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Research Article

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Seed Treatment with Ethyl Methanesulfonate Generates Compact and Phenotypically Diverse M_1 Populations of Polka-Dot Plant (*Hypoestes phyllostachya* Baker)

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Abstract

Background Polka-dot plant (*Hypoestes phyllostachya* Baker) is a popular foliage pot plant valued for its pink-spotted leaves and compact habit, yet induced mutagenesis has not been explored in this species and the useful dose range of chemical mutagens is unknown. We examined how seed treatment with ethyl methanesulfonate (EMS) affects germination, growth and phenotypic variability in the M_1 generation, in order to define a working protocol for mutation breeding.

Results Seeds of the cultivar 'Confetti Compact Red' were soaked in 0.5% or 1% EMS for 30 or 60 min, and germination, plant architecture, biomass, stomata, pigments, leaf color and leaf metabolites of M_1 plants were compared with untreated controls. EMS reduced final germination from 97% to 78–94% and, more markedly, slowed it, halving the germination index and lowering the hazard ratio of germination to 0.27–0.43. EMS promoted a compact habit: 24–42% of treated plants reached the highest compactness score, compared with none of the controls. Shoot fresh weight decreased by 25–33% in all EMS treatments, and 60-min soaks also reduced root biomass. The 0.5% EMS for 30 min treatment produced the most leaves (+18%), the tallest plants, the longest stomata and the highest total phenolics (+36%). Phenotypic dispersion increased under EMS: the coefficient of variation of growth traits rose 1.4-fold on average, and 30–56% of treated plants were multivariate off-types, compared with 2% of controls. Ten M_1 variants with altered habit, leaf shape and pigmentation were selected.

Conclusions Seed treatment with EMS is an effective way to create new variation in polka-dot plant. A soak in 0.5% EMS for 30 min offers the best balance between induced variability and plant vigour and is recommended as a starting point for building mutant populations. The selected variants now need to be advanced to the M_2 generation to test whether their traits are heritable.

Keywords Acanthaceae, Chemical mutagenesis, Foliage plant, Germination kinetics, Induced variation, Mutation breeding, Ornamental breeding, Phenolic compounds, Plant architecture, Stomata

Background

The ornamental plant industry relies on a continuous supply of new cultivars, distinguished by variations in flower or foliage color, plant form, growth rate, and post-production performance. This constant innovation is crucial because novelty fuels consumer demand and provides growers with a competitive advantage [1,2]. Foliage plants constitute a significant and expanding segment of this market. Their value is almost entirely determined by the aesthetic qualities of their leaves and overall plant shape. Consequently, even minor heritable alterations in leaf pattern, size, or branching can lead to a marketable new cultivar. However, for many of these crops, the available gene pool is limited, with cultivated material often descending from a small number of founders. Furthermore, conventional hybridization methods are frequently slow or constrained by issues of sterility and incompatibility [2,3].

Induced mutagenesis offers a complementary route to generating new variation. It creates new alleles within an elite genetic background, allowing for the modification of one or a few traits while preserving the integrity of the cultivar. This approach is particularly attractive for ornamentals, whose commercial value relies on a comprehensive set of traits [3–5]. Ornamentals have benefited from this approach more than any other crop group, with induced or spontaneous mutants of chrysanthemum, carnation, rose, and many foliage plants being released as cultivars [3,6]. Furthermore, mutation breeding is free from the regulatory costs that have restricted the commercial release of transgenic ornamentals [1,2].

Ethyl methanesulfonate (EMS) is the most widely used chemical mutagen in plants. It alkylates guanine at the O^6 position, which then pairs with thymine and produces G/C to A/T transitions; in a large reverse-genetic screen in *Arabidopsis* more than 99% of EMS-induced lesions were of this type [7]. Because EMS induces dense point mutations with relatively few chromosomal rearrangements, it suits both forward screens for visible phenotypes and reverse-genetic approaches such as targeting induced local lesions IN genomes (TILLING) [8,9]. Its effects depend on the dose, which is the product of concentration and exposure time, as well as on temperature, pH and pre-soaking of the seed [9,10]. Too low a dose yields few mutations, whereas too high a dose kills seeds or causes severe physiological damage in the M_1 generation. For this reason, every new species needs its own dose–response study before large populations

are raised, and the reduction of germination or seedling growth is commonly used to choose a working dose [4,9].

EMS has been successfully applied to several ornamentals, primarily through *in vitro* explants. Examples include chrysanthemum floral pedicels and nodal segments [11,12], leaf explants of African violet [13], and seeds of *Chrysanthemum indicum* and carnation [14,15]. Our group previously treated four chrysanthemum cultivars with EMS *in vitro*, confirming induced changes with ISSR and IRAP markers [12], and building on a regeneration system developed for this purpose [16]. In these studies, low to moderate doses typically produced variants in plant height, leaf size, and flower traits, whereas high doses reduced germination, regeneration, and survival. For seed-propagated ornamentals, seed treatment remains the simplest option due to its capacity for treating large numbers of seeds simultaneously and its ease of repeatability [9].

Even in the M1 generation, EMS can alter growth, pigment content, and secondary metabolism. In pepper, EMS-treated M1 plants differed from the wild type in height, flowering date, and leaf architecture [17]. Similarly, in the ornamental *Celosia cristata*, putative M1 mutants exhibited wide variations in phenolic and flavonoid content [18]. Such biochemical shifts are expected, as mutagen-induced oxidative stress activates the phenylpropanoid pathway [19,20]. Enlarged trait variance, rather than a shift in the mean, serves as the most useful early signal that a treatment has generated variation worth selecting [21]. Multivariate summaries of this variance, such as principal component and cluster analyses, are routinely used to describe natural diversity in horticultural germplasm [22,23] and can be applied similarly to mutant populations.

