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Influence of Successive Subcultures and Hormone Concentrations on Somaclonal Variation in Tissue-Culture-Derived Pineapple (*Ananas comosus* var. MD2)

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ABSTRACT

Somaclonal variation is a major limitation in the micropropagation of pineapple (Ananas comosus var. MD2), as it reduces clonal fidelity and impacts commercial planting material. This study investigated the influence of successive subcultures and plant growth regulator (PGR) concentrations on morphological variation. Successive subcultures showed no abnormalities up to the 8th passage, but abnormal plantlets began to emerge at the 10th subculture (0.30%) and progressively increased to 0.90% at the 14th subculture. At the 7th subculture, increasing benzylaminopurine (BAP) concentrations caused a dose-dependent rise in abnormalities: 0% at 1 mg L^{-1}, 0.20% at 5 mg L^{-1}, 1% at 7.5 mg L^{-1}, and 3% at 10 mg L^{-1}. Higher BAP levels also shifted plant morphology from predominantly spineless to spiny forms, with full-margin spines reaching 12% at 10 mg L^{-1}. Abnormalities included stunted shoots, distorted leaves, and spiny margins. These findings indicate that extended subculturing and supra-optimal hormone levels induce somaclonal variation, compromising true-to-type regeneration. Limiting subculture cycles (≤8 passages) and maintaining lower PGR concentrations (around 1 mg L^{-1}) are recommended to preserve clonal fidelity in MD2 pineapple micropropagation.

INTRODUCTION

Pineapple (*Ananas comosus* L. Merr.) is one of the most economically important tropical fruits cultivated worldwide, with the MD2 variety being favored for its superior quality, sweetness, and global consumer demand. Commercial propagation of pineapple is traditionally constrained by the limited availability of suckers and crowns, making tissue culture an indispensable tool for large-scale production of uniform planting material. However, a major

challenge of tissue culture-based propagation is the occurrence of somaclonal variation, which can result in phenotypic instability and reduced field performance of regenerated plants (Krishna et al., 2016; Bairu et al., 2011).

Somaclonal variation arises from genetic and epigenetic modifications induced during extended periods of in vitro culture. Repeated subculturing, exposure to plant growth regulators (PGRs), and physiological stress have all been implicated in the induction of chromosomal rearrangements, mutations, and DNA methylation changes (Miguel & Marum, 2011; Khan et al., 2011). In pineapple, such variations often manifest as changes in leaf morphology, including spine formation, stunted growth, or distorted shoots, which reduce their desirability for cultivation and consumer markets (Liu et al., 1989). While earlier studies in banana and sugarcane have demonstrated that the frequency of somaclonal variation increases with prolonged subculturing and higher hormone exposure (Khan et al., 2011; Taylor et al., 1995), limited systematic data exist for MD2 pineapple. Understanding the relationship between subculture cycles, PGR concentration, and morphological variation is critical for optimizing micropropagation protocols that ensure genetic fidelity while minimizing off-types. This study was therefore conducted to (i) determine the effect of successive subcultures on abnormality incidence in MD2 plantlets, and (ii) evaluate the influence of different BAP concentrations on plantlet morphology during the 7th subculture stage.

MATERIALS AND METHODS

Plant Material and Explant Preparation

Pineapple suckers of MD2 variety, approximately 25–30 cm in length, were collected as the starting material. After removing outer leaves, suckers were trimmed to 5–8 cm, washed under running tap water with detergent, and soaked for 1 h in 5% (w/v) Benlate fungicide solution. The suckers were then rinsed with sterile distilled water. Under aseptic conditions in a laminar flow cabinet, the material was surface sterilized by immersion in 50% (v/v) Clorox™ (5.25% sodium hypochlorite) for 15 min, followed by 20% (v/v) Clorox™ for 10 min, with three sterile water rinses between treatments. The sterile cores were cut into 1–2 cm explants following the method of Zuraida et al. (2018).

Culture Medium and Subculture Cycles

Explants were cultured on Murashige and Skoog (MS) basal medium supplemented with 30 g L^{-1} sucrose, 5 mg L^{-1} benzylaminopurine (BAP), and 0.3% agar. Cultures were maintained at 25 °C under a 12 h light/12 h dark photoperiod using cool-white fluorescent lamps. Transfers to fresh medium were carried out every four weeks for three months.

