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Mutagenic effects of sodium azide on pineapple micropropagant growth and biochemical profile within temporary immersion bioreactors

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Summary

Sodium azide (NaN₃) is widely used to induce mutagenesis within *in vitro* plant systems. However, since this mutagenesis is un-directed, its unintended effects demand characterization. This study investigated the mutagenic effects of sodium azide (0-0.45 mM) on selected growth (shoot multiplication rate and shoot cluster fresh weight) and biochemical (aldehydes, chlorophylls, carotenoids and phenolics) parameters in pineapple micropropagants within temporary immersion bioreactors (TIBs). The content of soluble phenolics in the culture medium was also evaluated. Irrespective of the concentration NaN₃ decreased shoot multiplication rate (by 87% relative to the control at 0.45 mM) and fresh weight (by 66% relative to the control at 0.45 mM). Levels of chlorophyll *a* and *b*, and soluble phenolics in the culture medium were also negatively correlated with NaN₃ concentration. Interestingly, NaN₃ application increased shoot carotenoid and soluble phenolic levels but had no significant effect on a range of established plant stress biomarkers: cell wall-linked phenolic levels, malondialdehyde and other aldehydes. Given that 0.19 mM NaN₃ decreased shoot multiplication rate by 50% and resulted in propagants that displayed no morphologically abnormalities, increased levels of photoprotective pigments (relative to the control) and no significant increase in lipid peroxidation products, the mutagen can be used at this concentration to induce pineapple mutagenesis in TIB based studies aimed at producing agriculturally-useful mutants.

Keywords: *In vitro* stress; *in vitro* mutagenesis; plant metabolites; *Ananas comosus* (L.) Merr.; plant breeding

Abbreviations: Sodium azide (NaN₃); temporary immersion bioreactor (TIB).

Introduction

Plant breeders have generated new varieties using chemical mutagens for many decades (DUBEY et al., 2017). Sodium azide (NaN₃) is widely regarded as a relatively safe to handle and very efficient chemical mutagen that is both inexpensive and non-carcinogenic (SALVI et al., 2014). This common laboratory chemical is used widely in organic synthesis, agricultural and medical research (most often as bactericide, pesticide and nitrogen gas generator) but it is most famous for its mutagenic effects in plants and animals (MENDIONDO et al., 2016). This mutagenic capacity is based on NaN₃'s production of an organic metabolite, β-azidoalanine, which is valued for its ability to induce chromosomal aberrations at a rate much lower than other mutagens (DUBEY et al., 2017).

The concentrations of NaN₃ that are used for the treatment of plant tissues vary widely across species: e.g. 3-10 mM (CASTILLO et al.,

2001) or 0.5-4.0 mM in *Hordeum vulgare* (SZAREJKO et al., 2017); 1.0-3.0 mM in *Pisum sativum* (DIVANLI-TÜRKAN et al., 2006); 0.04-1.1 mM in *Phaseolus vulgaris* (CHEN et al., 2011); 15.5-77.6 mM in *Trigonella foenum-graecum* (SIDDIQUIA et al., 2007); and 31.1-124.3 mM in *Brassica napus* (HUSSAIN et al., 2017). However, we have not found any report on the use of NaN₃ during *in vitro* production of axillary buds. Despite the wide range of concentrations at which NaN₃ is used, the mutagen generally acts by creating point mutations in the genome, disturbing metabolic activity, growth and development, and inhibiting protein and DNA replication (RAGUNATHAN and PANNEERSELVAM, 2007). These effects, the severity of which are concentration dependent (VAINSTEIN, 2002), change the balance between growth promoters and their antagonists (GRUSZKA et al., 2012). Nevertheless, a number of traits have been improved in a variety of species using NaN₃, including days to germination, flowering and silique maturation in *Brassica napus* (HUSSAIN et al., 2017); related to quality, yield and disease resistance in wheat (DUBEY et al., 2017); germination, seedling survival, root length and height, height at maturity, number of leaves, and fruit yield in tomato (ADAMU and ALIYU, 2007); and groundnut yield (MENSAH and OBADONI, 2007). The mutagen has also been applied to a variety of explant types: e.g. cultures of *Pisum sativum* seeds (DIVANLI-TÜRKAN et al., 2006), tomato seeds (EL KAABY et al., 2015), *Brassica napus* cotyledons (HE et al., 2011), and *Hordeum vulgare* anthers and microspores (CASTILLO et al., 2001). Although the TIB technique is known to increase multiplication rates in many crop species (JIMÉNEZ et al., 1999), the use of this culture technique for the NaN₃ application has not been reported to date.

