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A Half-diallel *In vitro* Androgenesis Study Using Drought- and Yield-Adapted Eggplant Parents

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Abstract

Anther culture-based doubled haploid (DH) technology offers an efficient approach for accelerating homozygosity in eggplant, although its application is often limited by strong genotype dependency and inconsistent regeneration. In this study, a set of F_1 hybrids developed through a half-diallel mating design using drought-tolerant and high-yielding parental lines were evaluated for androgenic responsiveness under controlled *in vitro* conditions. Significant variation was observed among crosses for embryo-like structure (ELS) induction, green plantlet regeneration, and the composite Androgenic Index (AI). The diallel matrix revealed that several combinations, particularly RWE P11-2 × BP20-3**,** BP20-3 × RWE ©**,** B. Baria × BP20-3**,** and 3 (8) × BP20-3**,** showed superior ELS formation and regeneration efficiency, while albino production remained minimal across the population. Ranked AI values and Tukey groupings further identified RWE P11-2 × BP20-3 as the most promising cross for DH production. The results demonstrate strong parental effects on androgenesis, with BP20-3 and RWE P11-2 functioning as consistently responsive parents. Overall, this study highlights the potential of drought-adapted eggplant germplasm for efficient DH line development and provides valuable insights for integrating half-diallel-based selection with haploid breeding strategies.

Introduction

Eggplant (*Solanum melongena* L.) is an economically important vegetable crop widely cultivated across tropical and subtropical regions, including Bangladesh. The crop contributes significantly to nutritional security, smallholder income, and year-round

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vegetable supply. However, hybrid seed production and varietal development in eggplant remain constrained by its high degree of heterozygosity, severe inbreeding depression, and long breeding cycle, all of which hinder efficient genetic improvement (Khan and Isshiki 2018). In recent years, doubled haploid (DH) technology has emerged as a powerful tool to overcome these obstacles by enabling the rapid production of completely homozygous lines within a single generation, thereby accelerating hybrid development and genetic analysis (Seguí-Simarro 2015, Kumar et al. 2023).

Among the DH methods, anther culture is the most widely applied in Solanum species because it allows direct recovery of haploid microspore-derived embryos and regenerants. Successful androgenesis, however, is strongly genotype-dependent and influenced by microspore embryogenic competence, stress responsiveness, and regeneration potential (Corral-Martínez and Seguí-Simarro 2014). Eggplant, in particular, is known for high variability in ELS formation, high albino frequency, and inconsistent green plant regeneration, making the identification of responsive parents essential for improving DH-based breeding efficiency (Salas et al. 2012, Metwally et al. 2021).

Previous studies have documented significant differences among eggplant genotypes in callus induction, embryo-like structure (ELS) production, and green plant recovery, especially under abiotic stresses such as drought, salinity, and temperature fluctuations (Sharma and Millam 2016, Devi et al. 2022). Therefore, parental selection plays a critical role in determining androgenic success. The parental lines used in the present study were selected based on earlier evaluations demonstrating high yield potential and enhanced drought tolerance. Such drought-adapted genotypes have been identified as promising materials for both stress-responsive breeding and *in vitro* androgenesis (Farzana et al. 2021).

Despite the availability of androgenic protocols, limited information remains on how drought-tolerant and high-yielding parents behave in a diallel crossing framework, especially regarding ELS induction, albino formation, and green plantlet regeneration. Diallel analysis provides valuable insights into parental effects, heterotic patterns, and cross combinations influencing microspore embryogenesis (Ramesh and Gopal 2020). In addition, the use of quantitative indices such as ELS percentage, green regeneration efficiency, and green plantlets per anther helps to identify developmental bottlenecks across stages of androgenesis (Mitykó et al. 2019, Niu et al. 2022).

In this study, we conducted a systematic evaluation of androgenic responsiveness in a set of half-diallel F_1 eggplant hybrids derived from drought-tolerant and high-yielding parents. The experiment assessed multiple developmental stages, including ELS production, albino incidence, and green plantlet regeneration under controlled *in vitro* conditions. A composite Androgenic Index (AI) integrating early and late-stage responses was used to identify the most efficient cross combinations. The findings are expected to guide the selection of elite parents and F_1 combinations for DH production and support future breeding strategies aimed at developing stress-resilient eggplant varieties in Bangladesh and beyond.