Microshoots that developed were transferred to liquid MS medium containing 30 g L^{-1} sucrose, 1 mg L^{-1} BAP, and 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and maintained with monthly subculturing for another three months. Protocorm-like bodies (PLBs) were bulked in 250 mL Erlenmeyer flasks on a rotary shaker at 120 rpm and further propagated through 6–15 successive subcultures until robust plantlets were obtained.

Abnormalities were monitored at the 6th, 8th, 10th, 12th, and 14th subcultures. Morphological variations, including distorted shoots, stunted growth, and abnormal leaf formation, were recorded (Figure 1A), while normal plantlets (Figure 1B) were selected for acclimatisation and

field evaluation. Normal plantlets were transplanted to the field to evaluate spine distribution after 10 months of growth, across subculture cycles 6–15. The percentage of tissue-culture-derived pineapple plants exhibiting full-margin spines was recorded (Figure 2D).

Hormone Concentration Experiment

To evaluate the effect of plant growth regulator levels, protocorm-like bodies (PLBs) were cultured on MS medium supplemented with different concentrations of BAP (1, 5, 7.5, and 10 mg L^{-1}). Cultures were subcultured monthly until the 7th passage. At this stage, plantlets were assessed for morphological abnormalities such as stunted growth, twisted shoots, and distorted leaf development (Figure 1A).

Morphologically normal plantlets (Figure 1B) obtained from each treatment were transferred to the field. After 10 months of growth, leaf spine distribution was recorded and classified into five categories:

- A = spines at the base only
- B = spines at the tip only
- C = spines at both base and tip
- D = spines along the entire leaf margin
- Spineless = no spines present (Figure 2)

Additional field observations included the incidence of abnormal morphologies such as stunted growth, twisted shoots, and leaf distortion.

Acclimatisation

After in vitro culture, plantlets were carefully removed from their vessels and rinsed with sterile water to eliminate residual medium. They were transplanted into soil-filled polybags containing either sterilised peat moss alone or a peat moss–topsoil mixture (1:1). During the initial hardening stage, plantlets were maintained in a greenhouse under high humidity (>85%), moderate temperatures (24–26 °C), and low-intensity light (~50 μmol m^{-2} s^{-1}) for 1–2 weeks to minimise transplant shock. Substrate moisture was maintained by regular misting, avoiding waterlogging. Following this primary hardening phase, humidity was gradually reduced by opening vents or lowering plastic covers, while light intensity was increased stepwise over 7–10 days to promote cuticular development and stomatal regulation.

Data Collection and Analysis

The percentage of abnormal plantlets was calculated for each treatment. Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software to compare treatment responses.

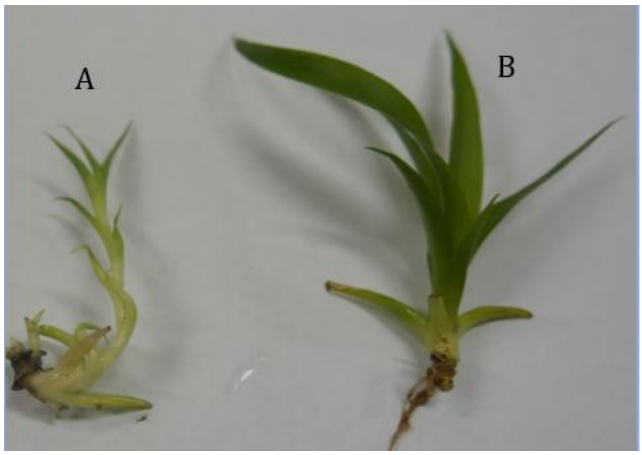

Figure 1: Morphological comparison between abnormal (A) and normal (B) pineapple plantlets derived from in vitro culture. The abnormal plantlet shows stunted growth and leaf distortion compared to the normal plantlet.

