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Original article

Profiling of black rot resistant *Dendrobium* 'Earsakul' induced by *in vitro* sodium azide mutagenesis

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Summary

In Thailand, *Dendrobium* 'Earsakul' is one of the most important commercial orchids. However, its production is negatively impacted especially by black rot disease caused by *Phytophthora palmivora*. Therefore, new orchid varieties with high quality and resistance to the disease are urgently needed. The objectives of this research were to assess the resistance levels in sodium azide (NaN_3) induced black rot resistant *D.* 'Earsakul' mutants by detached leaf assay and by characterizing the putative mutants using morphological characters, molecular markers, cytology, and protein profiles of black rot resistant mutants. The results revealed that two *D.* 'Earsakul' mutants, namely SUT17N05304 and SUT17N05308, were highly resistant and SUT17N05305 and SUT17N05501 were resistant. Their morphological characters including number of nodes, number of leaves, and number of roots differed from non-mutagenized controls showing susceptibility to the disease. Genetic variability by means of inter-simple sequence repeat (ISSR) analysis further demonstrated that these resistant mutants were genetically different from non-mutagenized controls (0.186 genetic dissimilarity; GD). Using flow cytometry, all of the resistant mutants were identified to be mixoploid ($2n+4n+8n$) and their DNA content and genome size, ranging from 3.77 to 3.90 pg $2C^{-1}$ and 3,640 to 3,764 Mbp, respectively, were higher than all the non-mutagenized controls. Moreover, SDS-PAGE showed up-regulation of a protein with a molecular weight of 39 kDa at 3 to 5 days after inoculation in four black rot resistant mutants, while that of non-mutagenized controls appeared at 4 to 5 days after inoculation. In addition, a 13 kDa protein was uniquely induced only in two of the black rot resistant mutants (SUT17N05304 and SUT17N05305). These results suggest the usefulness of these resistant mutants for future development of new black rot resistant *Dendrobium* varieties.

Keywords
flow cytometry, inter-simple sequence repeat, orchid, mutation, protein profiles

Significance of this study

What is already known on this subject?

- Earlier findings demonstrate the influence of NaN_3 on the mutagenesis of orchids.

What are the new findings?

- NaN_3 is effective in inducing black rot resistance and affects different morphological, genetic, DNA content and genome size, and protein profile characteristics of *Dendrobium* 'Earsakul' mutants.

What is the expected impact on horticulture?

- The morphological alterations of these black rot resistant mutants will also be evaluated at the flowering stage and evaluated for black rot resistance levels at the greenhouse level for potential commercialization in the future. In addition, NaN_3 mutagenesis will be beneficial for development and breeding orchids for resistance to black rot and other diseases in the future.

Introduction

Dendrobium, the third-largest genus in the family Orchidaceae, has become popular in the world as it is widely used in ornamental gardening and herbal medicine. In Thailand, *Dendrobium* 'Earsakul' is one of the most important commercial orchids (Kuehnle, 2007). However, there has been a reduction in its production due to several severe constraints. Of these, black rot caused by *Phytophthora palmivora* is one of the most serious foliar diseases that impacts production by causing root and crown rot (Aekaraj, 2008). This pathogen can potentially infect various host plants, i.e., almond trees, citrus trees, and cherry trees as well as ornamental plants including orchids (Uchida, 1994; Pane et al., 2006; Cacciola et al., 2008; Türkölmez et al., 2015). Moreover, all plant roots including rhizomes can also be infected which causes rapid plant death (Orlikowski and Szkuta, 2006; Cating et al., 2010). Therefore, new high-quality *D.* 'Earsakul' varieties with resistance to black rot are needed immediately. Black rot resistant orchids may minimize the use of severe chemicals harmful to humans and the environment. One effective approach to the development of black rot resistant orchids is mutation induction (mutagenesis). As orchid hybridization has faced the unavailability of a resistant source and trouble with sexual compatibility, orchid breeders have considered employing mutagenesis, especially *in vitro*, as an alternative approach to generate resistant orchids through enhancing genetic variability and randomly modifying gene(s) without altering the entire genetic profile of an organism (Ahmad et al., 2010; Wannajindaporn et al., 2014). In general, mutation includes two major types, spontaneous and induced mutation. Spontaneous mutation typically occurs in nature through several factors, i.e., radioactivity and heat etc., or even by transposable elements (transposons) with a

very low frequency. While induced mutation which occurs at a higher frequency is induced by several mutagens including physical mutagen (radiation), e.g., gamma rays, X-rays, and neutrons and chemical mutagens, e.g., ethyl methanesulfonate (EMS), 5-bromouracil (5-BU), and sodium azide (NaN_3) (Laosuwan and Thipyapong, 2007).

NaN_3 is highly mutagenic in several organisms by producing an organic metabolite of azide. It is well known that this mutagen induces lower frequency of chromosomal aberrations than other mutagens. However, it frequently induces point mutation (transition or transversion) through changing nucleotides from G-C → A-T, translocation, chromosome lagging, and chromosome bridge, which may lead to amino acid changes, thereby altering the function of proteins (Wannajindaporn et al., 2014; Hualsawat, 2019). Moreover, it is inexpensive, and mutagenesis by NaN_3 has effectively improved agronomic/horticultural characteristics and resistance/tolerance to abiotic/biotic stresses in several plants (Al-Qurainy and Khan, 2009; Wannajindaporn et al., 2014). Therefore, mutagenesis by this mutagen can alternatively be employed for the improvement of *D.* 'Earsakul', particularly for black rot resistance.

Historically, researchers have identified mutants through genotyping and phenotyping to discover the mutations associated with the improved agronomic/horticultural variants. Molecular markers are most useful for assessing genetic variability at DNA and/or RNA levels and are continually used without environmental constraints. Particularly, inter-simple sequence repeat (ISSR) markers with wide genome coverage are now preferred because of their high polymorphic information content (PIC) with multi-locus nature and no prior sequence information required. These properties make them useful for the identification of polymorphisms which have occurred from mutation. In addition, ISSR markers are simple, rapid, cost-effective, and environmentally independent (Wannajindaporn et al., 2014; Hualsawat, 2019). Therefore, they have been widely used in several plants, i.e., *Tulipa* L., banana, and Persian Poppy (Kiani et al., 2012; Kharadi et al., 2014; Hadipour et al., 2020). For *Dendrobium* mutants induced by NaN_3, Wannajindaporn et al. (2016) observed genetic profile variations in 20 of 24 putative mutants (83.33%) using ISSR markers, and some putative mutants were morphologically altered compared with controls including reduced height, a higher number of nodes, reduced node length, shorter and thicker leaves, and shorter and fewer roots. Moreover, characterization of mutants has been conducted using cytology based on flow cytometry. This method can be employed to detect structural chromosomes and ploidy level changes as well as DNA content, and it is also more rapid, accurate, and convenient than chromosome counts. Using this method for investigating the effects of colchicine on survival rate and ploidy level of orchid hybrids, Choopeng et al. (2019) found that the DNA content of orchid mutants could increase from 33 to 50% of the original DNA content. Moreover, Jones et al. (1998) also used this method to reveal the DNA content of 26 orchid genera, ranging from 1.53 to 4.23 pg $2C^{-1}$.

Under pathogen infection, various molecules including pathogenesis related (PR) proteins, e.g., chitinase and β-glucanase and phytoalexins, are potentially generated for plant defense mechanisms. Jayasankar et al. (2000) reported that anthracnose resistant lines from *in vitro* selection which derived from embryogenic grapevine culture had an up-regulated protein band of about 36 kDa that was not found in non-selected control, which may be related to chitinase. Qiu et al. (2004) reported that benzothiazole (BTH) considered as an abiotic elicitor could increase resistance to *P. palmivora* causing root rot disease in papaya by inducing the accumulation of β-1,3 glucanase and chitinase. Therefore, these methods may be used to effectively characterize putative resistant mutants of *D.* 'Earsakul'.

