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Investigation of 2-Aminopurine Effect on Mutation Induction in Hoya Hearts (*Hoya kerrii*)

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Abstract

Hoya hearts (*Hoya kerrii*) is a decorative plant with beautiful leaf shapes and long-lasting flowers. Creation of new clones of this plant through *in vitro* mutagenesis has potential for genetic diversity and economic development. **Aim:** This study aims to investigate the impact of 2-Aminopurine (2-AP) on mutation induction in *Hoya kerrii* and assess its effects through morphological, anatomical, and molecular evaluations. **Materials and Methods:** *In vitro*-cultured segments of *Hoya kerrii* stems were treated in 2-AP solutions of 0%; 0.15%; 0.25%; 0.35%; 0.45% concentrations for 1 hour before culturing into a shoot regeneration medium. Regenerated shoots were subcultured in root formation medium before acclimatisation. An anatomical analysis of leaf petioles and genotypic variation of mutant plants was carried out using double-dyeing with *Carmin vert d'iode* and RAPD and ISSR markers. The highest shoot regeneration rate (100%) was observed in the control and 0.15% 2-AP treatments, while the lowest was at 0.45% 2-AP (81%). *In vitro* root formation using 0.25% 2-AP achieved the greatest root quantity and length, while 0.35% had the lowest. The 0.25% 2-AP treatment also resulted in the highest acclimatisation rate of 98.41%, alongside noticeable floral changes, such as distinct petal morphology and pistil colouration. **Result:** An anatomical analysis of leaf petioles showed that increasing the concentration of 2-AP increased petiole diameter, epidermal thickness, and vascular tissue area. Genotypic analysis using RAPD and ISSR markers indicated that the 0.25% 2-AP sample showed the greatest genetic divergence compared to the wild type.

Major Findings: These findings suggest that the 0.25% 2-AP concentration is optimal for inducing stable mutations in Hoya hearts, enhancing both phenotypic traits and genetic diversity for ornamental breeding.

Keywords: 2-AP, *Hoya kerrii*, *In vitro*, ISSR, Mutation, RAPD

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1. Introduction

The genus Hoya (Family: Apocynaceae) has more than 500 species, comprising mainly epiphytes and is geographically distributed in South America, Southeast Asia, Indo-Malesia and Australian regions. Most of the species are cultivated for their ornamental, aromatic and showy flowers[1]. The commonly cultivated ornamental plant *Hoya kerrii*, also known as "Hoya hearts", has gained increasing popularity due to its star-shaped flowers and distinctive, wax-coated, heart-shaped succulent leaves[2]. In Vietnam, tissue-cultured *Hoya kerrii* was regarded as a reliable source of plantlets that can provide large quantities for horticulturists. Additionally, developing new clones of *Hoya kerrii* would enhance the diversity of this species, thereby better meeting the needs of horticulturists[3].

Biotechnological tools and approaches have emerged as viable alternatives to traditional breeding programs, particularly in the genetic improvement of crops. Among these approaches, *in vitro* mutagenesis stands out as a pioneering technique in creating variation among existing breeding material as well as screening of superior mutants. This technique has wide applications in bolstering both crop yield and quality and increasing resistance to different abiotic and biotic stresses[4]. One of the chemicals used in the mutagenesis process is 2-Aminopurine (2-AP), with a

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chemical formula of $C_5H_5N_5$, a purine analogue of guanine and adenine, which is capable of promoting mismatch pairing between base pairs during DNA synthesis. This interaction induces DNA damage and potentially leads to molecular-level mutations by causing errors in nucleotide incorporation[5,6]. Unlike potent physical agents such as gamma rays, X-rays, and other chemical agents like alkylating compounds (which may induce both beneficial and detrimental mutations in plants), 2-AP is considered a profitable mutagen, generating predominantly point mutations and fewer lethal mutations in plants[7].

Botanical anatomical studies enable the observation of internal changes in plants from tissue to organ levels, allowing morphological trait identification in the mutant types[8-11]. In this study, the anatomical structures (counting and analysing specific tissues of certain organs) of mutant *Hoya kerrii* clones were examined to observe differences between mutants and wild type. Besides morphological investigation, surveys of genotypes are also conducted. Successful genetic population studies and detection of genetic diversity in various plant species have been achieved using RAPD (Random Amplified Polymorphic DNA), ISSR (Inter-Simple Sequence Repeats), or a combination of both techniques[12,13]. RAPD and ISSR, PCR-based markers, were utilised as indicators, requiring minimal DNA samples and obviating the need for radioactive labelling, presenting simpler and quicker alternatives to other markers[12]. The RAPD technique, owing to its random primer sequences, is straightforward and easily executed[14]. ISSR has been demonstrated as a highly precise, rapid, simple, and cost-effective marker for assessing genetic diversity and delineating differences among closely related crop varieties, particularly in plant species with limited genomic sequence information. Neither marker necessitates prior knowledge of the genome sequence of the species studied, rendering them suitable for assessing mutation-induced changes. Therefore, RAPD and ISSR were used to evaluate the molecular-level changes in mutant clones and the wild type. However, there is a lack of scientific research to support the cultivation demand or to induce mutations for new phenotypic strains of *Hoya kerrii*. This study aimed to investigate the mutagenic effects of 2-AP on Hoya heart plants, and to apply morphological and anatomical observations, along with molecular markers (RAPD and ISSR), to assess the genetic diversity of mutant Hoya heart lines in response to the needs of the ornamental plant market and scientific research.

