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Pest resistance by molecular mechanism: effect of chemical mutagens on lemon butterfly (*Papilio demoleus*) incidence in acid lime (*Citrus aurantifolia* Swingle.)

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Abstract

Citrus cultivation, particularly acid lime (*Citrus aurantifolia* Swingle.), faces significant yield losses due to insect pests, of which the lemon butterfly (*Papilio demoleus* L.) is among the most economically damaging at nursery and juvenile plant stages. Conventional chemical control is increasingly constrained by environmental and regulatory concerns, necessitating investigation into alternative resistance mechanisms. The present study evaluated the potential of two chemical mutagens — Ethyl Methane Sulphonate (EMS) and Colchicine — at ten incremental concentrations (0.1% to 0.5%) on the incidence of *Papilio demoleus* larvae on acid lime seedlings under field conditions at Uttar Banga Krishi Viswavidyalaya, Pundibari, West Bengal (2018-2020). A Completely Randomized Design (CRD) with 10 treatments and 3 replications involving 50 plants per observation was used. Results demonstrated a clear and statistically significant inverse relationship between mutagen concentration and larval infestation. Colchicine at 0.5% concentration reduced infestation to as low as 4% compared to 66% in the untreated control, outperforming EMS (6% at 0.5%) across all concentration levels. Higher mutagen doses are hypothesized to alter secondary metabolite profiles, surface wax composition and other physico-chemical plant attributes that collectively enhance pest deterrence. These findings open avenues for chemically induced resistance as a component of integrated pest management in citrus.

Keywords: Acid lime, *Papilio demoleus*, EMS, colchicine, chemical mutagen, pest resistance, *Citrus aurantifolia*, induced resistance, molecular mechanism

1. Introduction

Citrus is among the most economically important fruit crops in tropical and subtropical regions of the world, cultivated across more than 140 countries with a global production exceeding 140 million metric tonnes annually (FAO, 2022) ^[5]. In India, citrus occupies a prominent position in horticulture, with acid lime (*Citrus aurantifolia* Swingle.) being one of the most widely grown species due to its high vitamin C content, flavour and broad culinary, medicinal and industrial utility (Singh *et al.*, 2019) ^[23]. Taxonomically, acid lime belongs to the division Magnoliophyta, class Magnoliopsida, order Sapindales, family Rutaceae, subfamily Aurantioideae, with a chromosome number of $2n = 2x = 18$ and is considered to have originated in North-Eastern India (Swingle and Reece, 1967) ^[24].

Despite its economic significance, citrus cultivation in India is severely constrained by a diverse range of insect pests. More than 250 species of insects and mites have been reported infesting various citrus species across the country (Patel *et al.*, 2020) ^[18]. Among these, the lemon butterfly (*Papilio demoleus* Linnaeus, Lepidoptera: Papilionidae), commonly known as the citrus butterfly or lime butterfly, is considered one of the most damaging pests at the nursery and juvenile growth stages. The larval stage of *P. demoleus* feeds voraciously on tender foliage and new flushes of grown-up trees, causing defoliation that can lead to stunted growth and significant economic losses (Jahnavi *et al.*, 2018; Ehsanullah *et al.*, 2017) ^[10, 4].

Conventional pest management in citrus predominantly relies on chemical insecticides. However, repeated and indiscriminate use of pesticides has led to insecticide resistance in pest populations, environmental contamination, disruption of natural enemy populations and growing consumer concerns regarding pesticide residues in fresh produce (Roditakis *et al.*, 2017; Sharma *et al.*, 2021) [20, 22]. This underscores the urgent need for sustainable, eco-friendly alternatives, including host plant resistance (HPR) as a component of integrated pest management (IPM).

Host plant resistance — the ability of a plant to reduce or tolerate pest damage — can be conferred through physical barriers (trichomes, surface waxes, leaf toughness), biochemical constituents (alkaloids, phenolics, terpenes, limonoids), or a combination thereof (Painter, 1951; Bergman *et al.*, 2020) [17, 2]. Induced resistance through mutagenic treatments represents a novel approach to alter the physiological and biochemical landscape of a plant, potentially enhancing pest deterrence.