In this investigation, we characterized *D.* 'Earsakul' black rot resistant mutants derived from *in vitro* NaN_3 mutagenesis of protocorm-like bodies (PLBs), followed by *in vitro* selection with *P. palmivora* culture filtrate (CF) (Khairum et al., unpublished data) by means of resistance evaluation against *P. palmivora* using detached leaf assay, morphological characters, ISSR markers, cytogenic analysis, and protein profiles before and after pathogen infection. This knowledge will be beneficial for further development and breeding orchids for black rot resistance.

Materials and methods

Evaluation of black rot resistance levels using detached leaf assay

NaN_3-induced *D.* 'Earsakul' PLBs via *in vitro* mutagenesis were obtained by Wannajindaporn et al. (2016). Briefly, PLBs from clonal propagation were treated with 0 (control), 0.1 (LD_{30}), and 0.5 mM NaN_3 (LD_{50}) in a 0.1 M sodium acetate buffer (pH 4) for 1 h. After the mutagen treatment, they were transferred to a modified Vacin and Went (VW) medium (Tantasawat et al., 2015), and incubated in a culture room at 27 ± 2°C under a 100 μmol·m^{-2} s^{-1} cool white florescent light for a 16-h photoperiod followed by an 8-h period of darkness. The mortality of PLBs was recorded at 3 days, 1 and 2 weeks. These mutants and non-mutagenized controls were challenged *in vitro* on selective pea sucrose broth (PSB) containing 0–60% of *P. palmivora* CF for 3 cycles (0, 30, and 50% (first 2 cycles) and 0, 40, and 60% (3^{rd} cycles)), and those which survived throughout 3 cycles of selection were selected as putative back rot resistant mutants (Khairum et al., unpublished data). In this current study, we used 35 putative resistant mutants including: SUT13N01301, SUT13N01302, SUT13N01303, SUT17N01501, SUT17N05501, SUT17N05301, SUT17N05302, SUT17N05303, SUT17N05304, SUT17N05305, SUT17N05307, SUT17N05308, SUT17N05309, SUT17N05310, SUT17N05311, SUT17N05312, SUT17N05313, SUT17N05315, SUT17N05316, SUT17N05317, SUT17N05319, SUT17N05321, SUT17N05322, SUT17N05323, SUT17N05324, SUT17N05325, SUT17N05326, SUT17N05327, SUT17N05329, SUT17N05330, SUT17N05331, SUT17N05333, SUT17N05335, SUT17N05337, and SUT17N05338. In addition, there were 5 non-mutagenized controls including: SUT16C001, SUT16C003, SUT16C007, SUT16C008, and SUT16C0012, which were randomly selected from a 0 mM NaN_3 treatment. All of the materials were cultured on a modified VW1 medium (Tantasawat et al., 2015) at 25 ± 2°C with a 16-h photoperiod provided by a cool-white fluorescent light (PPFD = 100 μmol m^{-2} s^{-1}) until reaching the 4 to 6 leaf stage.

Resistance levels were evaluated using detached leaf assay with the most virulent *P. palmivora* isolate NK-53-9 from Khairum et al. (2016). For inoculation, leaves were wounded by a pin, and a 3 μL of inoculum suspension with 10^6 zoospores mL^{-1} was used, while the controls were inoculated with reverse osmosis water (ROW). Inoculated and control leaves were kept at 25°C in the dark under controlled conditions. Three replicates were made. Disease symptoms were observed at 3 and 5 days after inoculation. A scale of disease severity from 0 to 5 was used as follows: highly resistant

Table 1. Score of disease symptom severity for detached leaf assay.

Disease rating	Severity of disease symptom for detached leaf assay
0	No symptoms
1	Very small, localized lesions
2	Yellow around lesions
3	Yellow around lesions with hyphae revealed
4	Brown lesions with hyphae expanded which covered entire leaf surface
5	Brown lesions with hyphae covered entire leaf surface and outside area

(0.00–0.49 scores), resistant (0.50–1.49 scores), moderately resistant (1.50–2.49 scores), moderately susceptible (2.50–3.49 scores), and susceptible (3.50–5.00 scores) (Table 1; Nyassé et al., 1995; Khairum et al., 2016). The mutants identified as highly resistant and resistant to black rot were used in further characterization based on morphological characters, ISSR markers, cytogenic analysis, and protein profiles before and after pathogen infection.

Morphological characterization

Morphological parameters including plant characteristics (plant height, number of nodes, and node length), leaf characteristics (number of leaves and leaf length), and root characteristics (number of roots and root length) of resistant and non-mutagenized plants were measured at six months of age. Measurement techniques followed as established by Wannajindaporn et al. (2014). A completely randomized design (CRD) with 10 replications was used. The SPSS v. 16.0 (Levesque and SPSS Inc., 2006) was used to perform the analysis of variance (ANOVA). The means among all materials studied were compared by Duncan's new multiple range test (DMRT) at $p < 0.05$.

Evaluation of genetic variation in black rot resistant mutants induced by NaN_3 using ISSR markers

For molecular characterization, the total DNA was extracted from fresh young leaves of each resistant mutant and non-mutagenized control via the modified cetyltrimethylammonium bromide (CTAB) method of Zhang et al. (2009). DNA purity and quantity were examined spectrophotometrically by Nanodrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.). A portion of the pure DNA was diluted to 40 ng μL^{-1} to be used as a template for polymerase chain reaction (PCR) analysis.

A total of 32 ISSR primers developed by the University of British Columbia with varied annealing temperatures were preliminarily tested among 2 representative resistant mutants and 2 non-mutagenized controls, and 11 polymorphic primers with microsatellite repeats (AC, AG, AT, CA, and GA) anchored at the 3'-end by 1–2 nucleotides producing clearly identifiable and reproducible bands were selected for the analysis (Table 2). All were found to be effective for the genetic identification of *D.* 'Earsakul' mutants (Wannajindaporn et al., 2014). Most of them have also been successfully used to assess the diversity in wild *C. goeringii* (Orchidaceae) populations (Yao et al., 2007). In addition, other 5 ISSR polymorphic primers with microsatellite repeats (GT, TC, TG, and ACC) were also used. For PCR analysis according to Wannajindaporn et al. (2014), PCR mix of 20 µL was made containing 40 ng DNA template, 1X buffer (75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphate (dNTP), 1U of Tag DNA polymerase (Vivantis, Selangor Darul Ehsan, Malaysia), and 4 µM of each ISSR primer.

The PCR program included an initial denaturation at 94°C for 5 min, 45 cycles of denaturing at 94°C for 45 s, annealing at 48–55.4°C for 45 s (Table 2), extension at 72°C for 90 s, and a final extension at 72°C for 7 min. The reaction was performed in a T100™ Thermal Cycler (Bio-Rad Laboratory, Inc., California, U.S.A.). The amplified DNA was revealed on 6% (w/v) denaturing polyacrylamide gel at 200 V using vertical electrophoresis for 70 min and silver nitrate staining (Sambrook and Russell, 2001). A 100 bp DNA ladder (Invitrogen, California, U.S.A.) was used as the DNA standard to calculate the molecular weights of the DNA bands. PCR was performed twice for each primer to confirm the DNA band pattern consistency.

The clearly reproducible amplified bands were sized and scored manually as 0 or 1 for their absence or presence, respectively. Polymorphic information content (PIC) was calculated as $PIC = 1 - \Sigma Pi^2$ where i is the total number of alleles detected for the ISSR primer, and Pi is the frequency of the i^{th} allele in the examined genotypes. Marker index (MI) was calculated as MI = PIC × EMR, where EMR is the effective multiplex ratio. EMR was calculated as $EMR = n \times \beta$, where n is the total number of loci amplified by a specific

Table 2. Information of ISSR primers used for the genetic evaluation of resistant mutant lines and non-mutagenized controls.