Polka-dot plant (*Hypoestes phyllostachya* Baker, Acanthaceae) is a small evergreen herb native to Madagascar [24]. It is cultivated worldwide as a pot, bedding, and indoor plant, valued for its leaves splashed with pink, red, or white spots. This distinctive pattern is linked to anthocyanin accumulation and is strongly influenced by light [25]. Commercial seed series, such as 'Confetti' and 'Splash Select,' have been bred for brighter colors and a compact habit. Compactness is a critical trait for this crop, so much so that growers often use growth regulators or manage light to prevent leggy growth [25,26]. The fact that at least one patented cultivar originated from a spontaneous branch mutation [27] demonstrates that natural mutation can yield marketable variation in this species. Despite this, we found no published studies on induced mutagenesis in *Hypoestes*. Consequently, the responses of its small seeds to EMS and the effective dose range remain unknown.

In this study, we treated seeds of *H. phyllostachya* 'Confetti Compact Red' with two EMS concentrations for two different exposure times. We then monitored the M1 plants from germination through to the mature pot-plant stage (Fig. 1). Our research addressed three primary questions: First, how do EMS concentration and exposure time, both individually and in combination, influence the extent and speed of germination? Second, how does EMS alter plant architecture, biomass, stomatal traits, pigmentation, and leaf metabolites in the M1 generation? Third, which treatment regimen optimally balances increased phenotypic variability with acceptable vigor, thereby providing a suitable foundation for developing mutant populations? To characterize the M1 response, we employed a time-to-event analysis for germination, factorial partitioning of concentration and duration effects, and multivariate measures of phenotypic dispersion, going beyond conventional comparisons of means.

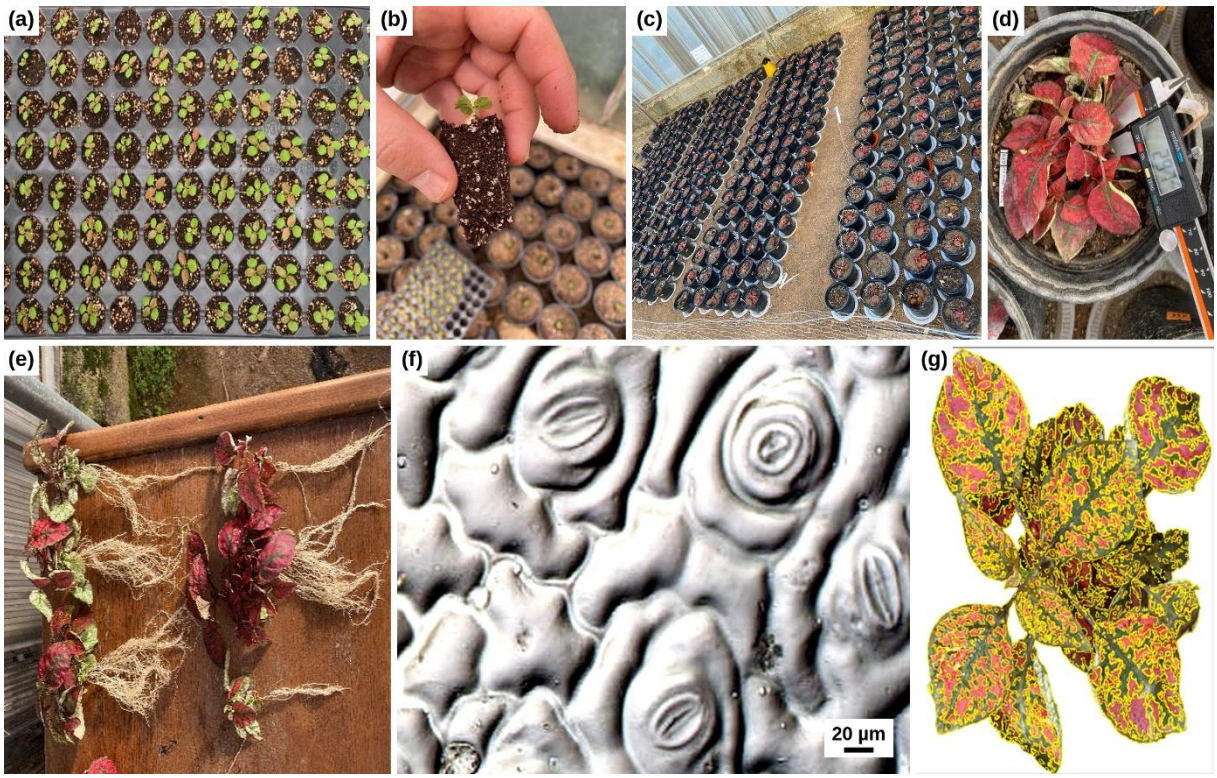


Fig. 1 Experimental workflow. (**a**) germinating seedlings in a plug tray; (**b**) seedling at the three- to four-leaf stage ready for transplanting; (**c**) M_1 plants in the tunnel greenhouse; (**d**) measurement of plant traits with a digital caliper; (**e**) harvested plants used for root and shoot biomass determination; (**f**) close-up of an epidermal impression of the abaxial leaf surface showing stomata (scale bar = 20 µm); (**g**) leaf area classified into green and pink-red fractions by color thresholding in ImageJ.

Methods

Plant material and EMS treatment

Untreated seeds of *Hypoestes phyllostachya* 'Confetti Compact Red' (article O0005126, lot O00084789; origin Sri Lanka) were obtained from Sakata Seed Corporation (Yokohama, Japan). Ethyl methanesulfonate (M0880; ≥ 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The experiment was carried out in the laboratories and greenhouses of the Department of Horticultural Sciences and Engineering, University of Kurdistan, Sanandaj, Iran, from August to December (Fig. 1). Seeds were pre-soaked in distilled water for 8 h and then immersed in EMS at 0.5% or 1% (v/v), prepared in 0.2 M potassium phosphate buffer (pH 7.0), for 30 or 60 min with occasional agitation. Control seeds were soaked in distilled water only. All EMS work was done in a fume hood. After treatment, seeds were rinsed three times for 2 min each in distilled water. The five resulting treatments (control; 0.5% for 30 min; 0.5% for 60 min; 1% for 30 min; 1% for 60 min) were arranged in a completely randomized design.

