culture needed a proportionately stronger stress signal to achieve the same reprogramming effect. The protocol that has not been standardized for pollen culture with respect to the media and the effective pre-treatment or thermal stress limit the success of the culture significantly due to a very highly specific response of the explant to the pre-treatment, media and growth conditions. The combined effect of these factors accounted for part of the non-responsiveness of pollen culture while taken together, they offer a more complete explanation than aseptic failure or unsuitable ambient conditions, both of which are ruled out by the concurrent success of ovule culture from the same flower buds.

2. Cold pre-treatment

The 48 hours of cold pre-treatment outperformed both the shorter and the longer duration for every parameter *viz*., number of days for callus formation, percentage of callus formation, number of days for embryogenesis and percentage of embryogenesis. A pre-treatment that is too brief did not deliver enough stress to redirect ovule cells

away from their normal developmental path, while one that runs too long risked the same stress and led to damage rather than reprogramming, due to a decline in membrane integrity and cell viability. A defined intermediate optimum of this kind has already been reported for cold conditioning of ovule cultures in sugar beet (Lux *et al*. 1990 [10]; Gürel *et al*. 2000) and gentian (Doi *et al*. 2011) [5], and the present result places Niger within that same pattern rather than as an exception to it.

3. Media composition

Callus and embryo formation increased and then fell as the concentration of 2,4-D and BAP increased through the M_9 to M_{14} series, a pattern consistent with a defined hormonal window rather than a simple dose response relationship in which lower concentrations of hormones (M_9) failed to trigger a response while too higher (M_{13}, M_{14}) became inhibitory rather than stimulatory. The complete failure of every 2,4-D + kinetin combination is a sharper finding. Bhat and Murthy (2008) [3] obtained a gynogenic response from Niger ovules using kinetin, but paired it with NAA rather than 2,4-D; the present results suggest that the stronger dedifferentiating action of 2,4-D specifically requires the more potent cytokinin activity of BAP to be channelled into organized callus formation, an interaction that kinetin could not support in this tissue. A similarly narrow dependence on the type and not just the concentration of the auxin and cytokinin pairing has been reported for ovule culture in *Gerbera jamesonii* (Sitbon 1981; Miyoshi and Asakura 1996) [11, 12].

4. Interaction between cold pre-treatment and media

The factors alone did not account for the strongest result recorded in this study. The most responsive medium (M_{11}) still gave a markedly lower response under a sub-optimal duration of cold pre-treatment while the optimal duration of pre-treatment could not raise the least responsive medium (M_9) to a useful level. This points to a synergistic, rather than additive, relationship between cold pre-treatment and hormonal environment in triggering gynogenic competence in Niger ovules, a similar pattern documented for isolated ovule and microspore systems in other species of rice (Gao *et al*. 2024) [7] and bread wheat (Springer *et al*. 2025) [13].

Conclusion

Cold pre-treatment for 48 hours combined with MS medium containing 2,4-D (2.0 mg/l) and BAP (2.5 mg/l) gave the highest percentage of callus and embryo from unfertilized Niger ovules among the conditions tested and offers a practical starting point for further development of a gynogenic haploid protocol in this crop. Isolated pollen culture, tested under the same range of pre-treatments and on the same fourteen media, did not respond, indicating that androgenesis in Niger will require a standardization of microspore stage for culture, pollen extraction and media combination.

Statements & Declarations

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