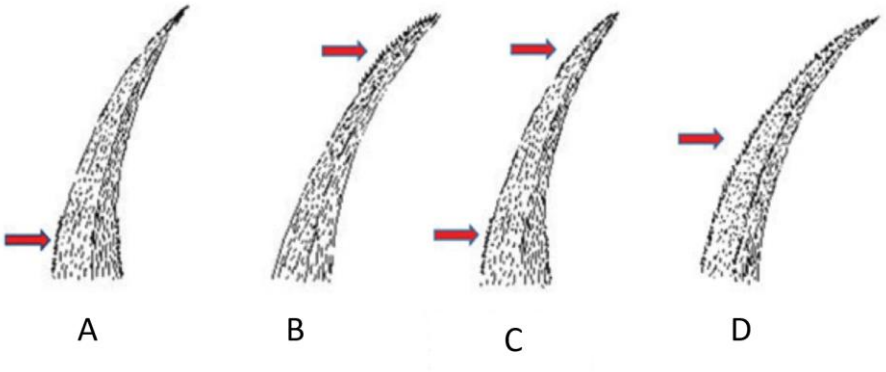

Figure 2: The arrangement or distribution of spines on pineapple leaves. A: at the base of the leaf only, B: at the tip of the leaf only, C: at both the base and the tip and D: along the entire leaf margin.

RESULT AND DISCUSSION

Influence of Plant Growth Regulator Concentrations

The effect of hormone concentration on the occurrence of abnormal plantlets was evaluated at the 7th subculture stage (Figure 1A). At the lowest concentration (1 mg/L), no abnormalities were observed (0%). However, as the concentration increased, a gradual rise in abnormal plantlets was recorded, with 0.20% at 5 mg/L, 1% at 7.5 mg/L, and the highest frequency of 3% at 10 mg/L (Figure 3). These results suggest that elevated hormone concentrations during subculturing may exacerbate the incidence of morphological abnormalities in pineapple plantlets.

The results indicate that higher concentrations of plant growth regulators (PGRs) are associated with increased somaclonal variation in pineapple tissue culture. While low concentrations of hormones maintained normal plantlet morphology, supra-optimal levels (≥7.5 mg/L) were correlated with higher frequencies of abnormality. This observation aligns with previous reports that excessive hormonal exposure, particularly cytokinins such as

benzylaminopurine (BAP) or auxins like 2,4-D, can trigger physiological stress and genomic instability in plant tissue cultures (Bairu et al., 2011; Krishna et al., 2016).

Abnormal morphologies at higher PGR concentrations may result from hormonal imbalances disrupting endogenous auxin–cytokinin ratios, which play critical roles in shoot and root differentiation (Thorpe, 2007). Such imbalances can alter gene expression, DNA methylation, and chromosomal stability, thereby promoting somaclonal variation (Miguel & Marum, 2011). Studies in banana (Khan et al., 2011) and sugarcane (Taylor et al., 1995) have similarly demonstrated that higher concentrations of growth regulators increase the likelihood of abnormal regenerants.

Although the overall percentages of abnormalities remain relatively low, the clear dose-dependent trend highlights the importance of optimizing hormone concentrations for clonal fidelity in commercial micropropagation. Reducing reliance on high PGR levels, or adopting alternative culture systems such as temporary immersion bioreactors, could minimize stress and maintain genetic stability (Etienne & Berthouly, 2002).

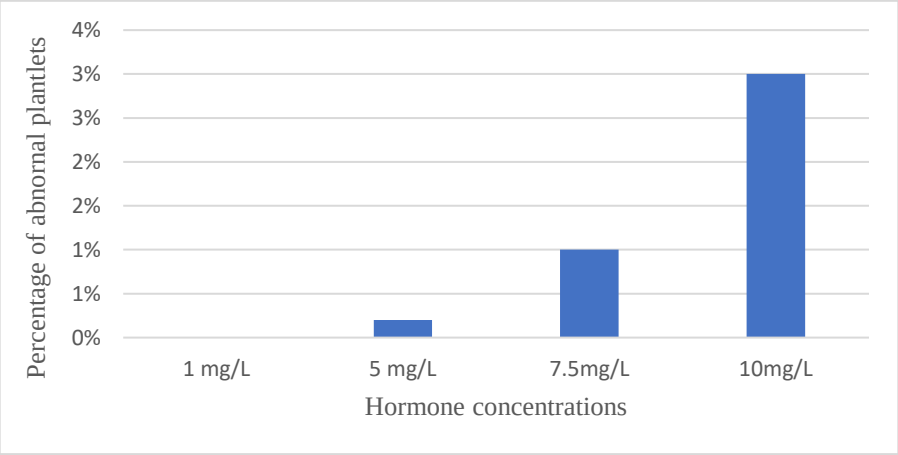

Figure 3: Influence of BAP concentration on abnormality incidence in MD2 pineapple plantlets at the 7th subculture.