Growth conditions

Greenhouse conditions throughout the experiment were 24/18 °C day/night temperature, a 16/8 h light/dark photoperiod, 60–70% relative humidity and a photosynthetically active radiation of 650–1200 µmol m^{-2} s^{-1}. Seeds were sown in plug trays and kept under mist in a glass greenhouse during germination (Fig. 1a). Seedlings with three to four true leaves were transplanted into 2 L plastic pots filled with field soil, leaf mould and sand (1:1:1, v/v) (Fig. 1b). Plants were drenched once with thiophanate-methyl (70% WP, 5 g L^{-1}, 50 mL per pot) to prevent damping-off and sprayed three times at 2–3-day intervals with abamectin (2 mL L^{-1}) against whitefly, aphids and mites. On 12 October the pots were moved to a polyethylene tunnel greenhouse (Fig. 1c) and irrigated when the substrate surface had dried, about every 3 days. Plants were fertilised with humic acid (1 g in 50 L; 100 mL per pot) on 9 October, with NPK 10-52-10 (5 g L^{-1}; 200 mL per pot) on 22 October and with NPK 20-20-20 (5 g L^{-1}; 200 mL per pot) on 3 November.

Germination

For each treatment, 100 seeds were sown in plug trays filled with peat moss and perlite (70:30, v/v) and covered lightly with substrate. Germinated seeds (emerged cotyledons) were counted daily for 10 days (30 August to 8 September). From the daily counts we calculated final germination percentage, time to 50% of final germination (T_{50}, by linear interpolation), mean germination time, germination index ($\Sigma\, n_i/t_i$, where n_i is the number of seeds germinated on day t_i), coefficient of velocity, synchronization index (uncertainty, U) [28], peak value and germination value [29].

Morphological measurements

Fifty plants per treatment were measured for plant width, plant height, leaf number, branch number, stem diameter (digital caliper; Fig. 1d) and the length and width of a fully expanded leaf. Plant compactness was scored visually on a four-point scale (0, open; 1, slightly compact; 2, moderately compact; 3, fully compact). Five plants per treatment were harvested (Fig. 1e) to determine root and shoot fresh weight on a digital balance (EK-300i, A&D, Tokyo, Japan) and dry weight after oven drying at 85 °C for 72 h. The whole M_1 population was screened visually, and plants with distinctive habit or foliage were labelled and photographed.

Stomata and leaf color

Epidermal impressions were taken from the abaxial surface of fully expanded leaves with clear nail varnish and transparent tape and examined under a light microscope (BX51, Olympus, Tokyo, Japan) (Fig. 1f). Stomatal length and width (n = 4 leaves per treatment) and stomatal density (n = 10 fields per treatment, expressed per mm^2) were measured in ImageJ [30]. Leaf color was quantified on digital images of 15 leaves per treatment by color thresholding in ImageJ, which separated the green and pink-red areas of each leaf blade; each class was expressed as a percentage of the leaf area (Fig. 1g).

Photosynthetic pigments

Leaf tissue (0.1 g FW) was ground in liquid nitrogen and then in 5 mL of 80% acetone with a pinch of $MgCO_3$, and the homogenate was centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was read at 470, 645 and 663 nm (UV-2100, Unico, NJ, USA). Chlorophyll *a*, chlorophyll *b* and total carotenoids were calculated with the equations of Lichtenthaler and Buschmann for 80% acetone [31], using A_{645} in place of $A_{646.8}$, and expressed as mg g^{-1} FW.

Phenolics and flavonoids

For total phenolics, 0.5 g of frozen leaf powder was extracted in 2 mL of 1% acidified methanol and centrifuged at 12,000 rpm for 10 min. A 25 µL aliquot of the supernatant was mixed with 100 µL of Folin–Ciocalteu reagent (diluted 1:9), followed after 6 min by 600 µL of 7.5% Na_2CO_3, and made up to 2 mL with water. Absorbance was read at 750 nm after 2 h at room temperature, and results were expressed as mg gallic acid equivalents (GAE) 100 g^{-1} FW from a gallic acid calibration curve (50–500 mg L^{-1}) [32]. Total flavonoids were determined in the same extract by the $NaNO_2$–$AlCl_3$–NaOH colorimetric method [33]: 500 µL of extract was diluted to 5 mL with water, 0.3 mL of 5% $NaNO_2$ was added, followed after 5 min by 0.6 mL of 10% $AlCl_3$, 2 mL of 1 M NaOH and 2 mL of water, and absorbance was read at 510 nm. Concentrations were determined from a quercetin standard curve (0–500 µg mL^{-1}, $R^2 = 0.98$) and expressed as mg quercetin equivalents (QE) g^{-1} DW.

Soluble protein and carbohydrates

Soluble protein was extracted from 0.25 g of leaf powder with 0.25 g polyvinylpyrrolidone and 1.5 mL of potassium phosphate buffer (pH 7.0) containing sodium metabisulfite, centrifuged at 15,000 rpm and 4 °C for 20 min, and measured by the Bradford method at 595 nm with bovine serum albumin as the standard [34]; it was expressed as mg g^{-1} FW. Soluble carbohydrates were extracted from 0.25 g of leaf tissue with 95% and then 70% ethanol and centrifuged at 3500 rpm for 10 min. A 1 mL aliquot of the extract was heated with 3 mL of anthrone reagent at 100 °C for 10 min, and absorbance was read at 625 nm against a glucose standard [35]; results were expressed as mg g^{-1} FW.

Statistical analysis

Final germination was compared among treatments with a χ^2 test and pairwise Fisher's exact tests with Holm adjustment. Within the EMS treatments, a binomial generalised linear model tested concentration, duration and their interaction. Germination time courses were analysed as time-to-event data, with ungerminated seeds right-censored at day 10 [36,37]. Curves were compared with the log-rank test, and hazard ratios were estimated with Cox proportional-hazards models. Cumulative germination was described with a three-parameter log-logistic model.

Continuous traits were analysed by one-way ANOVA across the five treatments, followed by Tukey's HSD for compact letter displays and Dunnett's test for comparisons with the control [38]. Effect sizes are given as ω^2. Because the four EMS treatments form a complete 2 × 2 factorial, they were also analysed by two-way ANOVA to partition concentration, duration and interaction effects, reported with partial η^2. Homogeneity of variances was tested with the Brown–Forsythe test, and plant compactness scores were also analysed with the Kruskal–Wallis test.