Primers	Primer sequences	Annealing temperature (°C)	Primers	Primer sequences	Annealing temperature (°C)
ISSR 807	$(AT)_8T$	52.3	ISSR 840	$(GA)_8YT$	48.0
ISSR 809	$(AG)_8G$	52.0	ISSR 841	$(GA)_8YC$	54.0
ISSR 811	$(GA)_8C$	53.0	ISSR 851	$(GT)_8YG$	54.0
ISSR 812	$(GA)_8A$	53.0	ISSR 857	$(AC)_8YG$	54.0
ISSR 822	$(TC)_8A$	50.0	ISSR 860	$(TG)_8RA$	52.0
ISSR 827	$(AC)_8G$	53.0	ISSR 861	$(ACC)_6$	54.0
ISSR 834	$(AG)_8T$	55.4	ISSR 888	$BDB(CA)_7$	51.0
ISSR 836	$(AT)_8YT$	52.0	ISSR 890	$VHV(GT)_7$	51.0

Y = T, C; R = A, T; V = A, C, G; B = T, C, G; H = A, T, C; D = A, T, G.

system marker (multiplex ratio), and β is derived from β = PB/(PB+MB), where PB is the number of polymorphic loci and MB is the number of non-polymorphic loci (Varshney et al., 2007). A pair-wise similarity matrix was determined using Jaccard, Dice, and simple matching (SM) similarity coefficients described by Nei and Li (1979) and Sneath and Sokal (1973). NTSYSpc software v. 2.2 (Rohlf, 2000) based on the unweighted pair group method with an arithmetic average (UPGMA) clustering algorithm was used to construct the dendrogram. The Mantel correlation (Mantel, 1967) was calculated to determine the goodness of fit for the materials studied to a specific cluster in the UPGMA cluster analysis. NTSYSpc software v. 2.2 was used again to generate a principal coordinate analysis (PCoA) to view their clustering pattern through multiple dimensions (Keim et al., 1992).

Investigation of chromosomal variation of black rot resistant *D.* 'Earsakul' mutants using flow cytometry

Intact cell nuclei from the fresh mature leaves of resistant mutants and non-mutagenized controls were extracted mechanically. The extraction and DNA staining were conducted according to Pfosser et al. (1995). Approximately 20–30 mg of the leaf samples were chopped with a sharp razor blade in a glass petri dish into 0.2–0.5 mm pieces in 1 mL of 1% (w/v) polyvinylpolypyrrolidone (PVPP) and 0.4 mL Quantum stain NA UV2 'component A' (extraction buffer) and incubated for 1 min. This homogenate was stained immediately with 0.4 mL of Quantum stain NA UV2 'component B' (staining reagent) and was then passed through a 50 µm nylon filter to remove cell fragments and tissue debris. After filtration, samples were incubated for about 30 s and analyzed using a flow cytometer (Meesawat et al., 2008).

Analyses were carried out using a CRD design with 5 replicates. The fluorescence of at least 10,000 stained nuclei per sample (across all peaks) was measured on a flow cytometer (Quantum analysis flow cytometer (QAFCMQP2), Friesoythe, Germany) at the Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand. The internal reference standard was *D. lindleyi* Steud. (2C = 2.40 pg $2C^{-1}$). The reference standard peak was set at channel 200 of relative fluorescence intensity for instrument calibration during analysis. The obtained histograms were computerized by software (CyPAD pantau). Mean DNA content was calculated based on *D. lindleyi* Steud. DNA using the following formula:

$$\text{2C DNA content} = \frac{\textit{Dendrobium} \text{ peak mean}}{\textit{D. lindleyi} \text{ peak mean}} \times \text{2C DNA of } \textit{D. lindleyi} \text{ Steud.}$$

The number of base pairs per haploid genome was estimated based on the equivalent of 1 pg DNA = 965 megabase pair (Bennett and Smith, 1976). DNA content and genome size were analyzed using the analysis of variance (ANOVA). Mean comparison was conducted by DMRT at $p < 0.05$. All analyses were performed using SPSS v. 16.0 (Levesque and SPSS Inc., 2006).

Characterization of protein changes following infection in black rot resistant mutants and non-mutagenized controls

Approximately 0.2 g of mature leaves of black rot resistant mutants and non-mutagenized controls inoculated with 10^6 zoospore mL^{-1} of *P. palmivora* at 0 to 5 days after inoculation were placed in a pre-chilled pestle and mortar and were immediately homogenized in chilled 1 mL of 0.1 M Tris HCl (pH 7), 0.1 M KCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg mL^{-1} leupeptin, 1 mM ethylene-diaminetetraacetic acid (EDTA), and 3% PVPP. The lysates were then clarified by centrifugation at 13,000 rpm for 30 min at 4°C. The supernatants of each sample were collected for protein profiling. Protein concentrations were measured according to Bradford (1976) through dye binding assay. A standard curve of absorbance at 590 nm versus 10–40 µg mL^{-1} of bovine serum albumin (BSA) was drawn. From this curve, the amount of protein was calculated and finally expressed as mg per g of leaves. The experiment was performed twice.

For SDS-PAGE, the protein concentration of each sample was adjusted to 0.4 µg μL^{-1} and was mixed with 4X loading dye containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue. The mixed samples of 10 µL were denatured at 95°C for 5 min and subjected to SDS-PAGE according to the method of Laemmli (1970) in a gel slab of 1 mm thickness (4% (w/v) stacking and 12% (w/v) resolving gels) by running at 100 V for 95 min. Staining of gels was carried out using silver nitrate solution (Laemmli, 1970). Protein standard (molecular weight range = 2–250 kDa) (Bio-Rad, California, U.S.A.) was used to determine the molecular size of the proteins.

Results and discussion

Evaluation of black rot resistance levels using detached leaf assay

The severity score for the black rot symptoms of 35 NaN_3 induced mutants and the 5 non-mutagenized controls displayed highly significant differences only at 5 days after inoculation ($p < 0.01$) but not at 3 days after inoculation ($p > 0.05$) (Table 3). The most virulent *P. palmivora* isolate NK-53-9 was pathogenic to all 5 detached non-mutagenized control leaves after 3 days of inoculation with severity scores of 3.67 to 4.33, resulting in visibly necrotic lesions and hyphae. While 35 NaN_3 induced mutants showed different disease symptoms with scores of 0.00 to 3.00. These symptom patterns were also observed at 5 days after inoculation. All non-mutagenized controls exhibited higher scores of 4.00 to 5.00, indicating their susceptibility to the disease. The mutants were identified with all disease responses which included highly resistant, resistant, moderately resistant, moderately susceptible, and susceptible with the scores of 0.00 to 4.67. Interestingly, the mutants SUT17N05304 and SUT17N05308 were the most resistant to the pathogen because they had no disease symptoms even at 5 days after inoculation. In addition, the mutants SUT17N05305 and SUT17N05501 were found to be potentially resistant with very small, localized lesions and had scores of 0.67 and 1.33, respectively. Thirteen mutants were moderately resistant with a yellowish halo around the lesions, giving scores of 1.67 to 2.33, while 12 mutants were moderately susceptible and had a yellowish halo around lesions with hyphae expanded (scores of 2.67 to 3.33), and only 6 mutants were susceptible, having brown lesions with hyphae expanded and scores of 3.67 to 4.67. It should be noted that induced mutagenesis via NaN_3, followed by *in vitro* selection with *P. palmivora* CF was highly effective for the induction of black rot resistant mutants. Four promising black rot resistant mutants, namely SUT17N05304, SUT17N05305, SUT17N05308, and SUT17N05501, were selected for further characterization.