2. Materials and Methods

2.1 Materials

One-year-old samples of *Hoya kerrii* plants were cultivated at the Institute of Food and Biotechnology, Can Tho University. Chemicals employed in tissue culture comprised commercial MS medium (Duchefa Biochemie - Netherlands), 96° Ethanol (NAFACO Manufacturing and Distribution Co., Vietnam), IBA (Indole-3-butyric acid), IAA (Indole-3-acetic acid), NAA (α-Naphthaleneacetic acid), BAP (6-Benzylaminopurine) (Duchefa - Netherlands), 2-Aminopurine (Sigma Aldrich - USA), sucrose (Bien Hoa Trading Co., Ltd. - Vietnam), agar (Robika Hai Long - Vietnam).

Chemicals used for staining and anatomical sectioning include sodium hypochlorite, acetic acid (VWR Chemicals - USA), carmin alum - vert d'iod, and glycerine. Chemicals utilized in DNA extraction and PCR reactions encompass PVP 40 - Polyvinyl pyrrolidone (Thermo Scientific - USA), SDS 10% (Clini Sciences - France), Isopropanol (VWR Chemicals - USA), purified 96% Ethanol (VWR Chemicals - USA), Chloroform (QReC - Malaysia), Isoamyl alcohol (VWR Chemicals - USA), agarose (Biobasic - Canada), Reaction buffer 5X, Taq DNA Polymerase, RAPD primers, ISSR primers (Bioline - Meridian Bioscience), and 100bp plus standard ladder (Bioline - Meridian Bioscience).

2.2 Methods

2.2.1 Effects of 2-AP on Regeneration Ratio and Variation Induction in Hoya kerrii Shoots in Vitro

Segments of *in vitro Hoya kerrii* stems (1-2 cm with axillary buds) were regenerated from one-year-old plants on basic MS medium supplemented with 1 mg/L IBA and 3.5 mg/L BAP. These nodal segments were immersed in solutions of 2-AP at concentrations of 0%, 0.15%, 0.25%, 0.35%, and 0.45% for one hour. Then they were rinsed three times with sterile distilled water and cultured on a basic MS medium supplemented with 1 mg/L IBA and 3.5 mg/L BAP. This experiment was laid out in completely randomized design, consisting of 5 treatments with 7 replicates, each containing 3 excised single-bud stem segments culturing in a vessel. The regeneration rate of the sample was observed and used as materials for the next experiment.

2.2.2 Root Formation

Hoya kerrii shoots regenerated from differenent concentrations of 2-AP treated (ranging 3-5 cm in

length), which were subcultured into basic MS medium supplemented with three different auxins (IBA, IAA, and NAA) at a consistent concentration of 0.8 mg/L. The experiment was carried out in two-factor design (2-AP treatment levels and type of auxin), replicated 5 times, with each plot containing 2 vessels and 3 regenerated shoots per vessel. The quantity and length of roots were observed at 40 days after culture.

2.2.3 *Acclimatization of Micropropagated Plants in the Nethouse*

Plantlet which had more than 2 roots, each root exceeding 1 cm in length, and attaining a plant height of 4 to 6 cm, were acclimatized in three different substrate formulations. (CT1) comprising 90% tribat soil + 10% composted manure; (CT2) composed of 45% tribat soil + 22.5% coconut coir + 22.5% rice husk + 10% composted manure; and (CT3) containing 45% coconut coir + 45% rice husk + 10% composted manure. The two-factor experiment design was set up with substrate formulations and plantlet at varying concentrations of 2-AP. The survival rate of plantlets at the 16-week was observed. The mean temperature under nethouse during experimental period ranged between 26-38°C at daytime and 23-26°C at night, and 30-80% relative humidity, photosynthetically active radiation 300 µmol m^{-2} s^{-1}.

2.2.4 *Investigating the Anatomical Differences in Leaf Petiole Morphology of Hoya kerrii Clones*

Sixteen-week-old *Hoya kerrii* plants grown in nethouse conditions were selected for this study. For each level of 2-AP treatment, one plant exhibiting distinct leaf morphologies from the control sample was chosen. Five leaves from each selected plant were subjected to sectioning (specifically, the second or the third leaf counting from the apex). Each leaf was cut at the petiole into ten thin transverse sections, which were stained doubly with carmine alum - vert d'iod. Then, three random slices were chosen to measure. Microscopic images were captured using a optical microscope equipped with a digital camera (Olympus CX23). Leaf petiole diameter (µm) was measured and observed at 60X magnification, vascular bundle area (µm²), epidermal thickness (µm), number of vascular bundles and number of calcium oxalate crystals were observed, photographed and recorded at 150X magnification.