To quantify phenotypic dispersion, the Mahalanobis distance [39] of each plant from the control centroid was computed over seven continuous growth traits using the control covariance matrix. Plants exceeding the 97.5% quantile of the χ^2 distribution with 7 df were classed as multivariate off-types and compared with the control by Fisher's exact test. Principal component analysis was performed on standardised architectural traits of all 250 plants, and differences among treatment centroids were tested by PERMANOVA on Euclidean distances with 999 permutations [40]. Spearman correlations were computed among plant-level traits, and treatment means of all traits were z-scored and clustered by Ward's method [41]. Analyses were run in Python 3 with SciPy, statsmodels, lifelines and scikit-learn [42–45]. $P < 0.05$ was considered significant.

Results

Germination extent and kinetics

Germination of untreated seeds was rapid and nearly complete: 26% of seeds germinated after one day, 82% after two days, and 97% after seven days (Fig. 2a). In contrast, only 1–6% of EMS-treated seeds germinated after one day. Final germination differed significantly among treatments ($\chi 2 = 25.3$, df = 4, $P < 0.001$). It was reduced to 82% by 0.5% EMS for 30 min, and to 80% and 78% by 1% EMS for 30 and 60 min, respectively. However, 0.5% EMS for 60 min (94%) did not significantly differ from the control (Fig. 2b; Table 1). Even the most severe treatment maintained final germination well above 50%, indicating that the LD50 for germination lies beyond the tested dose range.

EMS had a larger effect on the speed than on the extent of germination. All four treatments shifted the cumulative curves to the right (log-rank $\chi 2 = 92.6$, $P < 0.001$), increasing the time to 50% germination from 1.40 to 1.95–2.60 days and the mean germination time from 2.00 to 3.05–3.62 days. This also roughly halved the germination index, from 58.3 to 28.7–34.1 (Fig. 2c; Table 1). In a Cox model, the hazard ratio of germination relative to the control was 0.27–0.43 for the EMS treatments (all $P < 0.001$), which corresponds to a 57–73% lower daily rate of germination. The germination value, which combines speed and completeness, fell from 397.7 in the control to 128.7–206.8 in the EMS treatments. Additionally, the synchronization index rose from 1.62 to 2.18–2.58 bits, showing that germination of treated seeds was also less uniform (Table 1).

Within the EMS treatments, concentration and exposure time interacted. At 0.5%, the longer soak increased final germination (94% vs. 82%), whereas at 1% it did not (78% vs. 80%). This interaction was significant both in a binomial model of final germination ($P = 0.025$) and in a Cox model of germination time (hazard ratio of the interaction term 0.59, 95% CI 0.39–0.91, $P = 0.018$). Pairwise log-rank tests separated all EMS treatments from the control (Holm-adjusted $P < 0.001$) and the 0.5%/60 min treatment from the 1%/60 min treatment ($P = 0.003$). The slowest and least complete germination, and the highest germination uncertainty (2.58 bits), occurred at 1% for 60 min (Table 1).

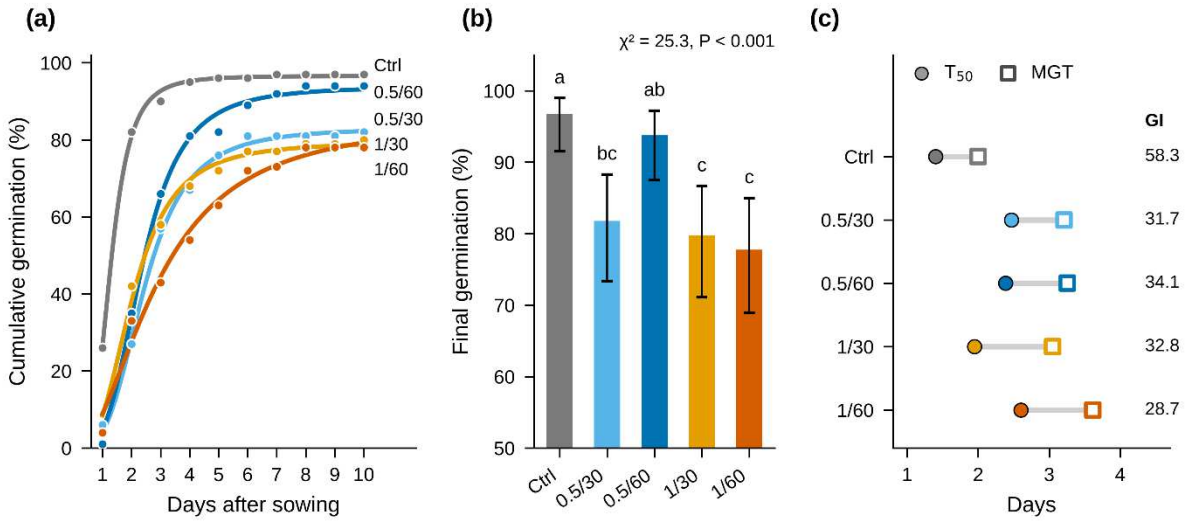


Fig. 2 Germination of *Hypoestes phyllostachya* 'Confetti Compact Red' seeds after EMS treatment. (**a**) Cumulative germination over 10 days (points) with fitted three-parameter log-logistic curves (lines). (**b**) Final germination; error bars are Wilson 95% confidence intervals, and bars sharing a letter do not differ (pairwise Fisher's exact tests, Holm-adjusted P > 0.05). (**c**) Time to 50% germination (T_{50}, circles) and mean germination time (MGT, squares); numbers on the right are the germination index (GI). Treatment codes give EMS concentration (%) and soaking time (min); Ctrl, untreated control. n = 100 seeds per treatment.

Table 1 Germination parameters of *Hypoestes phyllostachya* seeds treated with ethyl methanesulfonate (EMS).