The increased multiplication rates in TIBs might be caused by the combined advantages of using a solid and liquid culture medium. Micropropagation on solid culture medium allows plant air exchange but nutrient uptake is limited to the explant basal surface (ESCALONA et al., 1999). On the other hand, whilst micropropagation in liquid culture medium increases nutrient uptake, it often leads to hyperhydricity (ZIV, 1995). Hyperhydricity is characterized by various degrees of morphological and physiological disorders which can include a glassy, waterlogged-tissue appearance and disordered shoot growth (more specifically in the leaves). Unlike the classic liquid culture procedure (ALVARD et al., 1993), in a TIB explants are covered with the culture medium only for a few minutes and immersion allows nutrient uptake through the entire explant surface. The plant air exchange is restored after removal of the culture medium. Irrespective of the *in vitro* culture method, explant type or species, the mutations underlying the changes induced by chemical mutagens like NaN₃ are un-directed and as alluded to above can have negative effects on plant growth and performance. This necessitates the characterization of the unintended effects of NaN₃ exposure and identification of the optimum concentration for application within specific species.

Pineapple is the main commercial species of the Bromeliaceae globally (MARTÍN et al., 2015). Fruit production in pineapple reached 25.4 million tons in 2013 (FAOSTAT, 2016) but to maintain and/or improve the quality of pineapple production in many parts of the world, researchers are now developing new varieties (YABOR et al., 2016). Given that pineapple breeding through conventional techniques is extremely costly (LOISON-CABOT and LACOEUILHE, 1989), biotechnological approaches (e.g. induced mutagenesis) provide great potential for improving selected clones more affordably.

In vitro-induced mutagenesis has been employed to produce improved genetic variants in numerous crop species, including pineapple (IBRAHIM et al., 2009). Research on the efficacy of chemical mutagens, specifically NaN_3 , in inducing mutagenesis in pineapple is limited and hence, forms the focus of the present research. The study investigated the mutagenic effects of NaN_3 , applied at a range of concentrations (0–0.45 mM), on selected growth (shoot multiplication rate and shoot cluster fresh weight) and biochemical (aldehydes, chlorophylls, carotenoids and phenolics) parameters in pineapple micropropagants TIBs. Soluble phenolic content in the culture medium was also assessed. The selection of TIBs for the study is based on the high multiplication rate of pineapple (1:66) using this technique, compared with conventional micropropagation containers (1:8) (ESCALONA et al., 1999).

Materials and methods

Plant material, in vitro methods and growth measurements

Feld-grown pineapple plants (cv. MD2) served as the source of explants for initiating *in vitro* cultured buds (DAQUINTA and BENEGAS, 1997). These axillary buds, excised after removal of the crown leaves, were decontaminated (with 1% (w:v) $\text{Ca}(\text{ClO})_2$ for 10 min) followed by rinsing with tap water. The buds were excised with a portion of basal tissue and established in 300 ml glass containers with 5 ml of liquid culture medium per explant. MS salts (MURASHIGE and SKOOG, 1962), 100 mg l^{-1} myo-inositol, 0.1 mg l^{-1} thiamine-HCl, 30 g l^{-1} sucrose, 4.4 μM 6-benzyladenine (BA), and 5.3 μM naphthaleneacetic acid (NAA) were included in the medium. At 45 d of culture, shoots were transferred to the multiplication medium (original medium supplemented with 9.3 μM BA and 1.6 μM NAA). The shoots were subcultured and multiplied for 6 months, at 45-d intervals, and then placed in TIBs with 3.0 μM paclobutrazol (after ESCALONA et al. (1999)). Immersions (2 min in duration) occurred every 3 h for 30 d. Free shoots were located in the bottom of glass vessels (300 mL volume) filled with 200 mL of liquid medium with 5 explants within each of three containers per treatment (40 mL medium / explant).

At the beginning of the 30-d-long subculture, different levels of NaN_3 (0, 0.15, 0.30 and 0.45 mM) were supplemented in the culture medium. Culture conditions were as follows: 25 ± 1 °C and 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ cool fluorescent light for an 8 h photoperiod. Shoot multiplication rate, shoot cluster fresh weight and all biochemical parameters described below were measured after 30 d of culture.