Morphological characterization

The 6-month-old plants of 4 black rot resistant mutants SUT17N05304, SUT17N05308, SUT17N05305, and SUT17N05501 and 3 non-mutagenized controls, SUT16C003, SUT16C007, and SUT16C008, were evaluated for their morphological variability using a variety of parameters. All the characters studied were significantly different, except for plant height (Table 4; Figure 1).

Plant height: the resistant mutant SUT17N05308 tended to have the highest plant height (3.53 cm), followed by SUT16C007 (3.30 cm). While SUT16C003 showed the lowest plant height (2.60 cm) compared with the remaining lines. Although the resistant mutants had a significantly higher average number of nodes, they had a lower average length of nodes compared with the non-mutagenized controls, thereby resulting in the same height.

Table 3. Severity scores of symptoms at 3 and 5 days after inoculation with *P. palmivora* isolate NK-53-9 in 35 *D.* 'Earsakul' mutant lines and 5 non-mutagenized controls at four months.

Lines	Severity score of symptoms		
	3 days	5 days	Disease response[1]
SUT16C001[2]	4.33 ± 0.88	5.00 ± 0.58 a[3]	Susceptible
SUT16C003[2]	3.67 ± 1.00	4.00 ± 0.67 a-d	Susceptible
SUT16C007[2]	4.00 ± 0.33	4.33 ± 0.00 abc	Susceptible
SUT16C008[2]	3.67 ± 0.67	5.00 ± 0.00 a	Susceptible
SUT16C0012[2]	4.33 ± 1.33	5.00 ± 1.33 a	Susceptible
SUT13N01301	1.00 ± 0.58	3.33 ± 0.19 a-d	Moderately susceptible
SUT13N01302	1.67 ± 0.88	1.67 ± 0.88 c-f	Moderately resistant
SUT13N01303	2.00 ± 1.00	2.00 ± 1.00 b-f	Moderately resistant
SUT17N01501	1.00 ± 1.00	2.67 ± 1.33 a-e	Moderately susceptible
SUT17N05501	1.00 ± 1.00	1.33 ± 1.33 def	Resistant
SUT17N05301	1.00 ± 0.00	1.67 ± 0.54 c-f	Moderately resistant
SUT17N05302	1.33 ± 0.88	2.00 ± 0.58 b-f	Moderately resistant
SUT17N05303	2.00 ± 0.58	3.33 ± 0.88 a-d	Moderately susceptible
SUT17N05304	0.00 ± 0.00	0.00 ± 0.00 e	Highly resistant
SUT17N05305	0.33 ± 0.33	0.67 ± 0.10 ef	Resistant
SUT17N05307	0.00 ± 0.00	2.00 ± 0.00 b-f	Moderately resistant
SUT17N05308	0.00 ± 0.00	0.00 ± 0.00 e	Highly resistant
SUT17N05309	1.00 ± 1.00	2.33 ± 0.88 b-f	Moderately resistant
SUT17N05310	2.00 ± 1.00	3.00 ± 1.00 a-d	Moderately susceptible
SUT17N05311	0.67 ± 0.33	1.67 ± 0.33 c-f	Moderately resistant
SUT17N05312	3.00 ± 0.33	4.67 ± 0.33 ab	Susceptible
SUT17N05313	2.33 ± 0.67	4.00 ± 1.00 a-d	Susceptible
SUT17N05315	1.67 ± 0.67	3.00 ± 1.00 a-e	Moderately susceptible
SUT17N05316	2.33 ± 0.67	3.33 ± 0.33 a-d	Moderately susceptible
SUT17N05317	2.00 ± 1.00	3.67 ± 1.33 a-d	Susceptible
SUT17N05319	2.67 ± 0.33	4.00 ± 0.58 a-d	Susceptible
SUT17N05321	2.33 ± 0.67	3.00 ± 1.00 a-d	Moderately susceptible
SUT17N05322	2.00 ± 1.00	3.33 ± 0.67 a-d	Moderately susceptible
SUT17N05323	1.00 ± 0.00	2.00 ± 1.00 b-f	Moderately resistant
SUT17N05324	1.33 ± 0.88	2.00 ± 1.00 b-f	Moderately resistant
SUT17N05325	0.33 ± 0.33	3.00 ± 0.58 a-e	Moderately susceptible
SUT17N05326	2.00 ± 1.00	3.00 ± 1.00 a-e	Moderately susceptible
SUT17N05327	2.33 ± 0.67	3.00 ± 0.58 a-e	Moderately susceptible
SUT17N05329	3.00 ± 1.00	3.67 ± 0.88 a-d	Susceptible
SUT17N05330	2.00 ± 0.58	3.67 ± 1.20 a-d	Susceptible
SUT17N05331	1.33 ± 0.33	2.00 ± 0.00 b-f	Moderately resistant
SUT17N05333	1.00 ± 0.00	2.67 ± 0.33 a-e	Moderately susceptible
SUT17N05335	2.00 ± 0.58	2.33 ± 0.88 b-f	Moderately resistant
SUT17N05337	1.33 ± 0.88	2.00 ± 1.00 b-f	Moderately resistant
SUT17N05338	1.33 ± 0.88	2.00 ± 0.58 b-f	Moderately resistant

[1]: Disease response was observed at 5 days after inoculation. Scores were attributed according to the following scale: highly resistant (0.00–0.49 scores), resistant (0.50–1.49 scores), moderately resistant (1.50–2.49 scores), moderately susceptible (2.50–3.49 scores), and susceptible (3.50–5.00 scores), modified from Nyassé et al. (1995) and Khairum et al. (2016).

[2]: Non-mutagenized controls.

[3]: Means in the same column with different letters are significantly different among lines (DMRT) at $p < 0.05$.



Figure 1. Morphological traits of resistant mutants and non-mutagenized controls at 6 months.

Number of nodes: all resistant mutants showed a higher number of nodes which varied from 3.8 to 4.30 nodes. SUT17N05501 showed the highest number of nodes (4.30 nodes), followed by SUT17N05308 (4.00 nodes). However, the number of nodes of other resistant mutants (SUT17N05304 and SUT17N05305) were not significantly different from all non-mutagenized controls. The non-mutagenized control SUT16C003 had the lowest number of nodes (1.90 nodes).

Length of nodes: SUT17C007 showed the highest length of node (0.42 cm), but this was not significantly different from SUT16C003 (0.31 cm) and SUT16C008 (0.33 cm). While the resistant mutant SUT17N05501 had the lowest node length (0.21 cm), but it was not significantly different from the other resistant mutants SUT17N05305 (0.24 cm), SUT17N05304 (0.26 cm), and SUT17N05308 (0.28 cm). Three of these (SUT17N05304, SUT17N05305, and SUT17N05308) showed no significant difference in the node length from SUT16C003 and SUT16C008.

Number of leaves: SUT17N05305 showed the highest number of leaves (6.20), but it was not significantly different from SUT17N05308 (5.90) and SUT17N05304 (5.70). However, the number of leaves for these mutants was significantly higher than for SUT17N05501 and all the non-mutagenized controls. The lowest number of leaves was found in SUT16C003 (3.20 leaves).

Length of leaves: the highest leaf lengths were found on the non-mutagenized controls with the longest being (SUT16C008 (1.11 cm), followed by SUT16C007 (0.94 cm) and then SUT16C003 (0.93 cm)). However, these were not significantly different from SUT17N05304 (0.88 cm) and SUT17N05308 (0.86 cm). By contrast, SUT17N05305 and SUT17N05501 had significantly shorter leaves than those of the non-mutagenized controls.