Treatment	GP (%)	T_{50} (d)	MGT (d)	GI	CVG	U (bit)	GV	HR (95% CI)
Control	97 a	1.40	2.00	58.3	50.0	1.62	397.7	1 (reference)
0.5% EMS, 30 min	82 bc	2.47	3.21	31.7	31.2	2.35	155.8	0.32 (0.24–0.43)
0.5% EMS, 60 min	94 ab	2.39	3.26	34.1	30.7	2.18	206.8	0.43 (0.32–0.58)
1% EMS, 30 min	80 c	1.95	3.05	32.8	32.8	2.24	168.0	0.33 (0.24–0.44)
1% EMS, 60 min	78 c	2.60	3.62	28.7	27.7	2.58	128.7	0.27 (0.20–0.36)

GP, final germination percentage (letters from pairwise Fisher's exact tests with Holm adjustment, P < 0.05); T_{50}, time to 50% of final germination; MGT, mean germination time; GI, germination index ($\Sigma\, n_i/t_i$); CVG, coefficient of velocity of germination; U, synchronisation index (uncertainty) [28]; GV, germination value [29]; HR, hazard ratio of germination relative to the control from a Cox proportional-hazards model. n = 100 seeds per treatment.

Plant architecture

All eight architectural traits differed among treatments (Additional file 1: Table S1), although the effect sizes were modest (ω^2 = 0.02–0.11) because plant-to-plant variation was large. The 0.5%/30 min treatment produced the most vigorous plants: compared with the control, it increased leaf number by 18% (80.2 vs. 67.7 leaves per plant; Dunnett P < 0.001) and plant height by 12% (5.79 vs. 5.18 cm; P = 0.042) and gave the widest leaves (2.42 cm; Fig. 3; Additional file 1: Table S2). It also tended to produce more branches (12.1 vs. 11.3 per plant)

and longer leaves (3.76 vs. 3.63 cm), although these differences were not significant. In contrast, the 0.5%/60 min treatment reduced stem diameter by 13% (1.96 vs. 2.26 mm; P = 0.009) and gave the shortest leaves (3.38 cm). Plant width differed globally (P = 0.012), with the narrowest plants after the 60-min soaks (12.95–13.02 cm vs. 14.03 cm), but no pair of treatments was separated by Tukey's test.

The most consistent architectural response was a shift towards a compact habit. The mean compactness score rose from 1.32 in the controls to 1.70–2.22 in the EMS treatments (Kruskal–Wallis H = 30.9, P < 0.001), and the contrast between the control and the pooled EMS treatments was highly significant (P < 0.001). None of the control plants reached the highest score, whereas 24%, 42%, 36% and 28% of plants did so after the 0.5%/30, 0.5%/60, 1%/30 and 1%/60 treatments, respectively (Fig. 3h). Conversely, open-habit plants (score 0) made up 16% of the controls but only 2–6% of the populations from the 0.5%/60, 1%/30 and 1%/60 treatments.