The influence of hormone concentration on spine distribution in tissue-culture-derived pineapple plants after the seventh subculture and ten months in the field is summarised in Table 1 and Figure 1. Leaf spination was classified as A = spines at the base only, B = spines at the tip only, C = spines at both base and tip, and D = spines along the entire leaf margin. At 1 mg L^{-1}, the majority of plants (70 %) were spineless, with 29 % showing partial spines (Types A/B/C) and only 1 % fully spined along the margin (Type D). Increasing the hormone concentration to 5 mg L^{-1} reduced the spineless proportion to 46 %, while partial spines rose to 49 % and Type D to 5 %. At 7.5 mg L^{-1}, the proportion of spineless plants fell further to 35 %, with partial spines increasing to 57 % and Type D to 8 %. At the highest concentration, 10 mg L^{-1}, spineless plants were at their lowest level (30 %), while partial spines peaked at 58 % and Type D reached its maximum of 12 % (Figure 4).

This data clearly demonstrates a dose-dependent shift from spineless to spiny morphotypes as hormone concentration increased. Spineless plants, which are the desirable phenotype for

cultivation, decreased steadily from 70 % at 1 mg L^{-1} to 30 % at 10 mg L^{-1}, representing more than a 50 % reduction across the concentration range. In contrast, partial spination (Types A/B/C) almost doubled, from 29 % to 58 %, while full-margin spines (Type D) increased more than tenfold, from 1 % to 12 %. The progressive loss of spineless plants and the corresponding increase in spiny phenotypes are consistent with the occurrence of somaclonal variation in prolonged tissue culture. Elevated exposure to plant growth regulators, particularly benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D), has been reported to destabilise the genome and induce epigenetic changes that manifest as morphological alterations, such as spine development (Krishna et al., 2016; Khan et al., 2011). Similar results were reported in 'Red Spanish' pineapple, where spineless plants reverted to spiny forms during acclimatisation, with 14.1 % of spineless plantlets becoming spiny and 32.7 % of spiny plantlets reverting to spineless (Liu et al., 1989).

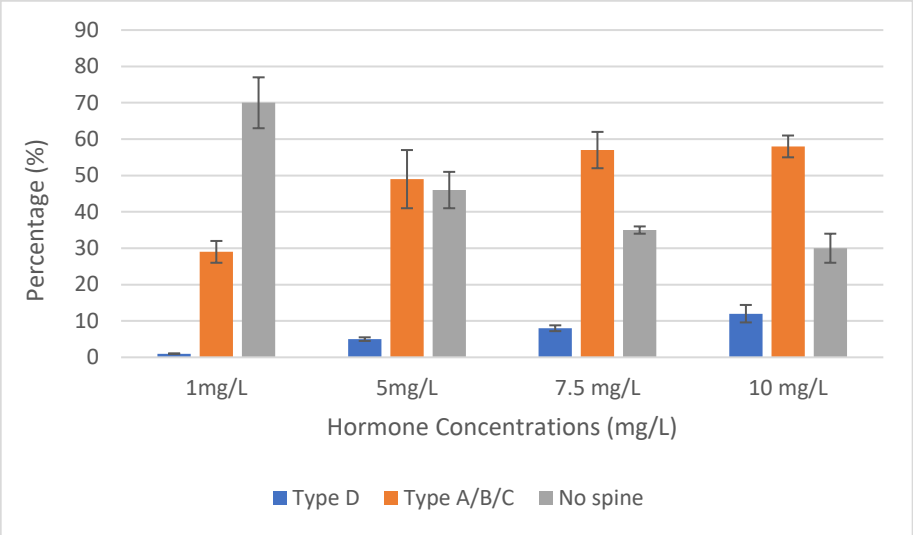

Figure 4: Influence of growth regulator concentration on the incidence and distribution of leaf-margin spines in seventh-subculture, tissue-culture-derived pineapple plants after ten months in the field. A: at the base of the leaf only, B: at the tip of the leaf only, C: at both the base and the tip and D: along the entire leaf margin.