Ethyl Methane Sulphonate (EMS) is a potent alkylating agent that induces point mutations predominantly via G/C to A/T transitions and is widely used in plant mutation breeding for generating genetic diversity (Kim *et al.*, 2006; Haughn and Bhatt, 2002) [11, 8]. Colchicine, an alkaloid derived from *Colchicum autumnale*, inhibits spindle fibre formation during mitosis, leading to polyploidization, altered cell morphology and modified secondary metabolite biosynthesis (Morejohn, 1991; Dhooche *et al.*, 2011) [16, 3]. Both agents have the potential to modify plant architecture and chemistry in ways that could influence pest behaviour and feeding preference.

The present investigation was therefore undertaken to evaluate the effect of EMS and Colchicine at graduated concentrations on the incidence of lemon butterfly (*Papilio demoleus* L.) larvae on acid lime (*Citrus aurantifolia* Swingle.) seedlings, with the broader objective of understanding chemically induced resistance mechanisms as a sustainable pest management strategy.

2. Materials and Methods

2.1 Experimental Site and Duration

The experiment was conducted during the years 2018 to 2020 at the Instructional Farm of the Department of Fruit Science (Pomology and Post-Harvest Technology), Uttar Banga Krishi Viswavidyalaya (UBKV), Pundibari, Cooch Behar, West Bengal, India. The experimental site is located at approximately 26°19'N latitude and 89°22'E longitude, at an altitude of about 43 m above mean sea level, with a sub-tropical humid climate characterized by hot humid summers and mild winters, typical of the sub-Himalayan terai zone of West Bengal (Kumar *et al.*, 2015) [14].

2.2 Plant Material

Uniform seedlings of acid lime (*Citrus aurantifolia* Swingle.) var. Kagzi lime were raised from seeds in nursery beds and subsequently used as the experimental material. Seeds were surface-sterilized with 0.1% mercuric chloride solution for 3 minutes, rinsed thoroughly in sterile distilled water and sown in germination trays containing sterilized sand and soil (1:1, v/v). Seedlings of uniform size (15-20 cm height, approximately 6 weeks old) were selected for treatment.

2.3 Mutagen Treatments

Two chemical mutagens were employed: (i) Ethyl Methane Sulphonate (EMS) (Hi-Media, India) and (ii) Colchicine (SRL Chemicals, India). EMS solutions were freshly prepared in phosphate buffer (pH 7.0) at the time of treatment. Colchicine solutions were prepared in distilled water. Seeds and seedlings were treated by soaking in respective mutagen solutions for 6 hours at room temperature (25±2 °C), followed by thorough washing with running tap water to remove residual chemical. The treatment schedule comprised 10 levels for each mutagen, ranging from 0.1% to 0.5% in increments of 0.05%, along with an untreated control (T₁). Seeds/seedlings for the control treatment were soaked in distilled water for an equivalent duration. All mutagen handling was performed with appropriate personal protective equipment following biosafety protocols.

2.4 Experimental Design

The experiment was laid out in a Completely Randomized Design (CRD) with 10 treatments and 3 replications per treatment. Each replication comprised 50 seedlings, yielding a total of 150 plants per treatment and 1,500 plants per mutagen series. The treatments are summarized in Table 1 below. Seedlings were maintained under standard nursery conditions with uniform irrigation, nutrition and weed management protocols throughout the experimental period.

Table 1: Treatment Details for EMS and Colchicine Mutagen Series

Treatment	EMS Concentration	Colchicine Concentration
T ₁	Control (distilled water)	Control (distilled water)
T ₂	0.10% EMS	0.10% Colchicine
T ₃	0.15% EMS	0.15% Colchicine
T ₄	0.20% EMS	0.20% Colchicine
T ₅	0.25% EMS	0.25% Colchicine
T ₆	0.30% EMS	0.30% Colchicine
T ₇	0.35% EMS	0.35% Colchicine
T ₈	0.40% EMS	0.40% Colchicine
T ₉	0.45% EMS	0.45% Colchicine
T ₁₀	0.50% EMS	0.50% Colchicine

2.5 Data Recording

Pest incidence was recorded as the percentage of infested seedlings per treatment per replication. Observations were made at fortnightly intervals throughout the experimental period. The incidence of *Papilio demoleus* larvae was assessed by direct visual counting of larvae on each plant. A plant was considered infested if one or more larvae or characteristic feeding damage (irregular notching, excised leaf margins, frass deposition) were observed. Monthly data were pooled seasonally and overall mean percentages were calculated for statistical analysis. Special attention was paid to the period July to August, which was identified as the peak infestation window.