Biochemical parameters

Plant tissues (100 mg per replicate) were sampled from each of three bioreactors. Phenolics were quantified according to GURR et al. (1992), chlorophylls following PORRA (2002) and malondialdehyde and other aldehydes after HEATH and PACKER (1968). For chlorophyll pigments *a* and *b*, the tissue was extracted in 5.0 ml acetone (80%, v:v), centrifuged (14086.8 g, 4 °C, 15 min) and the subsequent supernatants read for absorbance at 646.6 and 663.6 nm using a spectrophotometer (RAYLEIGH, VIS-723G). Similarly, carotenoids levels were measured by reading the absorbance of acetone (80%, v:v) extracts at 470 nm (LICHTENTHALER, 1987).

Phenolic compounds were extracted and quantified (mg gallic acid equivalents per g fresh weight) using a colorimetric assay which involves the reaction of phenols with Folin Ciocalteu reagent (GURR et al., 1992). The reaction of malondialdehyde and other aldehydes with thiobarbituric acid formed the basis of the colorimetric method used to quantify the products of lipid peroxidation (molar extinction coefficient: $1.57 \cdot 10^5 \text{M}^{-1} \text{cm}^{-1}$) (ALBRO et al., 1986; HEATH and PACKER, 1968). Phenolic exudation was quantified using a modification of the HOAGLAND (1990) procedure. This involved mixing the culture medium (0.5 ml; one sample per TIB) with 4.5 ml distilled water, after which 0.5 ml Folin Ciocalteu reagent (50% v/v) was added. This mixture was shaken and then left to stand for 5 min before saturated sodium carbonate (1 mL) was added. The mixture was then shaken again, left to stand for 60 min, and then read for absorbance at 725 nm. Phenolic concentration was calculated based on a gallic acid standard curve.

Statistical analysis

All statistical analyses were performed using SPSS (Version 8.0 for Windows, SPSS Inc., New York, NY). Where data was parametric, tested for normality using a Kolmogorov-Smirnov test, means were compared using a One-Way analysis of variance (ANOVA) in combination with a Tukey post-hoc test ($p \leq 0.05$). Additionally, the overall coefficients of variation (OCV) were calculated as follows: (standard deviation/average) * 100. In this formula, the average values of the four NaN_3 levels were compared (treatments) to calculate the standard deviation and average. The higher the difference between the four treatments compared, the higher the OCV (LORENZO et al., 2015). OCVs from 2.94 to 30.00% were classified as *Low*, from 30.00 to 57.06% as *Medium* and from 57.06 to 84.12% as *High*.

Results

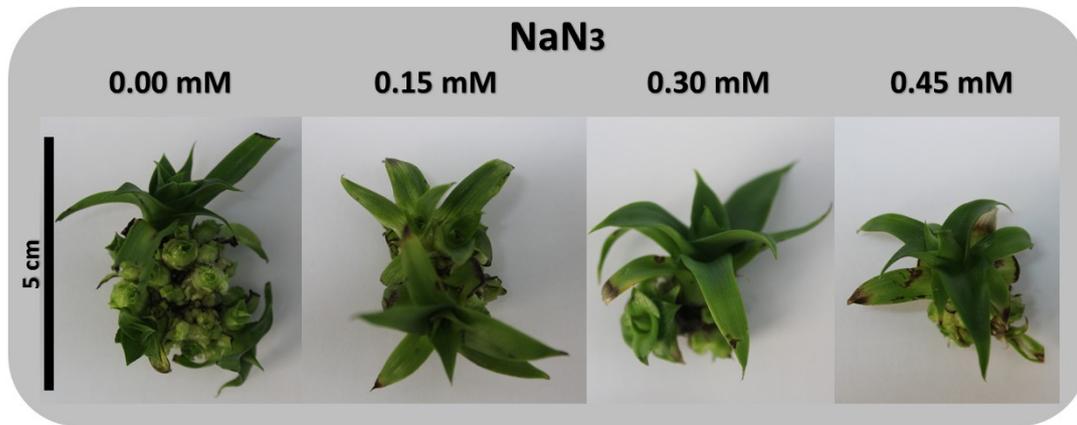
Exposure to NaN_3 decreased pineapple shoot multiplication rate and fresh weight in a concentration-dependent manner (Fig. 1A, 1B, 1C). At the maximum concentration (0.45 mM) shoot multiplication rate was 12.65% of that obtained in the control, while fresh weight was reduced by 66.42% relative to the control. Despite this reduction in growth and multiplication rate, shoot production was observed across all NaN_3 treatment concentrations and the shoots displayed no morphological abnormalities when compared to the control (Fig. 1A). It should also be noted that the inhibitory effects of increasing NaN_3 concentration were more severe on shoot multiplication rate than cluster fresh weight.