Materials and Methods

A set of eggplant (*Solanum melongena* L.) parental lines, identified as high-yielding and drought-tolerant in one of our earlier experiments, were used to develop experimental materials. Half-diallel crosses were performed among eight selected lines following standard emasculation and hand-pollination procedures (Acquaah 2012). Mature fruits were harvested at physiological maturity, and seeds were surface-sterilized and germinated in seedling trays. Healthy, vigorous F_1 plants were grown under natural photoperiod.

Flower buds containing predominantly uninucleate to very early binucleate microspores-the most responsive stages for androgenesis were selected based on sepal-petal morphology as described in the classical eggplant protocol by Rotino (2016). Buds approximately equal in height between fused sepal tips and petal tips were harvested early in the morning and immediately brought to the laboratory.

For sterilization, buds were washed in sterile water containing a drop of Tween-20, treated for 30s in 70% ethanol, and surface-sterilized for 20 min in 30% commercial bleach. Buds were then rinsed three times in sterile distilled water.

Inside a laminar-flow hood, the petal cone was removed using a transverse cut below the anther filament, followed by a longitudinal cut along the sepal to expose the anther cone. Individual anthers were excised by gently pushing them from the base without squeezing, a critical step to avoid callus-type responses.

Ten to twelve anthers were placed, convex (external) side down, on Petri dishes containing standard induction medium C, prepared according to Rotino (2016) with slight modification. Plates were sealed with parafilm and incubated at 35 °C in complete darkness for 8 days, a high-temperature shock known to enhance embryogenesis in eggplant anther cultures.

Culture Media: The following three media were used:

1. Induction medium (C): MS-based salts with 30 g/l sucrose, 8 g/l agar, and a genotype-responsive PGR combination (Rotino 2016).
2. Regeneration medium (R): Cytokinin-based medium (R1K or R1Z) corresponding to the induction medium type.
3. Plantlet development medium (V3): PGR-free MS medium with standard MS vitamins.

All media were adjusted to pH 5.8 before autoclaving at 121°C for 20 min.

After the 8-day high-temperature pretreatment, cultures were transferred to a growth room at 25°C, 16 hrs photoperiod. On day 13, anthers were transferred to regeneration medium (R). Subculture onto fresh regeneration medium was carried out every 5-6 weeks until embryo emergence.In this periodEmbryo-like structures (ELS), albino plantlets, and green regenerants were counted across genotypes following the scoring criteria described by Rotino (2016). Embryos larger than 4-6 mm were transferred to V3 medium for rooting and shoot elongation.

Rooted plantlets with well-developed foliage were gently washed to remove residual agar and transplanted into a soil mixture consisting of soil : cowdung at a 1 : 1 ratio. Hardened plants were shifted to shade until full establishment.

For each cross, the following parameters were recorded: ELS, albino plantlets, green regenerants, regeneration efficiency, green plantlets and Androgenic Index (AI).

AI was calculated as:

$$AI = \log_{10}(1 + \text{ELS}) + \log_{10}(1 + \text{green regenerants}) - \log_{10}(1 + \text{Albinos})$$

The experiment followed a completely randomized design (CRD). Data were analyzed using ANOVA in R. Tukey's HSD test ($p \leq 0.05$) was used for pairwise comparison. Bubble plots, correlation heatmaps, and ranked bar plots were generated using *ggplot2* and *reshape2*. Significance lettering was added using *multcomp*.

Results and Discussion

Highly significant variation was observed among the F_1 hybrids in embryo-like structure (ELS) induction, highlighting the strong genotype-dependence characteristic of eggplant androgenesis. The half-diallel matrix (Fig. 1) showed that certain combinations, particularly RWE P11-2 × BP20-3, BP20-3 × RWE ©, B. Baria × BP20-3, and 3 (8) × BP20-3, produced substantially higher ELS numbers than other crosses. These superior responses align with earlier reports of wide genotypic variation in microspore embryogenesis in eggplant and related Solanaceae species (Khan and Isshiki 2018, Ramesh and Gopal 2020). The consistently strong performance of BP20-3 as a male parent suggests the presence of alleles that promote early microspore reprogramming, a pattern also noted in studies by Metwally et al. (2021) and Seguí-Simarro (2015), who emphasized parental influence as a central determinant of androgenic competence.