2.6 Statistical Analysis

Data were expressed as percentage infestation and subjected to analysis of variance (ANOVA) appropriate for a CRD. Treatment means were compared using Critical Difference (CD) at 5% level of significance. Standard Error of Mean [SE(m)], Standard Error of Difference [SE(d)] and Coefficient of Variation (CV) were computed using standard statistical procedures (Gomez and Gomez, 1984).

3. Results and Discussion

3.1 Seasonal Pattern of Infestation

The incidence of *Papilio demoleus* larvae on acid lime seedlings was recorded throughout the two-year experimental period (2018-2020). Infestation occurred in all months, consistent with the year-round reproductive activity of *P. demoleus* in sub-tropical conditions. However, peak infestation was observed during July to August, coinciding with the monsoon flush of new growth that provides tender foliage for larval feeding. This seasonal pattern aligns with findings reported by Ehsanullah *et al.* (2017) [4], who documented peak lemon butterfly larval incidence in citrus nurseries of Sylhet, Bangladesh, during the monsoon season. Jahnvi *et al.* (2018) [10] similarly recorded heightened larval activity of *P. demoleus* on acid lime during periods of active vegetative growth. The positive correlation between new flush development and larval population density has been attributed to the higher nitrogen content and softer cell walls of juvenile foliage (Awasthi, 2001) [1].

3.2 Effect of EMS on Lemon Butterfly Incidence

Treatment with EMS at varying concentrations resulted in a progressive and statistically significant reduction in lemon butterfly larval infestation relative to the untreated control (Table 2). The control treatment (T₁) recorded the highest infestation at 66%, which is consistent with the naturally high susceptibility of acid lime seedlings to *P. demoleus* in the absence of any management intervention. Application of 0.1% EMS (T₂) reduced infestation to 60%, representing only a modest 9.09% reduction. As concentration increased through T₃ (0.15%, 54%), T₄ (0.20%, 50%), T₅ (0.25%, 42%), T₆ (0.30%, 38%), T₇ (0.35%, 32%) and T₈ (0.40%, 22%), a consistent dose-dependent decline in infestation was evident. The lowest infestation in the EMS series (6%) was recorded at T₁₀ (0.5% EMS), representing a 90.91% reduction from control. The CD values (2.544) confirmed that differences between treatments were statistically significant at $p \leq 0.05$.

EMS-induced mutations primarily function through alkylation of guanine residues in DNA, leading to mispairing and subsequent G:C → A:T transitions (Haughn and Bhatt, 2002) [8]. At higher concentrations, EMS may induce mutations in biosynthetic pathways governing secondary metabolite production, including limonoids, flavonoids and essential oils — compounds known to possess insect-antifeedant and toxic properties (Roy *et al.*, 2016; Rehman *et al.*, 2014) [21, 19]. Additionally, EMS treatment at higher concentrations has been reported to increase stomatal density and epicuticular wax deposition in some crops, which may physically impede larval feeding (Kulkarni *et al.*, 2000) [13]. The relatively lower resistance induced by EMS compared to Colchicine in the present study may reflect differences in the specific mutation spectrum and their downstream effects on plant defence chemistry.

3.3 Effect of Colchicine on Lemon Butterfly Incidence

Colchicine treatments demonstrated a stronger and more consistent reduction in *P. demoleus* infestation across all concentration levels compared to EMS (Table 2). Starting from T₂ (0.1% Colchicine, 62%), infestation declined progressively through T₃ (0.15%, 56%), T₄ (0.20%, 48%), T₅ (0.25%, 40%), T₆ (0.30%, 34%), T₇ (0.35%, 22%), T₈ (0.40%, 18%), T₉ (0.45%, 10%), to the minimum of 4% at T₁₀ (0.5% Colchicine), a 93.94% reduction from control. At

every comparable concentration, Colchicine consistently resulted in lower infestation than EMS and this divergence became more pronounced at higher concentrations (e.g., at 0.35% and above).