2.2.5 *Evaluation of Genetic Differences between Hoya kerrii Mutant and Control Samples using RAPD and ISSR Markers*

Hoya kerrii individuals displaying distinct morphologies were utilized for DNA extraction through an improved CTAB (Cetyltrimethylammonium bromide) method (Rogers and Bendich, 1988). Ten RAPD primers (OPF 04, OPF 03, OPF 02, OPF 05, OPD 05, OPS 11, OPB 11, OPK 14, OPT 05, OPC 05) following Williams' approach[14] and four ISSR primers (ISSR 13, ISSR K2, ISSR 22, ISSR 27) were employed. For RAPD-PCR the thermal cycle consisted of an initial denaturation at 94°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 45 seconds, annealing at 35°C for 15 seconds, and extension at 72°C for 1 minute and 20 seconds. A final extension step was performed at 72°C for 10 minutes. For ISSR-PCR, the thermal cycle began with an initial denaturation at 94°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 45 seconds, primer-specific annealing for 45 seconds (56.6°C for ISSR13, 53.5°C for ISSR K2 and ISSR 22; 47.9°C for ISSR 03), and extension at 72°C for 80 seconds. The final extension was conducted at 72°C for 10 minutes. Each sample underwent PCR with two replicates. The RAPD-PCR and ISSR-PCR products were examined on a 2% agarose gel supplemented with 0.01% safeview at 50V using a 100 bp plus ladder. The DNA bands observed on the gel were visualized using a BIO-RAD UV device. Images of the DNA bands post-electrophoresis were analyzed using GelAnalyzer v2019 software[15] and binary encoded for genetic grouping via NTSYS 2.1 software using UPGMA clustering method. These data would construct a similarity matrix to generate dendrograms reflecting the genetic relationships among the samples[16]. The data obtained from the experiments were entered and processed using Excel 2013. One-way ANOVA and two-way ANOVA were conducted, and differences (between the mean values of the treatments) were compared using Tukey's test in Minitab 16 software at a 5% significance level.

3. Results and Discussion

3.1 Effects of 2-AP on Regeneration Ratio and Variation Induction in *Hoya Kerrii In vitro*

After 40 days of culture period, mutation induction experiments using 2-AP at different concentrations showed regeneration rates ranging from 81% to 100%,

with a statistically significant difference at the 5% level. The 0.15% 2-AP treatment and the control (without 2-AP) showed the highest regeneration rate (100%), significantly different from the 0.45% 2-AP treatment (81%) but not from the other treatments. This result is consistent with previous publications on 2-AP, which is considered a point mutagen that causes mispairing by binding to nucleotides. Point mutations are easily accumulated because they are less lethal in experimental subjects[7].

3.2 Root Formation

The survival rate of plants when transferred from *in vitro* culture to nursery conditions relies heavily on the root system quality, where auxin plays a pivotal role in efficient root formation. Particularly, the two critical factors for root systems are their quantity and length. Table 2 shows significant differences in root quantity between conditions with and without 2-AP supplementation at all levels. This suggested the influence of 2-AP on root development in *in vitro Hoya kerrii*. The control treatment exhibited the highest average root quantity at 6.86 roots/shoot. Regarding 2-AP concentrations, except for the treatment of 0.25% 2-AP, all other treatments displayed lower root quantities compared to the control (Table 2). The 0.25% 2-AP treatment achieved the highest average root quantity at 5.29 roots/shoot, statistically similar to the control (5.21 roots/shoot) but significantly different from other treatments. In terms of root induction, IBA demonstrated the most efficient root-stimulating capabilities, with an average root quantity of 5.08 roots/shoot. This was statistically higher than treatments supplemented with NAA (4.80 roots/shoot) and IAA (3.69 roots/shoot). There is an interaction between the two studied factors, 2-AP concentration, and root-inducing agents. Without 2-AP, the NAA root-inducing agent achieved the highest root quantity at 6.86 roots/shoot in the control. However, in the presence of 2-AP, IBA and NAA had varying effects on root quantity across different treatment levels, including 0.15% 2-AP combined with IBA (6.09 roots/shoot), 0.25% 2-AP combined with IBA (5.67 roots/shoot), and 0.25% 2-AP combined with NAA (5.62 roots/shoot).

Similar to root quantity, the maximum root length was observed in the control, measuring 4.39 cm, statistically differing from other treatments. Root lengths in the mutated treatments fluctuated without consistency. Conditions involving the root-inducing agent IBA exhibited the longest roots at 3.45 cm, while the shortest were recorded at treatment with IAA induction. However, these differences were statistically insignificant when compared to treatment containing NAA. From both the results of root quantity and length of mutated and control *Hoya kerrii* clones, IBA proved effective in stimulating root formation. This result is consistent with the study by Don (2021), which showed that 1000 mg/L IBA was the most effective concentration for Hoya stem cuttings, resulting in the highest number and length of roots in both *Hoya imperialis* and *Hoya coronaria*[17]. Roots serve as vital nutrient absorption and transport organs in plant life, responsible for water and mineral uptake and facilitating nutrient translocation throughout the plant, anchoring it firmly in the soil. Longer and well-distributed roots enhance a plant's adaptability and survivability. As Table 2 showed that the sample treated with 0.25% 2-AP displayed higher root quantities and lengths compared to other mutated samples. This suggested a higher potential for adaptability and improved survival compared to other experiments with different 2-AP concentrations when acclimated in nursery conditions. The morphology of 40-day-old plantlets (Figure 1) depicts healthy growth, with some treatments displaying multiple shoots, and no abnormal morphological traits were noted in conditions treated with 2-AP.