Influence of Successive Subculture Cycles

Successive subculture of pineapple explants in vitro revealed a progressive increase in the frequency of morphological abnormalities (Figure 1). No abnormal phenotypes were detected at the 6th and 8th subcultures (0%). However, abnormal plantlets started to emerge at the 10th subculture with a frequency of 0.30%. This proportion further increased to 0.60% at the 12th subculture and reached 0.90% at the 14th subculture. The abnormalities observed included stunted growth, twisted shoots, and distorted leaf morphology compared to normal plantlets (Figure 5).

The results clearly demonstrate that prolonged cycles of subculturing in pineapple tissue culture increase the risk of somaclonal variation. This phenomenon has been widely documented in several plant species, where extended in vitro culture induces genetic and epigenetic instability, often leading to abnormal phenotypes (Krishna et al., 2016; Khan et al., 2011). In pineapple, the absence of abnormalities during the earlier subculture cycles (up to

the 8th passage) suggests that the plantlets retained genetic fidelity during the initial propagation stages. However, the gradual increase in abnormality frequency beyond the 10th subculture cycle reflects the accumulation of somaclonal variations with prolonged culture duration. Morphological aberrations such as stunted shoots, reduced leaf expansion, and abnormal root growth (Figure 2) are typical manifestations of somaclonal variation. These changes may arise from chromosomal rearrangements, point mutations, transposable element activation, or altered DNA methylation patterns (Miguel & Marum, 2011). Previous studies on banana (Khan et al., 2011) and sugarcane (Taylor et al., 1995) similarly reported an increase in variant phenotypes after extended subculturing, indicating that the phenomenon is not species-specific but rather a common limitation of tissue culture.

The relatively low percentage of abnormal plantlets (less than 1% by the 14th subculture) indicates that while somaclonal variation in pineapple is present, the rate remains manageable if propagation cycles are limited. Nonetheless, to ensure clonal fidelity in large-scale commercial micropropagation, molecular marker analysis such as RAPD, SSR, or AFLP could be employed to complement morphological screening (Bairu et al., 2011). Additionally, limiting the number of subculture passages and adopting cryopreservation or temporary immersion bioreactors could help reduce the accumulation of genetic variations (Rout et al., 2006).

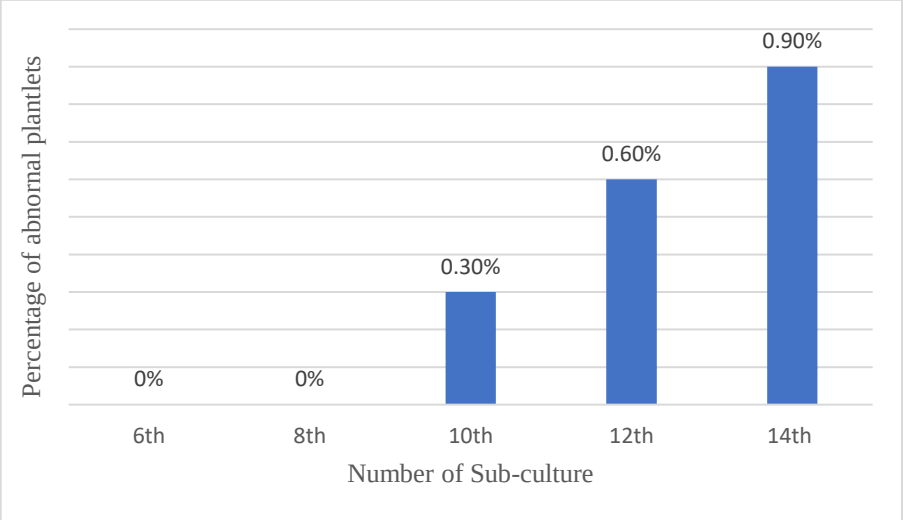

Figure 5: Frequency of abnormal pineapple plantlets observed across successive subculture cycles (6th, 8th, 10th, 12th and 14th subcultures).

This study assessed how successive subcultures influence the appearance of marginal spines in tissue-culture-derived pineapple plantlets during acclimatisation. The results showed that no spiny plants were observed at the sixth subculture (0 %; 0/1 510). However, the proportion of plants with spines increased progressively with the number of subculture cycles: 0.57 % (20/3 500) at the seventh subculture, 1.02 % (52/5 100) at the ninth subculture, and reaching 4.86 % (350/7 200) and 4.60 % (301/6 500) at the fourteenth and fifteenth subcultures (Figure 6). This trend mirrors general observations in plant tissue culture that prolonged culture duration and a greater number of subculture cycles lead to the accumulation of somaclonal variation.