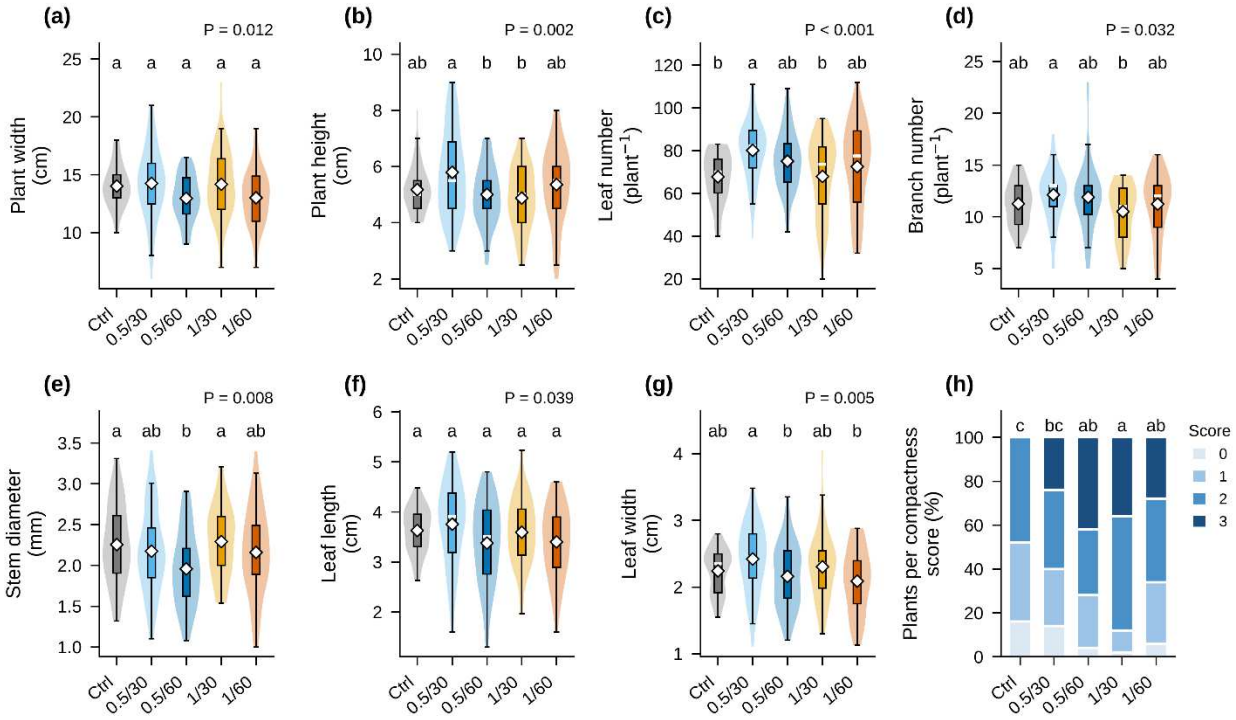


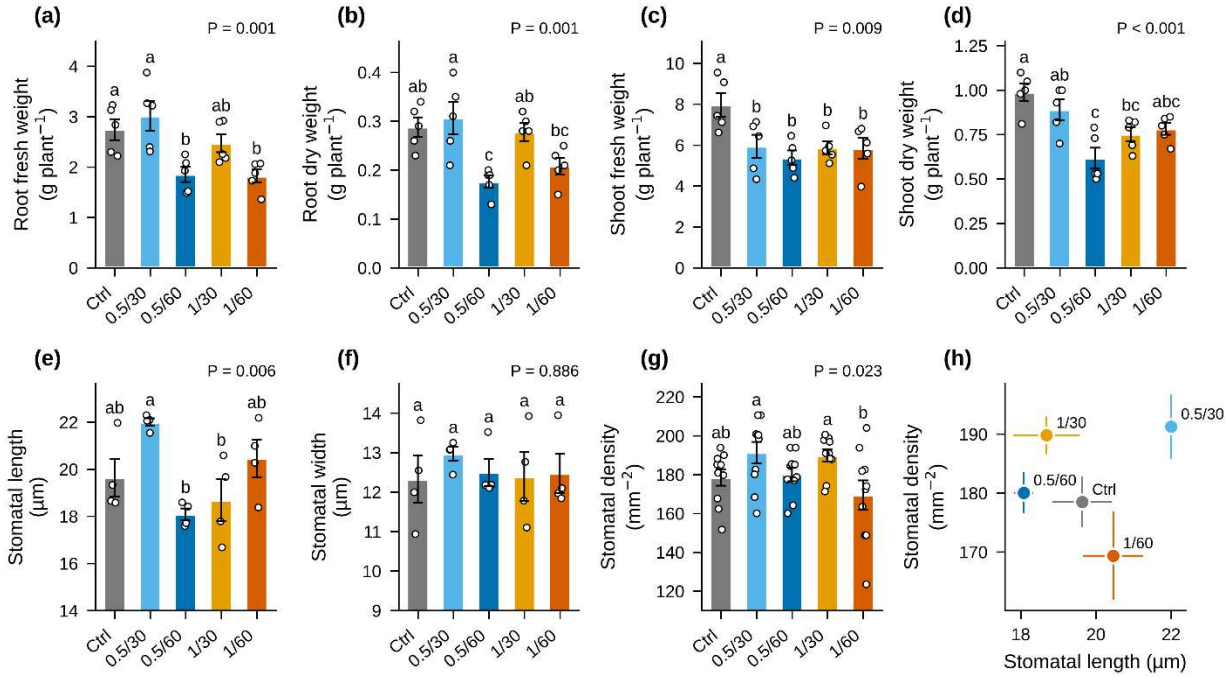
Fig. 3 Architecture of M$_1$ *Hypoestes phyllostachya* plants raised from EMS-treated seeds. (**a**–**g**) Violin plots with box plots (median, white line; mean, diamond) of plant width, height, leaf number, branch number, stem diameter, leaf length and leaf width; n = 50 plants per treatment. (**h**) Distribution of plant compactness scores (0, open habit; 3, fully compact). Treatments sharing a letter do not differ (Tukey's HSD for **a**–**g**; Tukey's HSD on scores for **h**; P > 0.05). P values are from one-way ANOVA.

Within the EMS treatments, exposure time was the main factor for plant width, stem diameter and leaf length and width, with 60-min soaks giving smaller organs, whereas concentration affected leaf and branch number. Concentration and duration interacted for plant height ($P = 0.001$), compactness ($P = 0.003$) and leaf number ($P = 0.041$) (Additional file 1: Table S1). For height, for example, the longer soak reduced plants at 0.5% (5.79 to 5.00 cm) but increased them at 1% (4.88 to 5.36 cm).

Biomass and stomata

Shoot biomass was the trait most consistently depressed by EMS. Shoot fresh weight fell by 25–33% in all four treatments, from 7.96 g in the controls to 5.37–5.94 g (Dunnett $P = 0.004$–0.024), and shoot dry weight fell by up to 38% at 0.5%/60 min (0.62 vs. 0.99 g; $P < 0.001$) (Fig. 4c,d). Root biomass depended mainly on exposure time: 60-min soaks reduced root fresh weight by 32–34% and root dry weight by 28–39%, whereas 30-min soaks did not (duration partial $\eta^2 = 0.56$–0.57, $P < 0.001$). The 0.5%/30 min treatment maintained root biomass at or slightly above the control level (3.02 vs. 2.74 g fresh weight; Fig. 4a,b), so it was the only treatment in which the root system was not weakened.

Stomatal length differed among treatments ($P = 0.006$) and was largest at 0.5%/30 min (22.0 µm) and smallest at 0.5%/60 and 1%/30 min (18.1–18.7 µm) (Fig. 4e). This pattern reflected a strong concentration × duration interaction (partial $\eta^2 = 0.64$, $P = 0.001$). Stomatal width was unaffected (12.3–13.0 µm; $P = 0.89$; Fig. 4f). Stomatal density ranged from 169 mm^{-2} at 1%/60 min to 191 mm^{-2} at 0.5%/30 min ($P = 0.023$), and 60-min soaks gave fewer stomata than 30-min soaks ($P = 0.005$; Fig. 4g). None of the stomatal traits differed from the control in Dunnett's test, so the stomatal effects mainly reflect contrasts among EMS treatments. Notably, the 0.5%/30 min treatment combined the longest stomata with the highest density, whereas the 1%/60 min treatment had relatively long but sparse stomata (Fig. 4h). Representative micrographs of the abaxial epidermis of each treatment are shown in Fig. 5.