These superior results with Colchicine may be attributed to its well-documented polyploidizing effects. Colchicine inhibits tubulin polymerization, thereby arresting cells at the metaphase stage and resulting in chromosome doubling (polyploidization) in treated cells (Morejohn, 1991; Dhooghe *et al.*, 2011) [16, 3]. Polyploid plants are generally known to exhibit enhanced secondary metabolite production — including increased concentrations of limonoids, flavonoids and acridone alkaloids in Rutaceae species — due to gene dosage effects and amplified flux through biosynthetic pathways (Lavania, 2005; Kour *et al.*, 2022) [15, 12]. The characteristic limonoids of citrus (e.g., limonin, nomilin) are well-established insect antifeedants and growth regulators for Lepidopteran species, including *P. demoleus* (Roy *et al.*, 2016; Hasegawa *et al.*, 2000) [21, 7]. Furthermore, polyploidization often results in altered stomatal size, increased cuticular thickness and modified trichome morphology — physical traits that can independently reduce larval attachment and feeding efficiency (Lavania, 2005) [15].

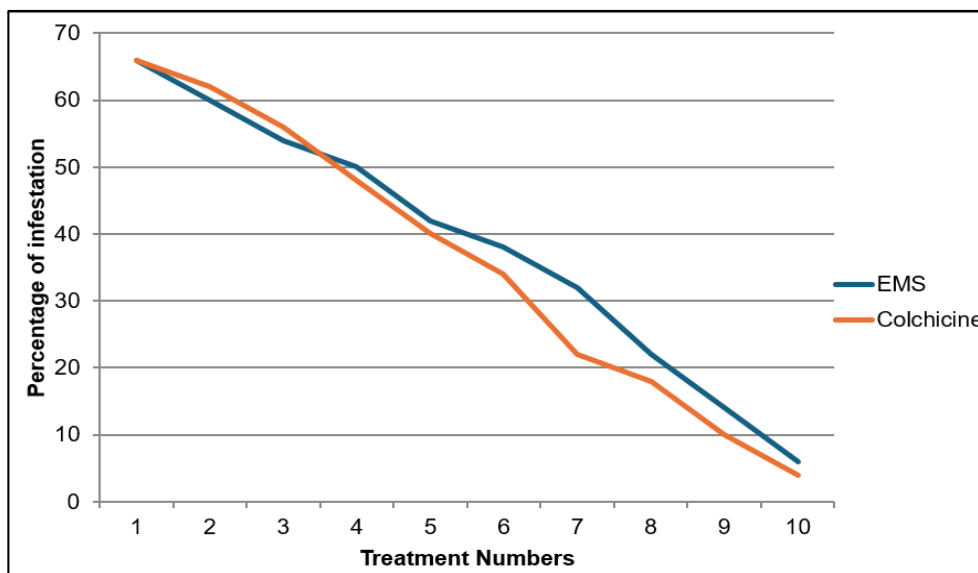
3.4 Comparative Analysis and Statistical Validation

The overall trend across both mutagen series is unambiguous: higher chemical mutagen concentration is associated with significantly lower larval infestation. The dose-response relationship was monotonically decreasing for both mutagens, with no reversal or plateau observed within the tested concentration range, suggesting that even higher concentrations might confer even greater resistance — though phytotoxicity thresholds would need to be assessed. The CD values (EMS: 2.544; Colchicine: 2.712) confirm that adjacent treatment means are significantly different, indicating a fine-grained, stepwise increase in resistance with concentration. The low coefficient of variation values (EMS: 3.863%; Colchicine: 4.392%) indicate high precision and reliability of the experimental data.