Krishna et al. (2016) note that variant karyotypes tend to accumulate as callus ages, and Khan et al. (2011) found that the number of somaclonal variants in banana increased after the eighth subculture while the multiplication rate of propagules decreased. These increases may stem from oxidative stress during in vitro culture and the effects of plant growth regulators such as benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D), both of which have been implicated in DNA methylation changes and mutations in cultured tissues. The relatively low incidence of spiny variants (<5 %) observed here, compared with higher rates reported for some cultivars, suggests that the pineapple genotype used is fairly stable. Nevertheless, the rise from zero to almost five per cent after nine to fifteen subculture cycles indicates that somaclonal changes accumulate over time. Liu and colleagues (1989) reported that, among 40 000 meristem-derived plantlets of the 'Red Spanish' pineapple, about half were spineless; 14.1 % of spineless plantlets reverted to spiny and 32.7 % of spiny plantlets became spineless. In their study, 72.9 % of spiny plantlets remained spiny and 85.8 % of spineless plantlets remained spineless after three to four months of acclimatisation, indicating that phenotype stability increases after acclimation. Comparatively, our data show lower but steadily rising variation, consistent with the concept that somaclonal variation accrues with each subculture cycle.

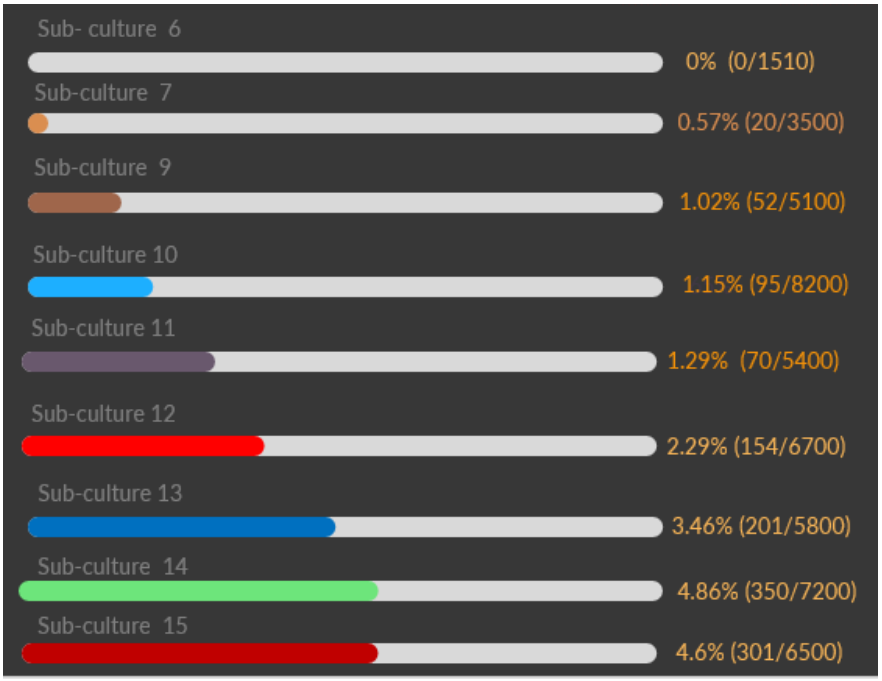

Figure 6: Effect of subculture on the percentage of tissue-culture-derived pineapple plants exhibiting spines along the full leaf margin during acclimatization.

The acclimatisation phase (Figure 7) was essential to ensure high survival and stable growth of MD2 pineapple plantlets. By gradually adjusting environmental conditions and optimising substrates, plantlets established robustly, enabling reliable assessment of morphological fidelity. After ten months in the field, clear differences in spine distribution and abnormal morphologies were observed among treatments, directly reflecting the influence of both subculture cycles and hormone concentrations. Thus, acclimatisation served as a critical bridge between in vitro propagation and field performance, allowing the identification of true-to-type

MD2 pineapple plants and demonstrating the long-term impact of tissue culture protocols on phenotypic stability.