In terms of plant pigment contents, NaN_3 decreased chlorophyll *a* (Fig. 2A) levels at the maximum concentration and chlorophyll *b* (Fig. 2B) at the two highest concentrations, relative to the control. In contrast, NaN_3 increased carotenoid levels, compared with the control, at all concentrations tested (Fig. 2C). OCV values were, however, *Low* (13.65–24.13%) across all three plant pigments and the shoots produced on NaN_3 supplemented media showed no signs of chlorosis (Fig. 1A) throughout the growth period.

While tissue soluble phenolics (Fig. 2D) increased relative to the control, soluble phenolics levels in the culture medium (Fig. 2F) decreased within increasing NaN_3 concentrations, but OCVs were again *Low* (18.15 and 28.22%, respectively). Interestingly, NaN_3 had no significant effects on the levels of cell wall-linked phenolics (Fig. 2E), malondialdehyde (Fig. 2G) or other aldehydes (Fig. 2H).

Discussion

In light of a predicted reduction in agricultural productivity in many parts of the world due to climate change, induced mutation technology for crop improvement has become an increasing important area of research over the last few decades (DUBEY et al., 2017). In this



A Typical pineapple shoots produced in temporary immersion bioreactors with mutagen sodium azide (NaN_3) at 30 d of culture.

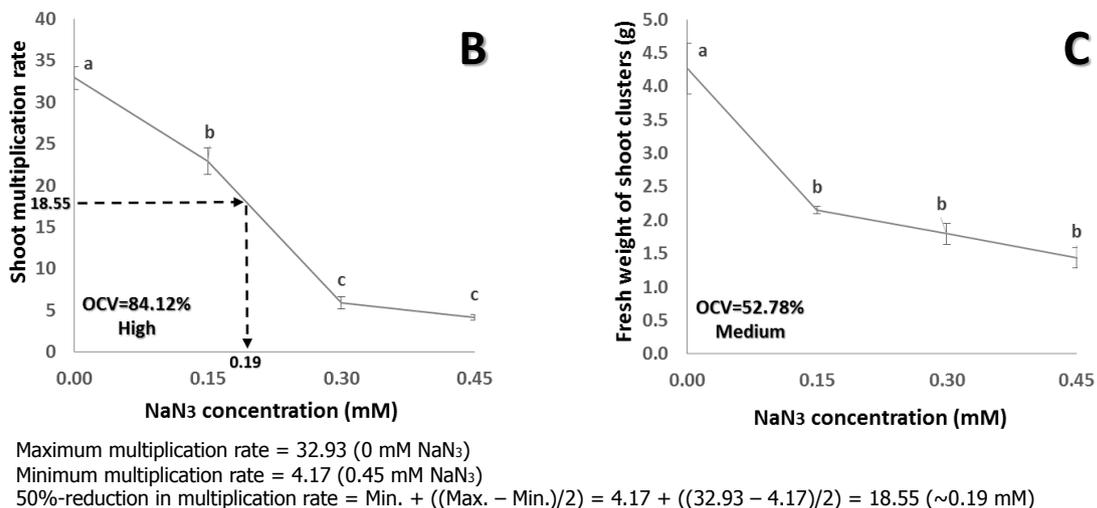


Fig. 1: Effects of sodium azide exposure on pineapple micropropagation in temporary immersion bioreactors. Values represent means \pm SE ($n=3$) and are significantly different when labelled with different letters (ANOVA, Tukey, $p \leq 0.05$). Overall coefficient of variation (OCV) = (Standard deviation/Average)*100. To calculate this coefficient, the four average values were considered. The higher the difference among results, the higher is the overall coefficient of variation: *Low* = 2.94 to 30.00%, *Medium* = 30.00 to 57.06% and *High* = 57.06 to 84.12%.

regard, chemical mutagens are now useful tools in crop improvement and have been used to produce abiotic stress tolerance and disease resistance in various susceptible crops, improving their yield and quality traits (OLAWUYI and OKOLI, 2017). There are several mutagens available for crop improvement and like NaN_3 , the mutagen investigated here, each has its own positive or negative effect on plants (HESLOT, 1977).