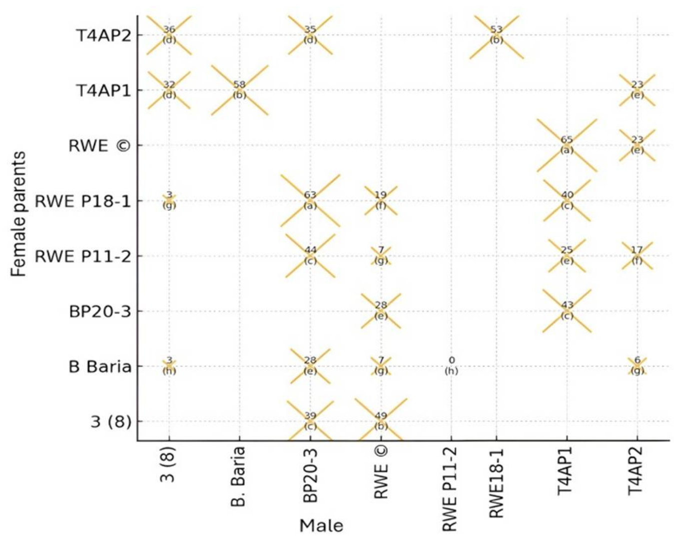

Fig. 1. Bubble plot showing ELS induction across half-diallel combinations with Tukey HSD grouping.

Albino formation remained exceptionally low across the half-diallel population (Fig. 2). Most hybrids produced either no albinos or only a single regenerant, and Tukey groupings revealed no significant differences among most crosses. This is encouraging, as albinism has traditionally been one of the most serious bottlenecks to eggplant doubled-haploid production (Mennella et al. 2020). The low frequency recorded here may be attributed to both the genetic background of the parents several being drought-tolerant and the regeneration medium used, which has previously been shown to reduce plastid degeneration and improve chlorophyll differentiation (Corral-Martínez and Seguí-Simarro 2014).

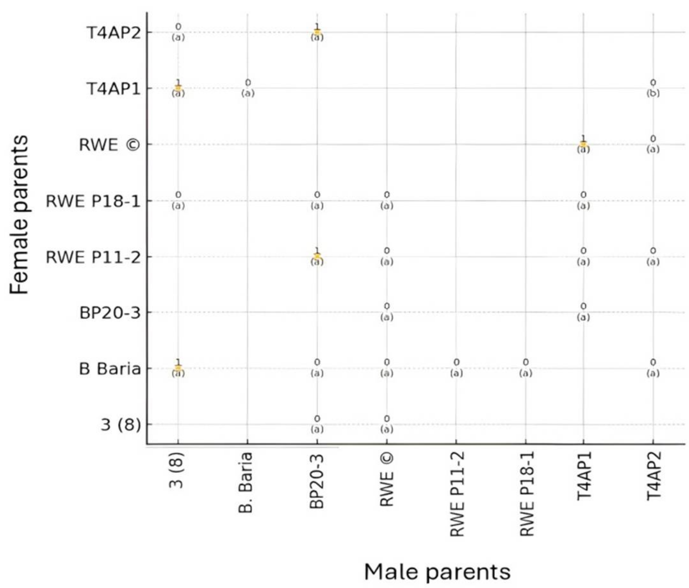

Fig. 2. Bubble plot showing production of albino plantlets across half-diallel combinations with Tukey HSD grouping.

Green plantlet regeneration (Fig. 3) exhibited moderate variability and was more discriminative than albino formation. Crosses involving RWE P11-2, BP20-3, and B. Baria displayed superior regeneration efficiency, whereas hybrids containing RWE © or RWE-P11-2 showed markedly lower conversion rates. The best-performing hybrid, RWE P11-2 × BP20-3, not only produced high ELS numbers but also maintained strong conversion into viable green regenerants, making it the most promising material for doubled haploid production. This aligns with findings in earlier eggplant studies showing that green regeneration efficiency is more influenced by genotype than by culture medium composition (Salas et al. 2012, Taha et al. 2019). The separation between high ELS induction and weak green conversion in certain hybrids, such as T4AP1 × BP20-3, reflects the developmental bottlenecks described previously in Solanaceae androgenesis (Corral-Martínez and Seguí-Simarro 2014).