Width of leaves: SUT17N05501 had the widest leaves (0.49 cm), but it was not significantly different from those of the other resistant mutants, except for SUT17N05308 and the non-mutagenized controls. In addition, these resistant mutants had visibly thicker leaves which were darker green than the non-mutagenized controls.

Number of roots: SUT17N05305 had the most roots (6.10 roots), which was significantly more than the others. It was followed by SUT17N05308 (4.30 roots) and SUT17N05304 (3.70 roots), but they were not significantly different from SUT16C007 (3.10 roots). While SUT16C003, SUT16C008, and SUT17N05501 had the least roots (2.30, 2.40, and 2.70 roots, respectively), but they were not significantly different from SUT16C007 (3.10 roots), and SUT17N05304 (3.70 roots).

Length of roots: SUT17N05304 had the maximal mean root length (1.23 cm), followed by SUT16C007 (1.13 cm) and SUT17N05501 (1.05 cm). By contrast, SUT16C003, SUT16C008, and SUT17N05308 had significantly shorter roots than the other lines (0.36, 0.41, and 0.48 cm, respectively).

NaN_3 appeared to induce favorable mutations in the morphological traits of the resistant mutants as it increased its number of nodes (SUT17N05308 and SUT17N05501), number of leaves (SUT17N05304, SUT17N05305, and SUT17N05308), and number of roots (SUT17N05305). While the non-mutagenized controls had a longer leaf length than the resistant mutants, except for SUT17N05304 and SUT17N05308. NaN_3 has been found to cause morphological changes in various plant species. Treatment of the *Gardenia jasminoides* Ellis. node with NaN_3 increased the number of leaves, number of branches, and leaf length (Tawfik and Fathy, 2020). On *D.* 'Earsakul' plantlets after the NaN_3 treatment, Wannajindaporn et al. (2016) revealed an increased number of nodes, shorter and thicker leaves, while node length, root length, number of roots, and plant height were reduced as compared with the controls. These morphological changes in plants induced by NaN_3 may be caused either by genetic changes from mutation or delay/inhibition in physiological and biological processes required for plant growth and development. Khan et al. (2009) found that mutation by the action of NaN_3 is mediated through the production of an organic metabolite of the azide compound which subsequently interacts with DNA and causes point mutation during DNA replication. This mutagen may disturb adenosine triphosphate (ATP) biosynthesis by acting as a strong inhibitor of the proton-translocating ATPase complex in the terminal segment of the electron transport chain, thereby reducing proton pumping and altering the mitochondrial membrane potential, respectively. Because of low energy in the form of ATP, the rates of biological processes, i.e., DNA, RNA, and protein synthesis in the plant cells decrease which may slow growth and development, leading to morphological changes (Srivastava et al., 2011). The morphological changes of these putative resistant mutants will also be evaluated at the flowering stage. In addition, the levels of black rot resistance will be evaluated at the greenhouse level in the future.

Table 4. Morphological characterization of resistant mutants and non-mutagenized controls at 6 months.

Lines	Morphological characters							
	Height (cm)	Number of nodes	Length of nodes (cm)	Number of leaves	Length of leaves (cm)	Width of leaves (cm)	Number of roots	Length of roots (cm)
SUT16C003	2.60 ± 0.12	1.90 ± 0.18 b[1]	0.31 ± 0.03 ab	3.20 ± 0.25 c	0.93 ± 0.07 a	0.46 ± 0.03 a	2.30 ± 0.58 c	0.36 ± 0.10 c
SUT16C007	3.30 ± 0.22	2.90 ± 0.46 b	0.42 ± 0.05 a	3.90 ± 0.46 bc	0.94 ± 0.13 a	0.48 ± 0.05 a	3.10 ± 0.35 bc	1.13 ± 0.20 a
SUT16C008	3.01 ± 0.13	3.30 ± 0.65 b	0.33 ± 0.04 ab	4.00 ± 0.47 bc	1.11 ± 0.10 a	0.46 ± 0.03 a	2.40 ± 0.73 c	0.41 ± 0.10 c
SUT17N05304	2.98 ± 0.12	3.80 ± 0.47 ab	0.26 ± 0.02 bc	5.70 ± 0.52 a	0.88 ± 0.07 ab	0.45 ± 0.03 ab	3.70 ± 0.60 bc	1.23 ± 0.17 a
SUT17N05305	2.90 ± 0.18	3.90 ± 0.41 ab	0.24 ± 0.03 bc	6.20 ± 0.53 a	0.74 ± 0.04 b	0.46 ± 0.03 a	6.10 ± 0.95 a	0.87 ± 0.18 b
SUT17N05308	3.53 ± 0.35	4.00 ± 0.73 a	0.28 ± 0.01 bc	5.90 ± 0.84 a	0.86 ± 0.05 ab	0.44 ± 0.03 b	4.30 ± 0.92 b	0.48 ± 0.10 c
SUT17N05501	3.02 ± 0.50	4.30 ± 0.90 a	0.21 ± 0.02 c	5.10 ± 0.86 bc	0.78 ± 0.03 b	0.49 ± 0.04 a	2.70 ± 0.76 c	1.05 ± 0.59 ab
F-test	ns	*	**	**	**	*	**	**

[1]: Means in the same column with different letters are significantly different among lines (DMRT) at $p < 0.05$.
ns = non-significant; * = significant ($p < 0.05$); ** = highly significant ($p < 0.01$).

Evaluation of genetic variation in black rot resistant mutants induced by NaN_3 using ISSR markers

In molecular characterization, 4 *D.* 'Earsakul' black rot resistant mutants and 3 non-mutagenized controls were analyzed using 16 ISSR primers (Table 2). A total of 16 ISSR primers were tested because they exhibited defined amplification patterns and high reproducibility. Of these primers, a total of 225 clear and reproducible DNA bands were produced among all the materials used (Table 5). The number of bands obtained ranged from 8 (ISSR 861) to 23 (ISSR 841) bands per primer. Of the total number of bands, 51 bands from all primers were identified to be polymorphic (22.67%). The size of all amplified bands varied from 204 bp (ISSR 834) to 2,000 bp (ISSR 807). Each ISSR primer generated an average of 14.06 bands per primer, and 3.19 of these were polymorphic. The highest and lowest number of polymorphic bands were obtained from ISSR 834 (9) and ISSR 812, 822, 861, 888, and 890 (1), respectively. The ISSR 834 showed the highest polymorphism percentage (50.00%), followed by ISSR 857 (45.45%), ISSR 827 (31.25%), ISSR 811 (28.57%), and ISSR 836 (28.57%), while the lowest polymorphism percentage was obtained from ISSR 812 (6.25%), with an average of 22.29% from all primers. This polymorphism percentage was lower than that of Wannajindaporn et al. (2016), who found a polymorphism percentage of 34.81% in 24 *D.* 'Earsakul' mutants induced by NaN_3. The polymorphism may largely depend on the sequences of primers and the number of materials being analyzed (Keim et al., 1992). The range of PIC values was 0.2449 (ISSR 807 and 861) to 0.4898 (ISSR 822, 851, 857, and 888) with an average of 0.4042. The highest and lowest MI were obtained from ISSR 834 (3.7548) and ISSR 861 (0.2449), respectively with an average of 1.2954 (Table 5). Four ISSR primers (ISSR 827, 834, 841, and 857) were found to have high MI (2.041–3.7548), indicating their usefulness for genetic differentiation of *Dendrobium* mutants. For the correlation coefficients determined between cophenetic and similarity coefficients, the highest coefficient was obtained from the Jaccard coefficient (0.969) as compared with the SM (0.939) and Dice coefficients (0.936); therefore the dendrogram was constructed by the Jaccard coefficient. In this study, all 4 resistant mutants exhibited the presence of genetic differences compared with the non-mutagenized controls, particularly the 6 unique bands observed in only resistant mutants (2 unique bands from SUT17N05304, 2 unique bands from SUT17N05501, and 2 unique bands from SUT17N05305). In addition, SUT16C008 (non-mutagenized control) was genetically different from SUT16C003 and SUT16C007. These results suggest the evidence of somaclonal variation in the absence of NaN_3 treatment during *in vitro* culture. Martínez-Estrada et al. (2017) reported that ISSR markers could detect the existence of somaclonal variation during micropropagation of sugarcane whose frequencies depended on *in vitro* establishment and the number of subcultures. These may include types of tissues and explants, types and components of culture media, and culture duration, as well as methods of culture that may possibly cause this variation through the alteration in DNA methylation patterns (Kaeppler et al., 2000; Rout et al., 2006; Leva et al., 2012; Wannajindaporn et al., 2016). On the other hand, the absence/addition of ISSR loci may result from the loss/gain of primer binding sites as a result of changes in the nucleotide sequences of SSR motifs in each genotype or the changes in distance between primer binding sites, thereby resulting in the polymorphism.