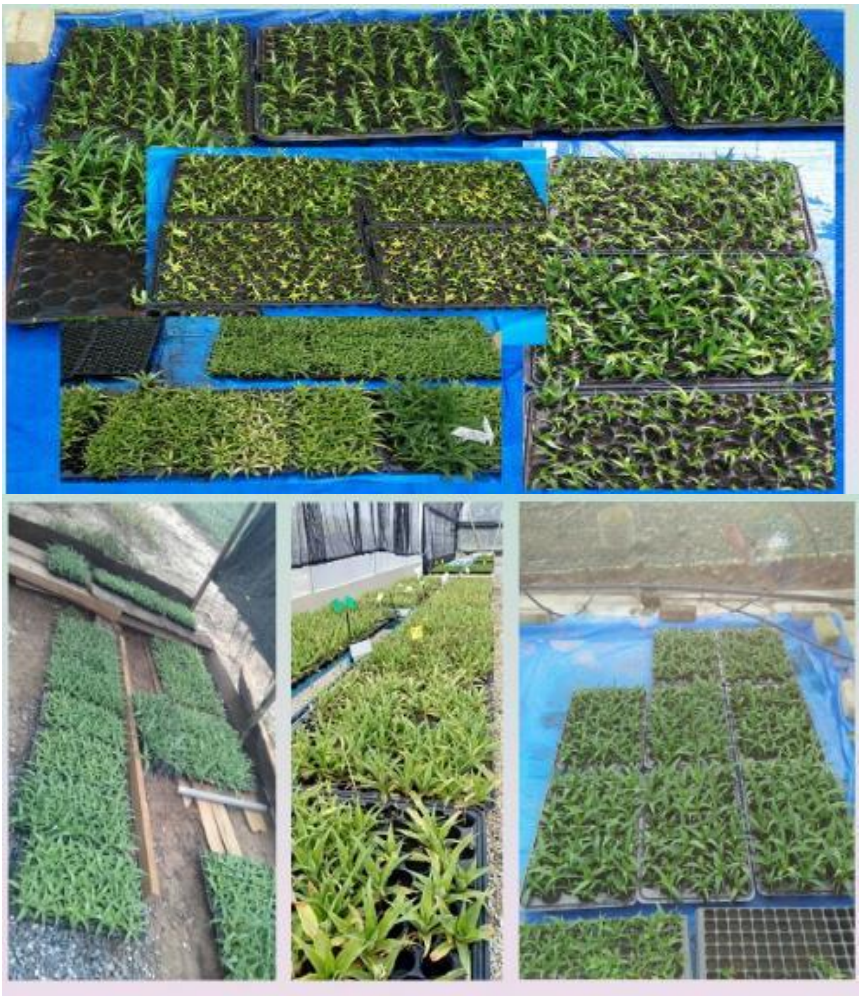

Figure 7: Hardening and acclimatisation of in vitro–derived MD2 pineapple plantlets under greenhouse conditions.

CONCLUSION

This study demonstrates that both the number of subculture cycles and the concentration of plant growth regulators (PGRs) significantly influence the occurrence of somaclonal variation in tissue-culture-derived MD2 pineapple. Abnormal phenotypes, including distorted shoots, stunted growth, and spiny leaf margins, were absent in the early passages but increased progressively after the 10th subculture, indicating that prolonged in vitro maintenance compromises clonal fidelity. Likewise, supra-optimal hormone levels (≥7.5 mg L^{-1} BAP) were strongly associated with higher frequencies of abnormal regenerants and a clear shift from spineless to spiny morphotypes.

These findings highlight the importance of optimising micropropagation protocols for commercial pineapple production. Restricting the number of subculture cycles to no more than eight and maintaining lower PGR concentrations (around 1 mg L^{-1}) can help minimise genetic

instability while sustaining regeneration efficiency. Routine morphological screening, complemented by molecular marker analysis, is recommended to ensure true-to-type plantlets and to prevent the accumulation of undesirable variants.

From an agronomic perspective, the observed reduction of spineless plants under extended subculturing and high hormone concentrations has direct implications for plantation uniformity, crop management, and consumer preference. As spineless variants are preferred for ease of handling and market acceptance, their stability must be preserved through conservative subculturing and careful hormone regulation. The adoption of advanced propagation systems such as temporary immersion bioreactors or cryopreservation could further enhance genetic stability and scalability for producing high-quality planting material.

In conclusion, while tissue culture remains a powerful tool for large-scale propagation of MD2 pineapple, meticulous regulation of subculture duration and hormone dosage is essential to minimise somaclonal variation and maintain clonal fidelity in commercial production systems.

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