Table 2: Incidence of Lemon Butterfly (*Papilio demoleus*) Larvae on Acid Lime Seedlings Treated with EMS and Colchicine at Varying Concentrations

Treatment	EMS Infestation (%)	Colchicine Infestation (%)	EMS Reduction (%)	Colchicine Reduction (%)
T ₁ (Control)	66	66	-	-
T ₂ (0.10%)	60	62	9.09%	6.06%
T ₃ (0.15%)	54	56	18.18%	15.15%
T ₄ (0.20%)	50	48	24.24%	27.27%
T ₅ (0.25%)	42	40	36.36%	39.39%
T ₆ (0.30%)	38	34	42.42%	48.48%
T ₇ (0.35%)	32	22	51.52%	66.67%
T ₈ (0.40%)	22	18	66.67%	72.73%
T ₉ (0.45%)	14	10	78.79%	84.85%
T ₁₀ (0.50%)	6	4	90.91%	93.94%
C.D.	2.544	2.712	-	-
SE(m)	0.856	0.913	-	-
SE(d)	1.211	1.291	-	-
C.V. (%)	3.863	4.392	-	-

Values represent mean percentage infestation across 3 replications. CD = Critical Difference ($p \leq 0.05$); SE(m) = Standard Error of Mean; SE(d) = Standard Error of Difference; C.V. = Coefficient of Variation (%)



3.5 Proposed Molecular and Physico-Chemical Resistance Mechanisms

Based on the present results and corroborating literature, the following molecular and physico-chemical mechanisms are proposed to underlie the observed pest resistance:

(i) Enhanced Secondary Metabolite Biosynthesis: Both EMS-induced point mutations and Colchicine-induced polyploidy may activate or amplify the phenylpropanoid, terpenoid and alkaloid biosynthetic pathways in citrus. Elevated concentrations of limonoids, flavanones (e.g., hesperidin, naringenin) and acridone alkaloids are known to reduce larval palatability and interfere with midgut digestive enzymes of Lepidopteran pests (Roy *et al.*, 2016; Rehman *et al.*, 2014) ^[21, 19].

(ii) Alterations in Cuticle and Surface Wax Composition: Mutagen treatments, particularly Colchicine, have been reported to modify epicuticular wax content and composition, which can reduce oviposition site selection by adult *P. demoleus* females and impede larval movement and feeding (Lavania, 2005; Bergman *et al.*, 2020) ^[15, 2].

(iii) Activation of Defence Gene Networks: EMS-induced mutations may produce loss-of-function or gain-of-function alleles in regulatory genes governing jasmonate (JA) and salicylate (SA) signalling pathways — the primary hormonal cascades mediating herbivore resistance in plants (Howe and Jander, 2008) ^[9]. Upregulation of JA-responsive protease inhibitors and oxidative enzymes (peroxidase, polyphenol oxidase) in mutant lines could contribute to reduced larval growth and survival.

(iv) Morphological Modifications: Polyploid plants arising from Colchicine treatment may exhibit increased leaf thickness, denser trichome coverage and more fibrous cell walls physical deterrents that mechanically impede leaf-chewing herbivores (Lavania, 2005) ^[15]. Biochemical characterization of treated plants in future studies will be essential to confirm these mechanistic hypotheses.

4. Conclusions

The present study conclusively demonstrates that treatment of acid lime (*Citrus aurantifolia* Swingle.) seedlings with chemical mutagens EMS and Colchicine induces a significant, dose-dependent reduction in the incidence of lemon butterfly (*Papilio demoleus* L.) larvae. The key findings are: (i) infestation was highest in the untreated control (66%) and declined progressively with increasing

mutagen concentration for both agents; (ii) Colchicine at 0.5% concentration induced the highest resistance, reducing infestation to 4% (a 93.94% reduction), while EMS at 0.5% reduced infestation to 6% (90.91% reduction); (iii) at every tested concentration, Colchicine outperformed EMS in pest resistance induction; and (iv) peak natural infestation occurs during the July-August monsoon flush period, suggesting that pre-season mutagen treatment protocols may be particularly valuable.

These results support the hypothesis that higher concentrations of chemical mutagens promote the development of pest-resistant phenotypes in citrus, likely through enhanced secondary metabolite production, altered physico-chemical surface properties and activation of molecular defence pathways. Future research should focus on: (a) biochemical characterization of treated plants to identify specific resistance-conferring metabolites; (b) histological analysis of leaf morphology under mutagen treatment; (c) evaluation of the heritability of induced resistance traits in subsequent generations; (d) phytotoxicity assessments to establish safe upper-concentration thresholds; and (e) multi-location trials to validate resistance stability across diverse agro-climatic conditions. The integration of chemically induced resistance into IPM frameworks holds considerable promise for sustainable citrus pest management.

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