A dendrogram based on cluster analysis was construct-

Table 5. Information of ISSR primers used for the analysis of 4 resistant mutant lines and non-mutagenized controls.

Primers	Primer sequences	Annealing temperature (°C)	No. of total bands	No. of polymorphic bands	Polymorphism (%)	Amplified band size (bp)	PIC	MI
ISSR 807	$(AG)_8T$	52.3	18	4	22.22	218-2,000	0.2449	0.9796
ISSR 809	$(AG)_8G$	52.0	11	2	18.18	235-600	0.3673	0.7346
ISSR 811	$(GA)_8C$	53.0	14	4	28.57	314-1,200	0.4286	1.7144
ISSR 812	$(GA)_8A$	53.0	16	1	6.25	559-1,500	0.4082	0.4082
ISSR 822	$(TC)_8A$	50.0	13	1	7.69	519-1,200	0.4898	0.4898
ISSR 827	$(AC)_8G$	53.0	16	5	31.25	221-1,200	0.4082	2.0410
ISSR 834	$(AG)_8YT$	58.5	18	9	50.00	204-600	0.4172	3.7548
ISSR 836	$(AG)_8YA$	52.0	14	4	28.57	234-1,500	0.3265	1.3060
ISSR 840	$(GA)_8YT$	48.0	12	3	25.00	360-1,000	0.3810	1.1430
ISSR 841	$(GA)_8YC$	54.0	23	5	21.74	278-1,200	0.4490	2.2450
ISSR 851	$(GT)_8YG$	54.0	11	3	27.27	235-500	0.4898	1.4694
ISSR 857	$(AC)_8YG$	54.0	11	5	45.45	245-700	0.4898	2.4490
ISSR 860	$(TG)_8RA$	52.0	11	2	18.18	236-1,200	0.4245	0.8490
ISSR 861	$(ACC)_6$	54.0	8	1	12.50	260-1,000	0.2449	0.2449
ISSR 888	$BDB(CA)_7$	51.0	15	1	6.67	242-800	0.4898	0.4898
ISSR 890	$VHV(GT)_7$	51.0	14	1	7.14	333-1,000	0.4082	0.4082
Total			225	51				
Average			14.06	3.19	22.29		0.4042	1.2954

Y = T, C; R = A, T; V = A, C, G; B = T, C, G; H = A, T, C; D = A, T, G.

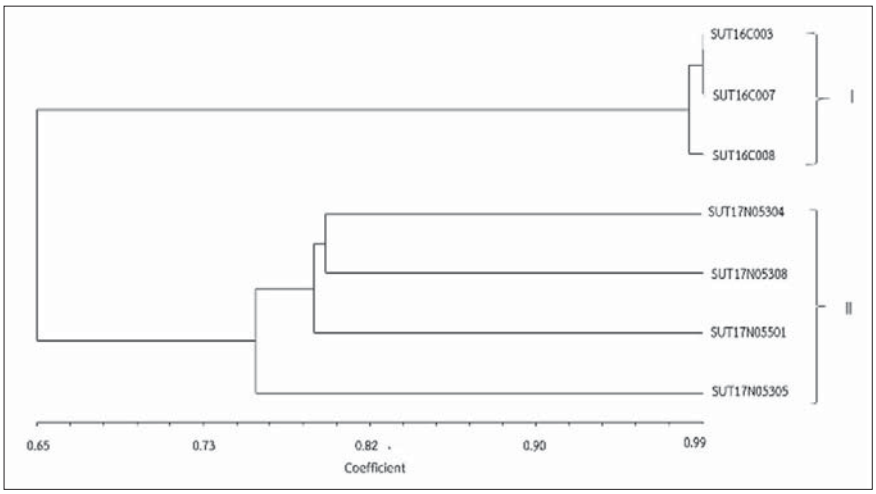


Figure 2. ISSR marker derived dendrogram of black rot resistant mutants and non-mutagenized controls.

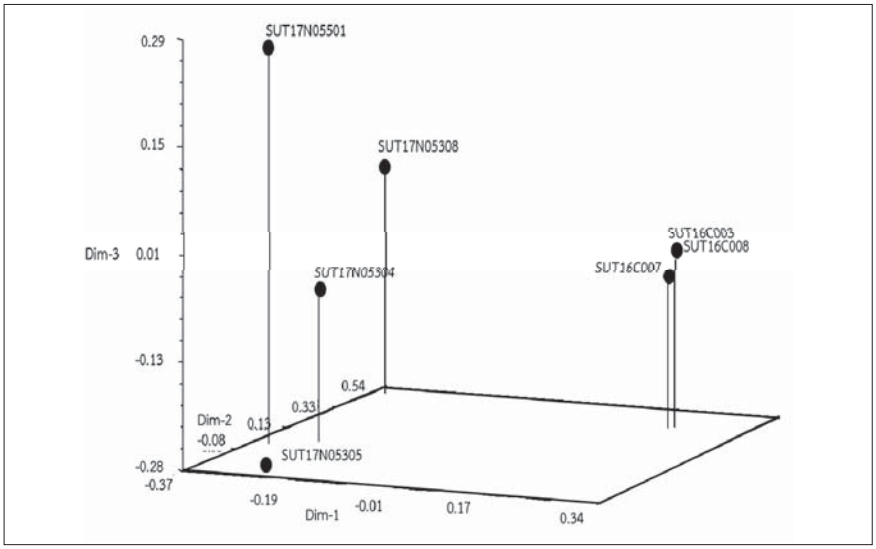


Figure 3. ISSR derived three-dimensional plot based on the first three principal coordinates from a principal coordinate analysis of black rot resistant mutants and non-mutagenized controls.

ed using all 51 polymorphic ISSR bands. Using the Mantel test, the cophenetic correlation coefficient value reached 0.903 ($p < 0.01$), indicating that the similarity matrix data were represented well by the dendrogram. Two different clusters were identified at the genetic similarity level of 0.73 (Figure 2). All three non-mutagenized controls (SUT16C003, SUT16C007, and SUT16C008) were classified as cluster I, while all the black rot resistant mutants (SUT17N05304, SUT17N05305, SUT17N05308, and SUT17N05501) were allocated to cluster II.

Among the pairwise combinations of materials studied, Jaccard's genetic similarity coefficients ranged from 0.722 to 0.990 with an average of 0.897. It was noted that these non-mutagenized controls and the black rot resistant mutants were also different in their morphological characters, i.e., number of leaves and number of roots. All the members of the non-mutagenized control group had higher genetic similarity with an average of 0.974. In the black rot resistant mutant group, the range of similarity coefficients was 0.845 (SUT17N05305 and SUT17N05501) to 0.898 (SUT17N05304 and SUT17N05501) with an average of 0.867.