Table 1. Survival rate of *Hoya kerii* after 40 days of cultivation

Treatment	Concentration of 2-AP (%)	Survival rate (%)
Control	0.00	100.0[a]
1	0.15	100.0[a]
2	0.25	90.5[ab]
3	0.35	85.7[ab]
4	0.35	81.0[c]
P-value		0.042
CV(%)		7.6

The data was converted to the square root for statistical analysis; numbers followed by the same letters a, b, c are not significantly different at 5%.

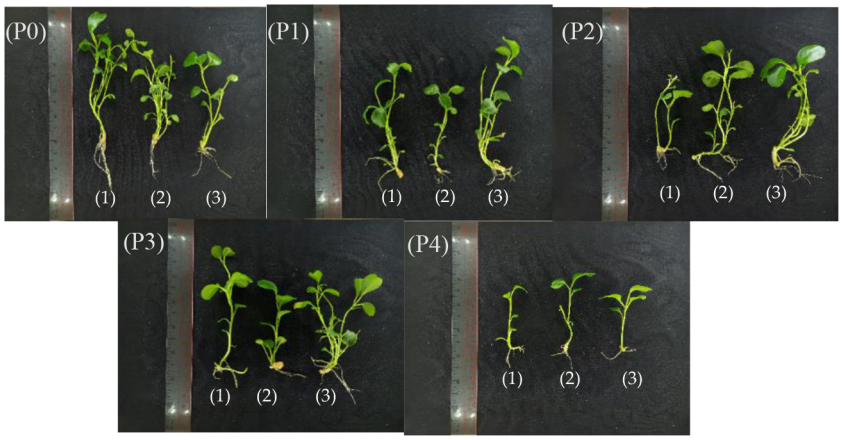
3.3 Acclimatisation of Micropropagated Plantlets in the Nethouse

After 4 weeks of acclimatisation, the survival rates of various *Hoya kerrii* clones were recorded. The 0.25% 2-AP treatment exhibited the highest survival rate at 98.41%,

Table 2. The quantity and length of roots in various clones of *Hoya kerrii* at the 40-day mark of *in vitro* cultivation

Sample	Quantity				Length			
	Auxin			Average (root/shoot)	Auxin			Average (cm)
	NAA	IAA	IBA		NAA	IAA	IBA	
Control	6.86^{a}	4.10^{fg}	4.67^{ef}	5.21^{A}	5.31^{a}	4.31^{b}	3.54^{cd}	4.39^{A}
0.15% 2-AP	3.57^{gh}	4.67^{ef}	6.09^{b}	4.78^{B}	2.47^{f}	1.64^{g}	2.55^{f}	2.22^{D}
0.25% 2-AP	5.62^{bc}	4.57^{ef}	5.67^{bc}	5.29^{A}	2.42^{f}	3.64^{c}	5.01^{a}	3.69^{B}
0.35% 2-AP	3.05^{hi}	2.57^{i}	3.52^{gh}	3.05^{D}	1.21^{h}	1.37^{gh}	2.62^{f}	1.74^{E}
0.45% 2-AP	4.91^{de}	2.52^{i}	5.43^{cd}	4.29^{C}	3.17^{de}	3.10^{e}	3.51^{cd}	3.26^{C}
Average (cm)	4.80^{B}	3.69^{C}	5.08^{A}		2.92^{B}	2.81^{B}	3.45^{A}	
P (Sample)				0.00				0.00
P (rooting inducer)				0.00				0.00
P (interacting)				0.00				0.00
CV (%)				7.41				6.48

In the same column or row, numbers followed by the same letters A, B, C, D, E are not statistically different at the 5% significance level. In the same column and row, numbers followed by the same letters a, b, c, d, e, f, g, h, i are not statistically different at the 5% significance level.
Note: NAA: Naphthalene acetic acid; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid



Notes: (P0): Control; (P1): 0.15% 2-AP; (P2): 0.25% 2-AP; (P3): 0.35% 2-AP; (P4): 0.45% 2-AP; (1): 0.8 mg/L NAA; (2): 0.8 mg/L IAA; (3): 0.8 mg/L IBA.

Figure 1. The root formation of *Hoya kerrii* after 40 days of culture.

statistically similar to the treatment of 0.45% 2-AP and the treatment of 0.15% 2-AP, which were recorded at 93.65% and 88.89%, respectively. The control treatment had the lowest survival rate at 58.73%, statistically differing from other treatments (Table 3).