Mutagenesis has been employed to introduce many useful traits, including plant size, fruit ripening and resistance to pathogens in a wide range of fruit crops (reviewed by LAMO et al. (2017)). However, studies on the effects of chemical mutagens on pineapple are scarce (PAULL et al., 2017), while no published reports on the effects of NaN_3 on the *in vitro* growth and biochemistry of pineapple explants were available at the time of this study. In the present study NaN_3 decreased shoot multiplication rate and cluster fresh weight (Fig. 1B, 1C). NaN_3 has been reported to have similar inhibitory effects on *in vitro* explant growth in other species (DUBEY et al., 2017; HUSSAIN et al., 2017; OLAWUYI and OKOLI, 2017; SZAREJKO et al., 2017). However, at 0.19 mM NaN_3 , the multiplication rate (1:19) in the TIBs used was lower than the control but still higher to that achieved for pineapple grown in the absence of NaN_3 using conventional culture methods (1:8) (ESCALONA et al., 1999). Furthermore the pro-

pagants generated displayed no morphological abnormalities, when compared to the control (Fig. 1A).

Sodium azide did, however, induce significant changes in a number of biochemical parameters relative to the control. Most plants grown *in vitro* have a different metabolism than *in vivo*. In the former, they are provided with a carbon source, growth regulators, high humidity, and less carbon dioxide and light, which promote proliferation but also change the autotrophic metabolism to heterotrophic or mixotrophic (CHANDRA et al., 2010). Once transferred to the *ex vitro* environment, micropropagated plantlets must then adapt their metabolism to the new conditions, which can represent a significant stress (TEIXEIRA et al., 2017). The biochemical profile of micropropagated plantlets can therefore influence, either negatively or positively, their survival and performance during acclimatization and subsequent *ex vitro* growth (HAZARIKA et al., 2006).

In the present study while *in vitro* exposure to NaN_3 decreased chlorophyll levels (*a* and *b*) in pineapple shoots, it increased carotenoid levels. Carotenoids are important components of the antioxidant system in photosynthetic organisms and their absence can increase the extent of photoinhibition (CABRERA, 2002). Both the decreased chlorophyll and increased carotenoid levels suggest that NaN_3 exposure may reflect a NaN_3 -stress-related photosynthetic down-regulation