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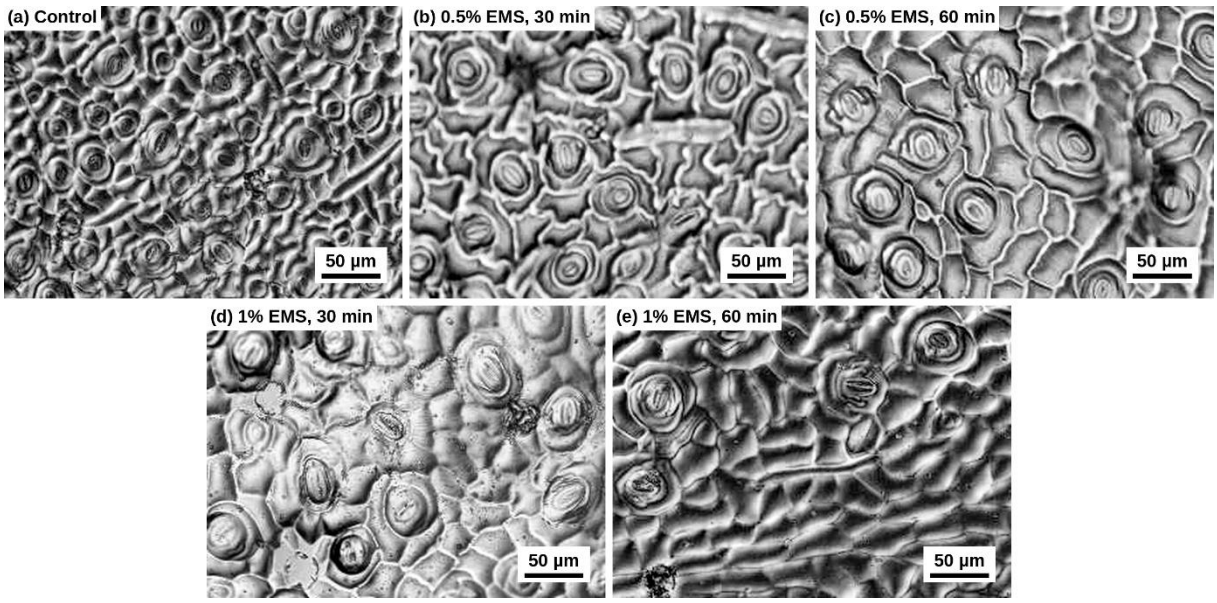
317 **Fig. 4** Biomass and stomatal traits of M1 *Hypoestes phyllostachya* plants. (**a**–**d**) Root and shoot
318 fresh and dry weights (n = 5 plants). (**e**–**g**) Stomatal length and width (n = 4 leaves) and stomatal
319 density on the abaxial epidermis (n = 10 fields). (**h**) Relationship between treatment means of
320 stomatal length and density (± SE). Bars show means ± SE with individual observations; bars
321 sharing a letter do not differ (Tukey's HSD, P > 0.05). In **e**–**g** the y-axis does not start at zero.



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323 **Fig. 5** Abaxial leaf epidermis of M1 *Hypoestes phyllostachya* plants showing stomata after EMS
324 seed treatment. (**a**) Untreated control; (**b**) 0.5% EMS, 30 min; (**c**) 0.5% EMS, 60 min; (**d**) 1%
325 EMS, 30 min; (**e**) 1% EMS, 60 min. Light micrographs of epidermal impressions; scale bars =
326 50 µm.

Pigments and leaf color

Leaf chlorophyll and carotenoid concentrations were low, as expected for a shade-tolerant variegated foliage plant. Chlorophyll *a* ranged from 0.18 to 0.23 mg g^{-1} FW, chlorophyll *b* from 0.064 to 0.079 mg g^{-1} FW, total chlorophyll from 0.25 to 0.30 mg g^{-1} FW and carotenoids from 0.053 to 0.063 mg g^{-1} FW, and none of these differed among treatments (P = 0.17–0.51; Fig. 6a–c). The 0.5% treatments had numerically higher chlorophyll *a* (+17–24%) and carotenoids (+18–19%) than the control. The ratios of carotenoids to chlorophyll (0.21–0.23) and of chlorophyll *a* to *b* (2.6–3.0) were stable, indicating that the composition of the photosynthetic pigment pool was not altered.

Image analysis showed that the green fraction of the leaf blade increased from 32.6% in the controls to 36.0% after 0.5%/60 min (P = 0.009), with a corresponding decrease in the pink-red fraction; the other treatments did not differ from the control (Fig. 6d). Representative leaves illustrate this variation (Fig. 7). Leaves from the 0.5% treatments ranged from deep red-spotted leaves with dense green venation to leaves dominated by green tissue with scattered red patches, and several were distorted or bilobed (Fig. 7b,c). Leaves from the 1% treatments included curled or folded blades with white or cream sectors and leaves with a pale pink background (Fig. 7d,e). Such sectorial and asymmetric color patterns were not seen on control leaves (Fig. 7a).

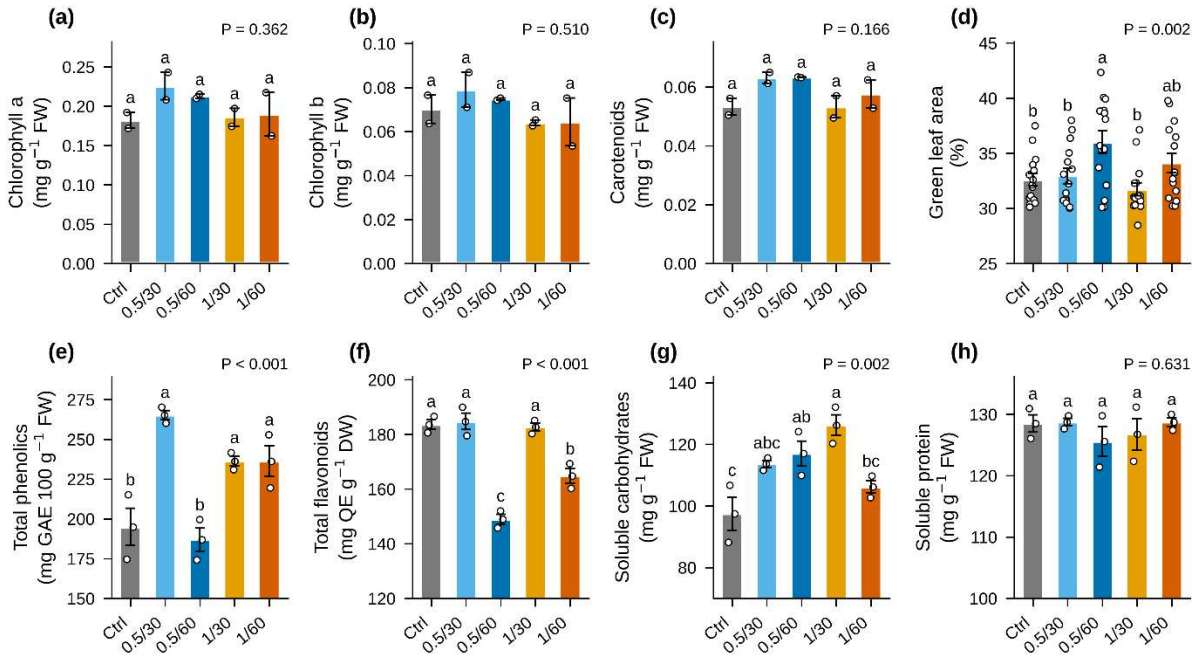

Fig. 6 Pigments, leaf color and leaf metabolites of M_1 *Hypoestes phyllostachya* plants. (**a**) Chlorophyll *a*; (**b**) chlorophyll *b*; (**c**) carotenoids (n = 2 extracts); (**d**) green fraction of the leaf area estimated by image analysis (n = 15 leaves); (**e**) total phenolics; (**f**) total flavonoids; (**g**) soluble carbohydrates; (**h**) soluble protein (n = 3). GAE, gallic acid equivalents; QE, quercetin equivalents. Bars show means ± SE with individual observations; bars sharing a letter do not differ (Tukey's HSD, P > 0.05). In **d**–**h** the y-axis does not start at zero.