The results indicated that although the control treatment demonstrated the highest root quantity and length in the *in vitro* root induction experiment, it exhibited the lowest survival rate under nursery conditions. Adaptability (survival rate) of a plant is influenced by multiple factors such as genotype, environment, and their interaction. Mutations can lead to variations in growth, development, and response to various environmental conditions of the plant (nutrition, light, temperature, etc)[10]. In this study, the survival rates of mutated clones were higher compared to the control under nursery conditions. CT1 (90% Tribat soil + 10% composted manure) showed a high survival rate (95.24%), suggesting it is a practical and affordable alternative to imported substrates. In contrast, CT2 and CT3, which lacked soil-based components, had significantly lower survival rates (below 45%), likely due to poorer nutrient retention. A previous study using a 1:1 peat moss and perlite mix reported 100% survival in *Hoya imperialis* and *Hoya coronaria*[17], confirming the importance of a rich, moisture-retentive medium for *Hoya* propagation. Thus, CT1 can be considered a suitable substitute when peat-based materials are limited.

3.4 Investigating the Anatomical Differences in Leaf Petiole Morphology of *Hoya kerrii* Clones

The anatomical analysis of mutated *Hoya kerrii* clones revealed notable differences in the diameter of leaf petiole, quantity of calcium oxalate crystals, the epidermal thickness, area of vascular tissue, and the quantity of vascular bundle between the 2-AP-treated mutated clones and the control clone (Table 4). Generally, an increase in the concentration of the 2-AP mutation treatment correlated with an increase in leaf petiole diameter, epidermal thickness, and area of vascular tissue. Concerning calcium oxalate crystals, the highest density was observed in the 0.15% 2-AP treatment, totalling 56.4 crystals per sample, followed by the 0.25% 2-AP treatment with 53.2 crystals/sample. The increase of calcium oxalate crystals within cells assists plants in regulating calcium in tissues, maintaining ion balance, detoxifying metals, and limiting herbivore damage, notably enhancing leaf rigidity[18]. Plants lack mobility like animals, thus requiring various protective measures against herbivory. Calcium is an essential micronutrient for plants, while oxalate aids in plant defence against herbivory as the presence of calcium oxalate irritates herbivores that consume them[19].

Regarding epidermal thickness, the 0.45% 2-AP treatment achieved the highest at 61.2 µm, while the lowest was observed in the 0.15% 2-AP treatment at 38.0 µm. Similarly, in the area of vascular tissue, the highest was recorded at 206.98 µm2 in the 0.45% 2-AP treatment, and the lowest was observed at 98.51 μm^2 in the 0.15% 2-AP treatment. As the concentration of 2-AP increased, both the epidermal thickness and the area of vascular tissue climbed. An increase in epidermal thickness and area of vascular tissue could support plant adaptation and tolerance to natural conditions[20,21]. The quantity of vascular bundles reached the highest in the control treatment at 25.6 bundles, with no statistically significant difference compared to the 0.45% 2-AP treatment. The lowest vascular bundle quantity was observed in the 0.35% 2-AP treatment, but it was not significantly different from the 0.15% 2-AP treatment. The presence of 2-AP appeared to reduce the vascular bundle quantity in the mutated treatments compared to the control. The size and quantity of vascular bundle might be linked to leaf rigidity, support capabilities, and water and mineral transport[16,21]. The impact of 2-AP at different concentrations on the morphology of these tissues varies, and the choice of 2-AP concentration will depend on the specific objectives of the study.

Observations of floral morphology in the mutated clones displayed remarkable differences compared to the wild type (Figure 3). The petal shapes in the 0.15% and 0.25% 2-AP mutated treatments appeared star-shaped, deeply lobed, and distinctly separated petals, in contrast to the 0.35% and 0.45% 2-AP mutated treatments, as well as the control. The morphology of petals with 0,45% 2-AP treatment is similar to 0,15% 2-AP treatment, whereas in the 0.35% 2-AP treatment, the petal shape

Table 3. The survival rate of various *Hoya kerrii* clones under nethouse conditions at the 4-weeks after acclimatisation

Sample	Substrate Formula			Average (%)
	(CT1)	(CT2)	(CT3)	
Control	95.24^{a}	38.09^{c}	42.86^{c}	58.73^{C}
0.15% 2-AP	100.00^{a}	85.72^{ab}	80.95^{ab}	88.89^{AB}
0.25% 2-AP	100.00^{a}	95.24^{a}	100.00^{a}	98.41^{A}
0.35% 2-AP	95.24^{a}	57.14^{bc}	90.48^{a}	80.95^{B}
0.45% 2-AP	100.00^{a}	95.24^{a}	85.72^{ab}	93.65^{AB}
Average (%)	98.10^{A}	80.00^{B}	74.29^{B}	
P (Variant sample)				0.00
P (Substrate formula)				0.00
P (interacting)				0.00
Cv (%)				18.54

In the same column or row, numbers followed by the same letters A, B, C, D, E are not statistically different at the 5% significance level. In the same column and row, numbers followed by the same letters a, b, c, d, e, f, g, h, i are not statistically different at the 5% significance level.

closely resembled that of the control. While the overall colour of the petals remained consistent across treatments, variations were observed in the colour of the floral pistils between the 2-AP mutated treatments and the control. Remarkably, distinct hues were observed: dark red-brown (control), red (0.15%, 0.25% and 0,45% 2-AP), and pink (0.35% 2-AP). As per the study by Abou-Dahab[22], flower morphology, leaf structure, chlorophyll content, and pigment concentrations of flowers may undergo alterations following mutagenic treatments in plants. External flower colour might be regulated by the concentration of pigments present within the flower[22]. Molecular biological analyses also indicate genomic changes in the mutated clones, leading to variations in floral morphology compared to the wild type[23]. Treatments with 0.15% 2-AP recorded changes in the number of petals. Specifically, in some flowers, the number of petals was reduced by one petal compared to the control treatment, which was five petals. The impact of 2-AP leads to phenotypic changes in the number of petals, leading to genetic diversity in this flower.