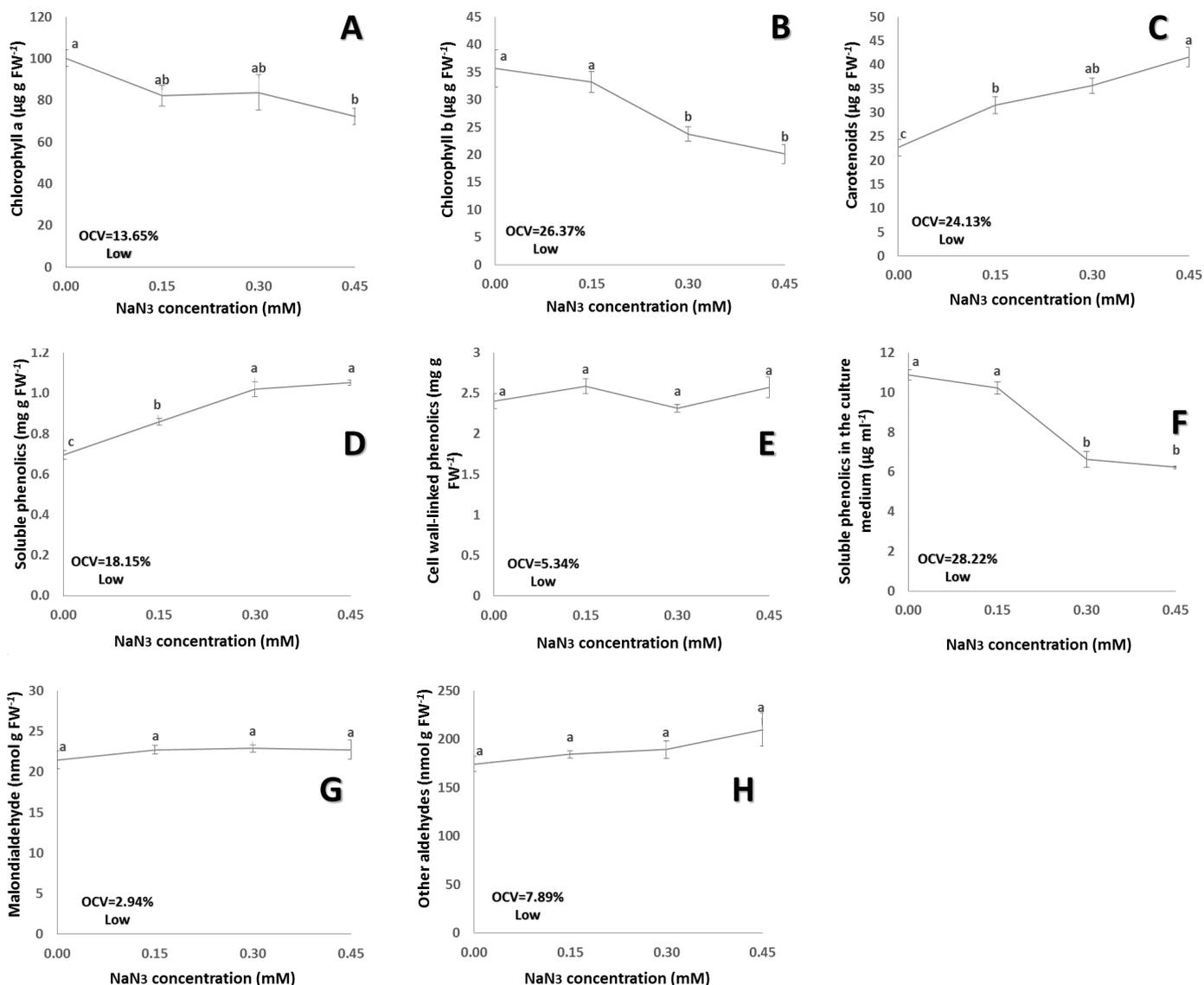


Fig. 2: Effects of sodium azide exposure on pineapple micropropagation in temporary immersion bioreactors. Values represent means \pm SE ($n=3$) and are significantly different when labelled with different letters (ANOVA, Tukey, $p \leq 0.05$). Overall coefficient of variation (OCV) = (Standard deviation/Average)*100. To calculate this coefficient, the four average values were considered. The higher the difference among results, the higher is the overall coefficient of variation: *Low* = 2.94 to 30.00%, *Medium* = 30.00 to 57.06% and *High* = 57.06 to 84.12%.

capacity and enhanced photoprotection. Increased carotenoid-based photoprotection is a commonly reported stress response (POMPELLI et al., 2010). This may have in turn decreased the potential for photo-oxidation, which can arise as a consequence of stress induced damage to the photosynthetic machinery. This suggestion is supported by the fact that NaN_3 exposure did not result an increase in malondialdehyde and other aldehyde levels (Fig. 2G, 2H) which are common products of cellular damage caused by (photo) oxidative stress (DUMET and BENSON, 2000). Under normal growth conditions, basal levels of aldehydes remain low in plant tissues but with exposure to abiotic stress they can accumulate to much higher levels (SERSHEN et al., 2016). Stress-induced aldehydes can act as generic signal molecules for plants under adverse environmental conditions, inhibiting developmental and metabolic processes (MOSTOFA et al., 2015). YADAV et al. (2005) for example, showed that the accumulation of aldehydes can inhibit shoot growth.

Phenolic compounds are some of the most common products of secondary metabolism in plants and some are essential for plant survival, given their involvement in defense mechanisms under stress

situations (SHARMA et al., 2012). The regulation of the biosynthesis of phenolics is generally brought about by biotic and abiotic stimuli (BETTAIEB et al., 2011) and generally accumulate in plant tissues during a stress (JOSEPH et al., 2015). In the present study, though NaN_3 exposure induced a reduction in growth, an unambiguous indication of stress, it did not increase phenolic levels in shoot tissues (Fig. 2D, 2E) and or the culture medium (Fig. 2F). This is encouraging, since cell wall-linked or insoluble phenolics, in particular, can make cell walls more rigid and less porous inhibiting nutrient uptake and growth (KABERA et al., 2014). Their accumulation in *in vitro* cultures is therefore not beneficial for growth (MACHAKOVA et al., 2008).

The results suggest that though NaN_3 decreased pineapple shoot multiplication rate within TIBs, propagants displayed no morphologically abnormalities, and when produced using 0.19 mM NaN_3 exhibited enhanced levels photoprotective pigments and no obvious signs of enhanced lipid peroxidation. The mutagen can therefore be used at this concentration to induce pineapple mutagenesis in TIB based studies aimed at producing agriculturally-useful mutants.

Author contribution

DG, LH, JM, JQ, BEZ, S, LY and JCL designed the research; DG, LH and JM conducted the experiments; DG, LH, JM, JQ, BEZ, S, LY and JCL analyzed the data and wrote the paper; and JCL had primary responsibility for the final content. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest Authors do not have any conflict of interests.

Human and animal rights This research did not involve experiments with human or animal participants.

Informed consent: Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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