The genetic distance between the resistant mutants and non-mutagenized controls was 0.186 GD. These results demonstrate that NaN_3 has high potential for inducing mutations and will be useful for a *Dendrobium* breeding program. The mechanism of NaN_3 is associated with the synthesis of organic metabolites such as an amino acid analogue L-azidoalanine [N_3-CH_2-CH (-NH_2)-COOH] or L-cysteine, and O-acetylserine sulfhydrylase is simultaneously found in this process. This enzyme can catalyze the binding of azide or sulfide with O-acetylserine to produce L-azidoalanine (N_3-CH_2-CH (NH_2)-COOH) or L-cysteine (Klášterská et al., 1976). This L-azidoalanine metabolite, an amino acid analogue, can induce point mutation during DNA replication (Kredich, 1971; LaVelle and Mangold, 1987; Owais and Kleinhofs, 1988), causing transversion, such as purine replacing pyrimidine base or vice versa (A or G to T or C) (Al-Qurainy and Khan, 2009).

PCoA with three-dimensional plots based on all of the polymorphic loci was generally consistent with the UPGMA cluster analysis with the three coordinates accounting for 58.9, 11.9, and 11.5% with a total of 82.3% of the total variance. PCoA could clearly separate the materials studied into two different groups (Figure 3), suggesting that it is effective in generating a good separation for these materials.

An ISSR marker can efficiently identify genetic differences among the resistant mutants and non-mutagenized controls. It should be noted that the variation of ISSR loci may not necessarily reflect the variation of plant phenotypes because ISSR loci may be located in non-coding regions of the genome, where the change of DNA sequences is not limited by the maintenance of gene function, resulting in a more rapid evolutionary change (Johns et al., 1997; Chawla et al., 2002). Using ISSR markers for the assessment of the genetic variability profiles of the resistant mutant and non-mutagenized control DNA proved to be useful for the improvement of *Dendrobium*.

Investigation of chromosomal variation of black rot resistant *D.* 'Earsakul' mutants using flow cytometry

The average DNA content and genome size of black rot resistant mutants (3.77 to 3.90 pg $2C^{-1}$ and 3,640 to 3,764 Mbp) were significantly higher than those of non-mutagenized controls (3.41 to 3.49 pg $2C^{-1}$ and 3,290 to 3,372 Mbp). Among these, the black rot resistant mutants (SUT17N05305 and SUT17N05501) had the highest DNA content (3.90 pg $2C^{-1}$) and genome size (3,762 and 3,764 Mbp, respectively), but with no significant difference from the remaining black rot resistant mutants (SUT17N05304 and SUT17N05308) (Table 6; Figure 4). These results are consistent with the previous study of Jones et al. (1998) who showed that the nuclear DNA content of 26 orchid genera ranged from 1.53 to 4.23 pg $2C^{-1}$ nuclei for *D. cruentum* and *D. spectabile*. Choopeng et al. (2019) observed that the DNA content of orchid mutants could increase from 33 to 50% of the original DNA content. It should further be noted that the genome size of our mate-

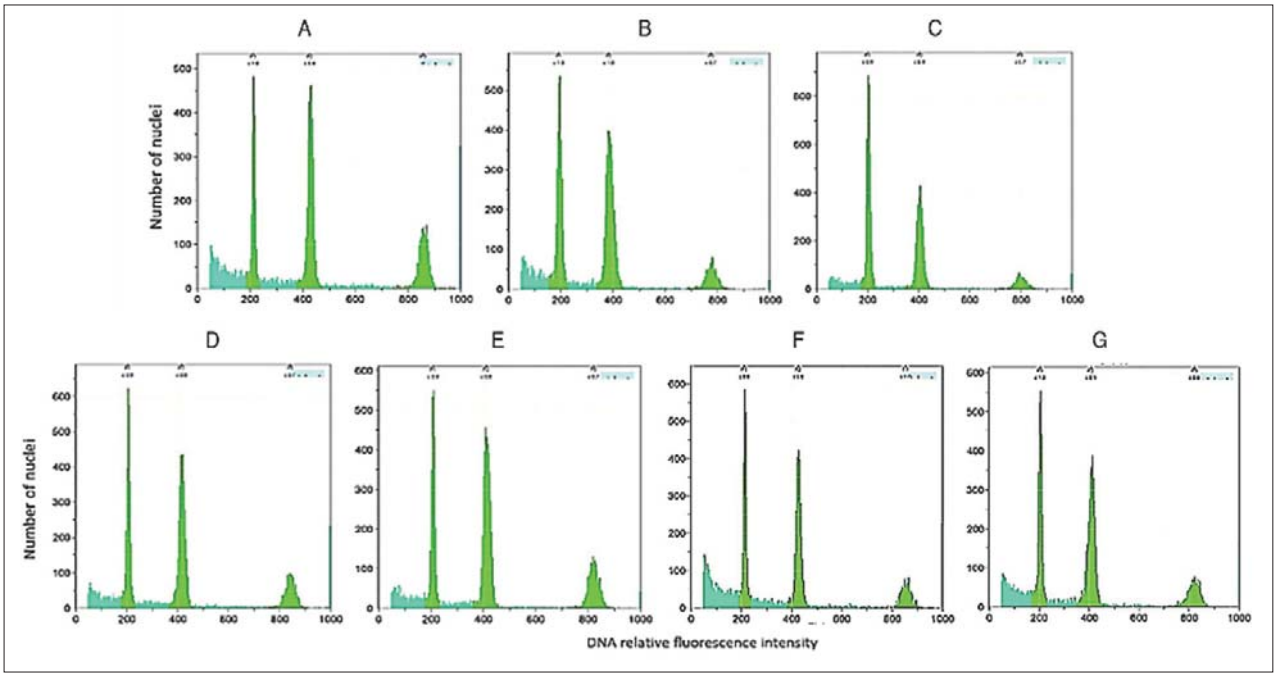


Figure 4. Flow cytometry histogram of non-mutagenized controls (A–C) and resistant mutants (D–G). A) SUT16C003; B) SUT16C007; C) SUT16C008; D) SUT17N05304; E) SUT17N05305; F) SUT17N05308, and G) SUT17N05501.

Table 6. DNA content and genome size in picograms and mega base pairs for black rot resistant mutants and non-mutagenized controls.

Lines	DNA content (pg 2C^{-1})	Genome size (Mbp)
SUT16C003	3.41 ± 0.17 b[1]	3,290 b
SUT16C007	3.47 ± 0.01 b	3,344 b
SUT16C008	3.49 ± 0.07 b	3,372 b
SUT17N05304	3.77 ± 0.04 a	3,640 a
SUT17N05305	3.90 ± 0.04 a	3,762 a
SUT17N05308	3.79 ± 0.05 a	3,660 a
SUT17N05501	3.90 ± 0.02 a	3,764 a
F-test	**	**

[1]: Means in the same column with different letters are significantly different among lines (DMRT) at $p < 0.05$.
**: Highly significant ($p < 0.01$).

rials studied may depend on DNA content. This is consistent with the results of Meesawat et al. (2008) who reported that genome size of *D. crumenatum* increased due to DNA content. The increasing/decreasing DNA content and genome size may result from multiple point mutation occurring in DNA or RNA in which nucleotide bases are added or changed, or duplication/deletion of chromosomes after NaN_3 mutagenesis. In addition, the mutation may cause cell division abnormalities in the phase of a cell cycle, especially in the G1/G0 phase, which result in higher DNA content and genome size in mutants than non-mutagenized plants (Jones et al., 1998; Khan et al., 2009).