3.5 Investigating and Evaluating the Genetic Differences of Mutant *Hoya kerrii* Samples and Control Samples Using RAPD and ISSR Markers

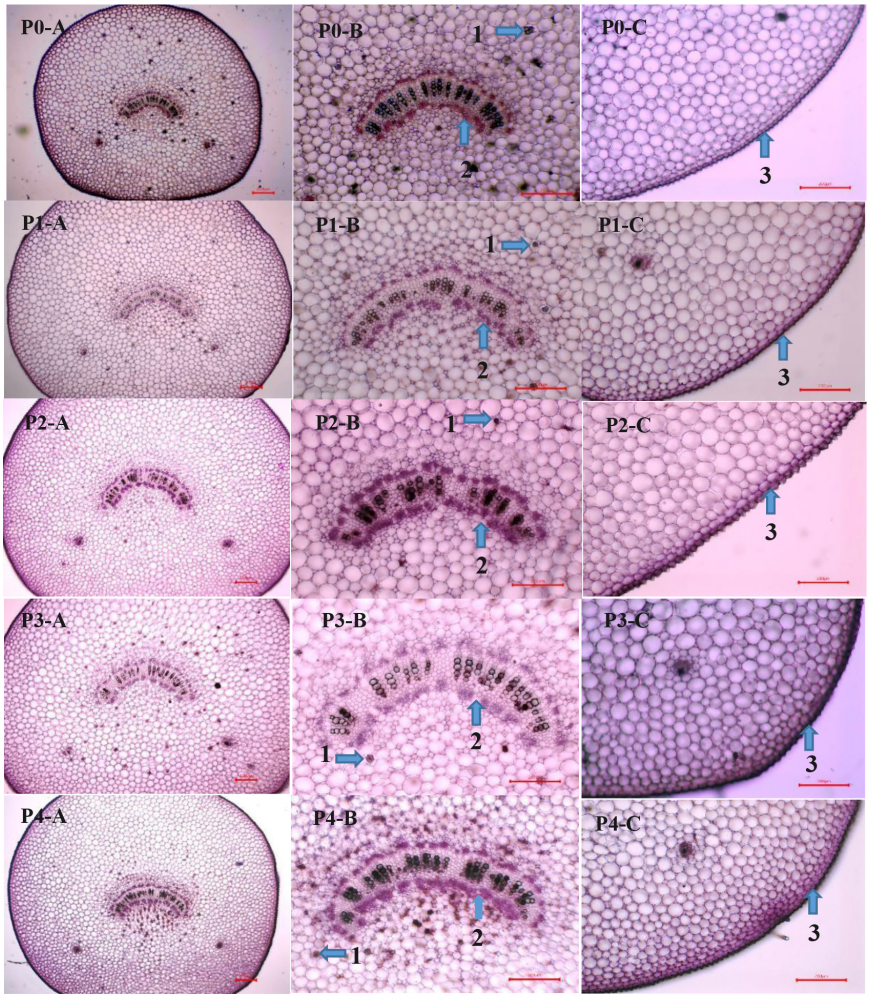
The individual *Hoya kerrii* underwent morphological examinations that were analysed genomically using 10 RAPD markers and 4 ISSR markers. The results showed that all primers showed polymorphism. The Polymorphic Information Content (PIC) ranged between 0.10 and 0.46, with an average of approximately 0.26. The use of RAPD and ISSR techniques to detect mutations in plants, bacteria, and microorganisms, when compared to wild types, has been considered efficient in genetic diversity studies[12,24,25]. For the OPF 02 primer, DNA bands of sizes 680bp and 300bp appeared in samples P1, P2, and P3 (0.15% 2-AP, 0.25% 2-AP, 0.35% 2-AP) and were absent in sample P0 (control). A single DNA band of 600bp appeared only in the control sample and was absent in the other samples. In terms of the primers OPB 11 and OPK 14, amplified DNA bands of 950bp, 1325bp, and 270bp, respectively, appeared in sample P2 and were absent in the other samples. The ISSR marker K2 identified polymorphic DNA bands in sample P4 (0.45% 2-AP) as shown in Figure 2.