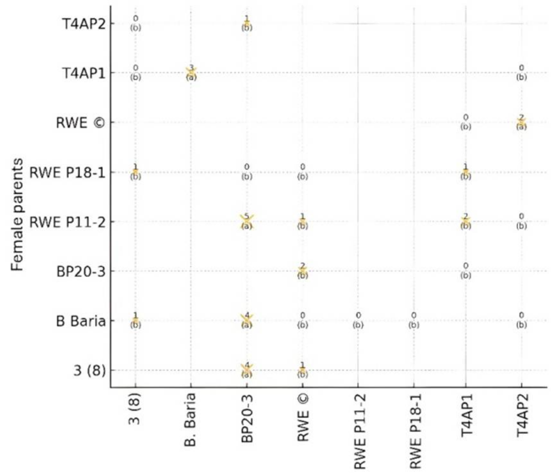

Fig. 3. Bubble plot showing regeneration of green plantlets regeneration with Tukey HSD grouping across the half-diallel combinations.

The composite Androgenic Index (AI), which integrates ELS/anther and green plantlets/anther, provided a more holistic assessment of androgenic responsiveness. The ranked AI distribution (Fig. 4) separated the hybrids into distinct performance classes, with RWE P11-2 × BP20-3, BP20-3 × RWE ©, B. Baria × BP20-3, and 3 (8) × BP20-3 forming the top-performing group. On the opposite end, combinations such as B. Baria × RWE P11-2, RWE © × T4AP1, and T4AP1 × T4AP2 consistently showed weak androgenic potential.

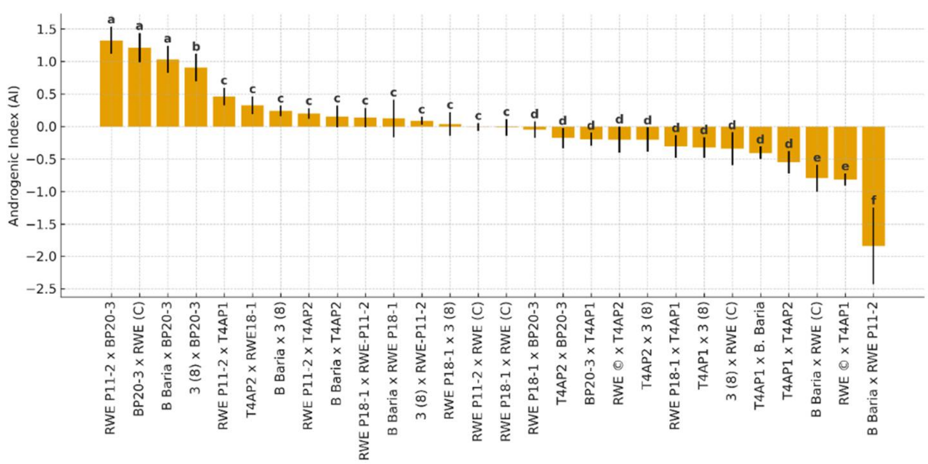

Fig. 4. Ranked bar plot of the Androgenic Index of F_1 hybrids.

The overall progression of androgenesis from cultured anthers to fully acclimatized plantlets is shown in Fig. 5, illustrating the sequential steps of anther culture, ELS formation, green shoot regeneration, and successful establishment of plantlets in soil.

Fig. 5. Developmental progression of eggplant androgenesis *in vitro*: (a) freshly excised anthers placed on the ELS induction medium initiating androgenesis, (b) formation of embryo-like structures (ELS) and emergence of early green shoots during the induction-regeneration transition, (c) regenerated green plantlets developing shoots and roots on regeneration medium in culture flasks, and (d) successfully acclimatized plantlet transferred to soil and exhibiting healthy vegetative growth.

Collectively, the results confirm that androgenic traits in eggplant ELS induction, albino frequency, green regeneration, and AI are strongly genotype-dependent and follow distinct parental patterns within the half-diallel combination. The consistently superior performance of parents such as BP20-3 and RWE P11-2 indicates that certain genotypes inherently possess a higher *in vitro* responsiveness, as also noted for other Solanaceae crops (Sharma and Millam 2016, Devi et al. 2022). The identification of RWE P11-2 × BP20-3 as the most responsive hybrid is particularly relevant for breeding programs aiming to accelerate homozygosity via doubled-haploid technology. These findings reinforce the importance of strategic parental selection and demonstrate that half-diallel-based assessment of *in vitro* traits can serve as a valuable complementary tool in hybrid development pipelines.

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