Flow cytometry analysis of black rot resistant mutants and non-mutagenized controls revealed that a peak at channel 200 (2*n*) was related to the G0/G1 of diploid plants, while the peaks at about 400 (4*n*) and 800 (8*n*) were tetraploid and octoploid, respectively. All black rot resistant mutants and non-mutagenized controls possessed all peaks of 200, 400, and 800, indicating the presence of diploid, tetraploid, and octoploid cells, called mixoploid (2*n* + 4*n* + 8*n*). The results obtained from this study confirmed the usefulness of flow cytometry as an efficient method for evaluating black rot resistant mutants since ploidy determination can be accomplished non-destructively with a small portion of tissues. Also, it can analyze large populations of cells, where mixoploidy is present (Doležel et al., 1998). Yenchon and Te-chato (2014) reported that this method could characterize the polyploidy induction of *D. formosum* PLBs by colchicine and showed that treatment with 0.20% colchicine for 48 h gave the highest number of tetraploid plantlets (75%). Based on chromosome observation, these tetraploid plantlets had a chromosome number of 2*n* = 4*x* = 76, whereas diploid plantlets had 2*n* = 2*x* = 38.

Characterization of protein changes following infection in black rot resistant mutants and non-mutagenized controls

An evaluation of changes in protein profiles of black rot resistant mutants and non-mutagenized controls following *P. palmivora* inoculation from 0–5 days was carried out.

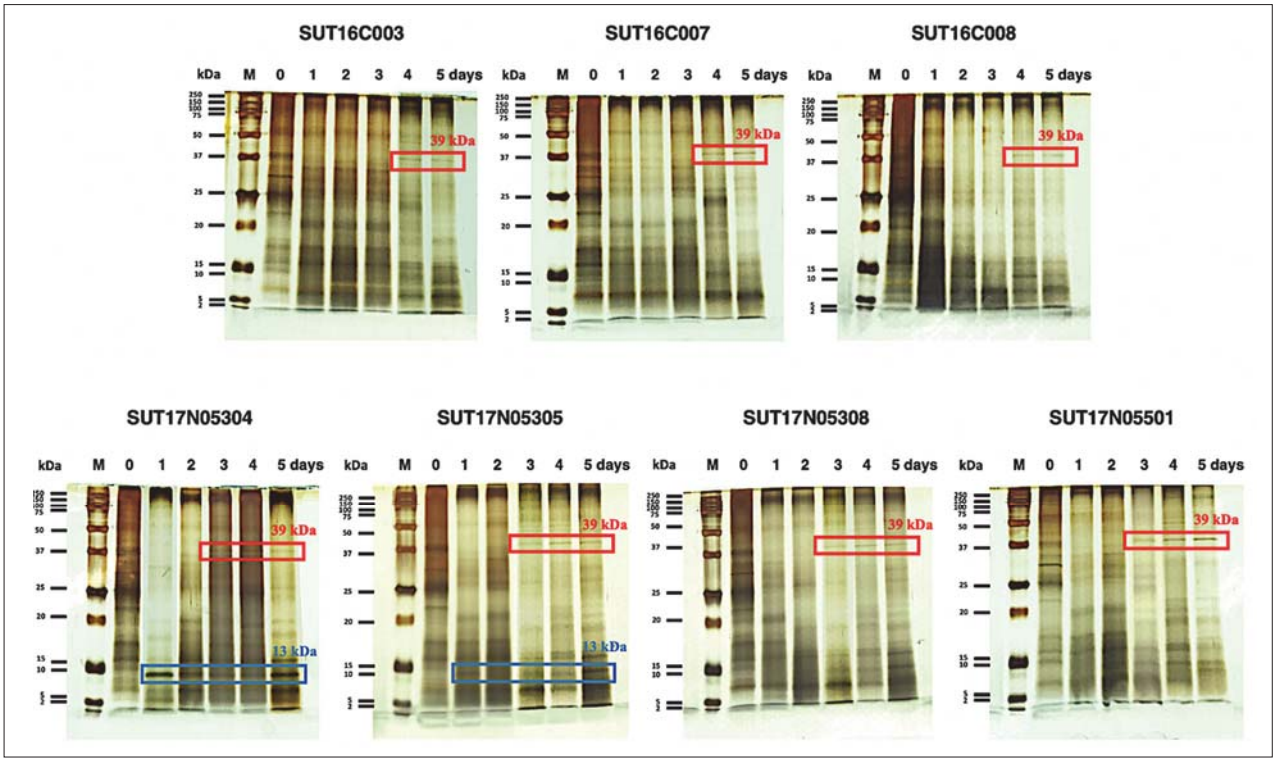


Figure 5. Protein profiles of black rot resistant mutants and non-mutagenized controls at 0–5 days following the *P. palmivora* inoculation using SDS-PAGE.

Leaves of non-mutagenized controls developed very small, localized lesions at the infection sites, and hyphae were also found on leaves after 1 day of spore inoculation, while very small, localized lesions were found on 4 black rot resistant mutants after 3 days. Lesion development on black rot resistant mutants was very slow, and minimal symptoms were observed after 5 days and even after several days. By contrast, non-mutagenized controls changed to brown lesions (rot) with hyphae covering the leaf surface and the surrounding area after 5 days. The infected leaves were collected every day after inoculation and extracted to reveal their protein profiles. These changes in protein profiles may be specific to plant pathogen interactions.

Plants have developed various resistant mechanisms to defend themselves against pathogens which include the production of low molecular weight secondary metabolites, proteins, and peptides having antifungal activities (Singh et al., 2014; Hualsawat, 2019). While considering the protein profiles of these materials using SDS-PAGE analysis with calibrating protein markers (2–250 kDa), two clearly up-regulated protein bands of ca. 13 and 39 kDa MW were observed. One up-regulated protein band of 39 kDa expressed in all black rot resistant mutants was detected in leaves at 3–5 days after inoculation. This protein band was also observed in all non-mutagenized controls but at 4–5 days after inoculation (Figure 5). In addition, a 13 kDa protein was only present in two of the black rot resistant mutants (SUT17N05304 and SUT17N05305), suggesting that the resistance mechanisms of these resistant mutants may differ. Both up-regulated proteins may be related to PR-proteins, i.e., β-1,3-glucanase and chitinase, which are believed to have vital roles in plant defense against fungal pathogen, as observed by Saikia et al. (2005) who showed the induction of β-1,3-glucanases with a molecular mass in the range of 23 to 39 kDa upon *Fusarium oxysporum* f. sp. *ciceri* infection in chickpea. In addition, most chitinases having molecular masses in the range of 15 to 43 kDa were also found when the plants respond to disease infection (Golshani et al., 2015). In grapevine resistant mutants derived from *in vitro* selection, Jayasankar et al. (2000) observed the induction of chitinase with a molecular mass of 36 kDa in response to an *Elinoe ampelina* infection. It is possible that the 13 kDa protein found in this study may be chitinase and the 39 kDa protein may be chitinase or β-1,3-glucanase, but further analysis would be needed to verify this hypothesis. These resistant *Dendrobium* mutants will be further evaluated for black rot resistance at the greenhouse level, and the alteration of their enzyme activities related to resistance mechanisms will also be analyzed. However, SDS-PAGE analysis can identify protein profile changes that may be related to defense mechanisms of black rot resistant mutants and non-mutagenized controls. This knowledge will be highly beneficial for *Dendrobium* breeding to improve black rot resistance in the future.

Conclusion

In summary, our results suggest that NaN_3 is effective in inducing black rot resistance and affecting different morphological, genetic, DNA content and genome size, and protein profile characteristics of *D.* 'Earsakul' mutants. The combined utilization of ISSR markers, flow cytometry, and SDS-PAGE together with *in vitro* mutagenesis will be beneficial for the future development of *D.* 'Earsakul' with black rot resistance.

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