The appearance of additional or missing DNA segments in the 2-AP mutation-treated sample compared to the control sample shows the impact of 2-AP on the *Hoya kerrii* genome, possibly including the region gene carrier and non-gene carrier[26]. Genetic changes may lead to the emergence of novel phenotypes in 2-AP-treated *Hoya kerrii* clones compared to the wild type[27,28]. In this study, the polymorphism of RAPD markers was higher than that of ISSR markers, as indicated by the average PIC value, aligning with the research of Nei and Li[16]. Several other studies also showed similar results, suggesting that RAPD markers exhibit higher polymorphism than ISSR markers in various plant species[29,30]. This discrepancy might be attributed to the proportion of coding and non-coding sequences within the genome and the difference in genomic composition of different plant species[12]. However, some studies suggest that ISSR markers are more polymorphic than RAPD markers[12,31]. This is explained by ISSR markers being repeat sequence regions distributed throughout the genome, with high mutation rates and association with important loci about function[18], while RAPD markers utilise random primers and neutrally select sequences[12].

Table 4. The criteria of the morphological and anatomical of leaf petioles of *Hoya kerrii* clones

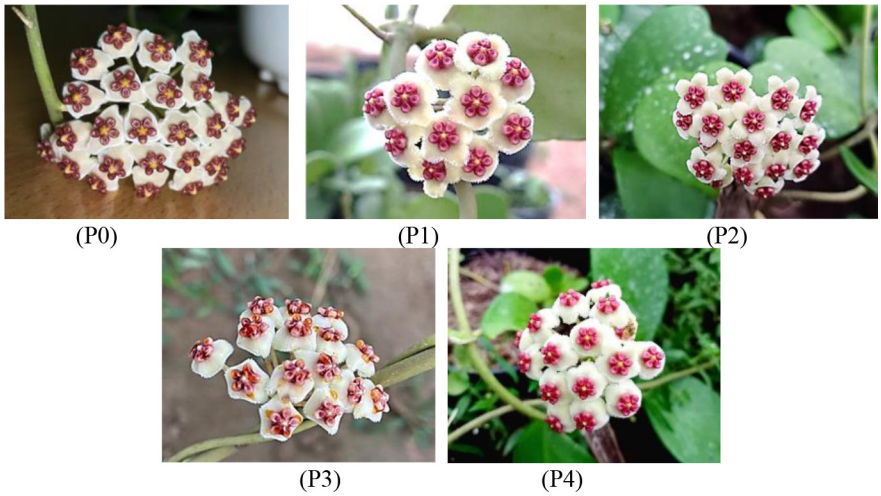
Sample	Diameter (µm)	Quantity of Calcium Oxalate	Epidermal Thickness (µm)	Area of Vascular Tissue (µm²)	Quantity of Vascular Bundle
Control	2783^{b}	46.0^{c}	43.8^{c}	151.48^{bc}	25.6^{a}
0.15% 2-AP	2355^{c}	56.4^{a}	38.0^{e}	98.51^{d}	19.6^{c}
0.25% 2-AP	2726^{b}	53.2^{b}	41.2^{d}	149.37^{c}	22.2^{b}
0.35% 2-AP	2844^{b}	15.2^{e}	52.8^{b}	160.65^{b}	18.0^{c}
0.45% 2-AP	3165^{a}	42.6^{d}	61.2^{a}	206.98^{a}	23.8^{ab}
P-value	0.00	0.00	0.00	0.00	0.00
Cv (%)	3.61	2.23	1.24	4.00	5.83

In the same column, numbers followed by the same letters a, b, c are not significant different at 5%.



Notes : (P0): Control ; (P1): 0.15% 2-AP ; (P2): 0.25% 2-AP ; (P3): 0.35% 2-AP; (P4): 0.45% 2-AP; (A): Leaf petiole taken using ocular micrometers E4; (B): Vascular bundle taken using ocular micrometers E10; (C): Dermal tissue taken using ocular micrometers E15; 1: Calcium oxalate crystals; 2: Vascular bundle; 3: Dermal tissue.

Figure 2. Anatomical morphology of leaf petioles of *Hoya kerrii.*



Notes : (P0): Control; (P1): 0.15% 2-AP; (P2): 0.25% 2-AP; (P3): 0.35% 2-AP; (P4): 0.45% 2-AP

Figure 3. Flower morphology of *Hoya kerrii* clones in *in vitro* conditions.

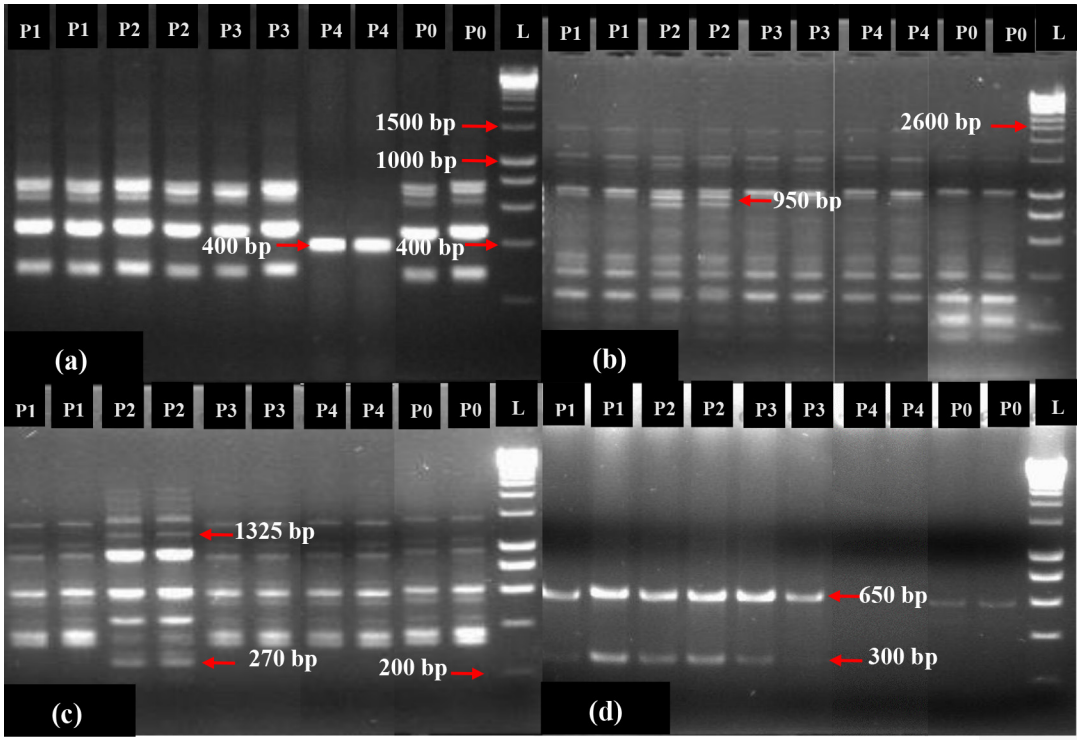


Figure 4. Electrophoretic images of RAPD and ISSR markers in *Hoya kerrii* samples treated with 2-AP compared to the control sample.

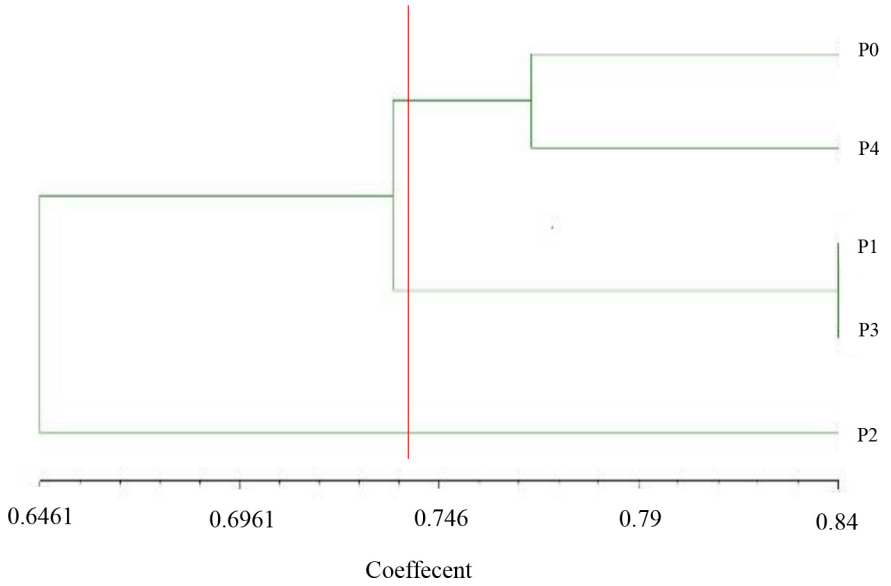


Figure 5. Phylogenetic diagram of *Hoya kerrii* samples treated with 2-AP compared to the control sample.

Based on the RAPD and ISSR analysis results, the phylogenetic diagram of the surveyed *Hoya kerrii* clones was divided into 3 distinct clusters, with similarity coefficients ranging from 0.65 to 0.85 (Figure 5). From the diagram, it can be observed that the increase in 2-AP concentration did not widen the genetic distance between the mutated and control clones. In the case of sample P2, the lowest similarity coefficient observed in comparison to the control sample was 0.55 and was separated into a distinct branch on the phylogenetic diagram. This difference might be attributed to sample P2 (treated with 0.25% 2-AP) showing more variations

in nucleotide sequences that directly affected the binding sites of RAPD and ISSR markers[25], resulting in differences in the genotypes and phenotypes of *Hoya kerrii*.

4. Conclusion

This study demonstrates that 2-Aminopurine (2-AP) is an effective agent for inducing both morphological and genetic variation in *Hoya kerrii*. Among all tested concentrations, 0.25% 2-AP produced the most promising results. Morphologically, it resulted in symmetrical, star-shaped flowers with distinct lobes and red pistils. Anatomically, the petiole diameter, epidermal thickness, and vascular tissue area were significantly increased. In terms of acclimatization, plants treated with 0.25% 2-AP achieved the highest survival rate (98.41%) across all substrates. Genetically, RAPD and ISSR marker analyses revealed that this treatment induced the greatest genetic divergence from the control, with the lowest similarity coefficient (0.55). These findings suggest that 0.25% 2-AP is an appropriate concentration for inducing stable mutations in *Hoya kerrii*, offering potential for the development of new ornamental varieties.

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6. References

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