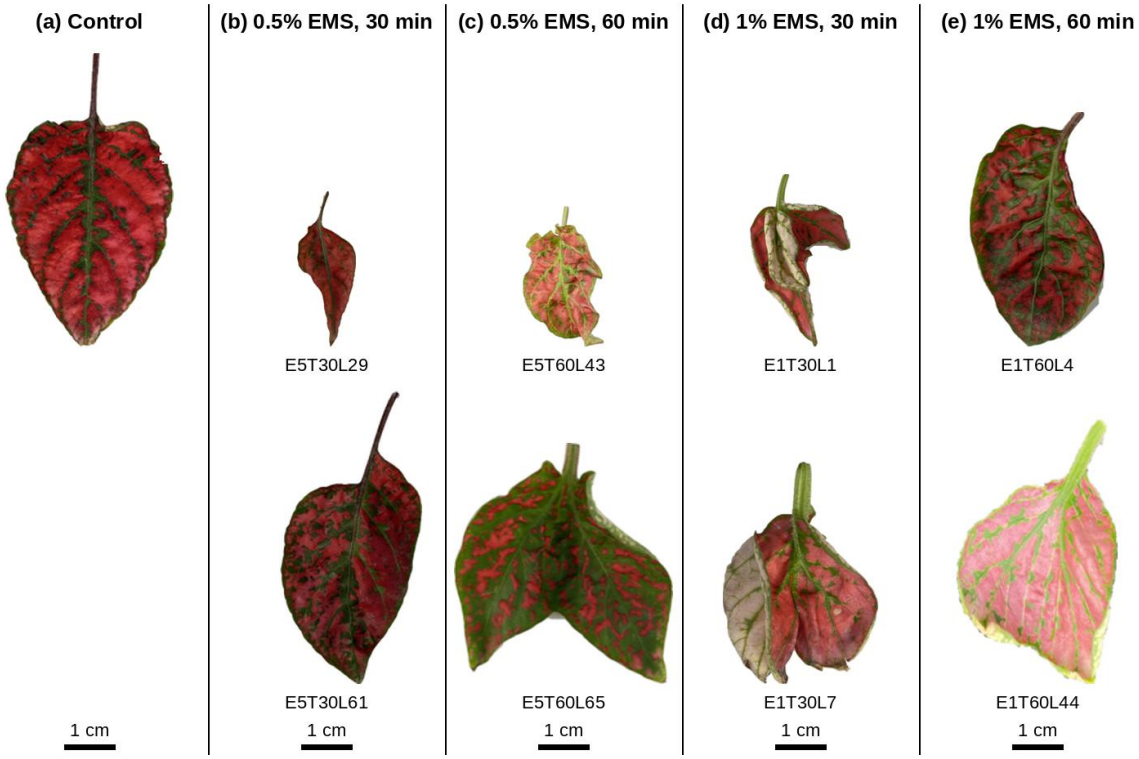

Fig. 7 Leaf color, spotting pattern and shape of M_1 *Hypoestes phyllostachya* plants. (**a**) Untreated control; (**b**) 0.5% EMS, 30 min; (**c**) 0.5% EMS, 60 min; (**d**) 1% EMS, 30 min; (**e**) 1% EMS, 60 min. Codes give the EMS concentration (E5, 0.5%; E1, 1%), the soaking time (T30, T60) and the plant number (L). All leaves are shown at the same scale; scale bars = 1 cm.

Leaf metabolites

Leaf metabolites responded more strongly than pigments. Total phenolics increased by 36% after 0.5%/30 min (265 vs. 195 mg GAE 100 g^{-1} FW; $P < 0.001$) and by 21% after both 1% treatments (236 mg GAE 100 g^{-1} FW; $P = 0.012$), but did not change after 0.5%/60 min (187 mg GAE 100 g^{-1} FW) (Fig. 6e). Total flavonoids showed the opposite pattern with respect to exposure time: they were unchanged after 30-min soaks (183–185 mg QE g^{-1} DW) but fell by 19% and 10% after 0.5%/60 and 1%/60 min, to 149 and 165 mg QE g^{-1} DW, respectively (both $P < 0.001$; Fig. 6f). Duration and the concentration × duration interaction explained most of the variance in both groups of compounds (partial $\eta^2 = 0.66–0.94$; Additional file 1: Table S1). Soluble carbohydrates rose by 17–30% in three of the four EMS treatments, with the largest increase after 1%/30 min (126 vs. 97 mg g^{-1} FW; $P = 0.001$) and no significant change after 1%/60 min (Fig. 6g). Within the EMS treatments, duration ($P = 0.021$) and its interaction with concentration ($P = 0.004$) were significant. Soluble protein was stable across treatments (125.6–128.7 mg g^{-1} FW; $P = 0.63$; Fig. 6h).

Treatment-specific response profiles

To compare treatments across all 25 traits, we expressed each EMS treatment as a percentage change from the control (Fig. 8a). Two features stand out. First, the 0.5%/30 min treatment had a mainly positive profile, with significant gains in leaf number, plant height, phenolics and soluble carbohydrates. Its only significant losses were in germination (−15%) and shoot fresh weight (−25%). Second, the 60-min treatments, especially 0.5%/60 min, had mainly negative profiles for biomass, stem diameter and flavonoids, but they also showed the largest shifts towards compactness and, at 0.5%, a greener leaf surface. The 1%/30 min treatment lay between these extremes, with a strong increase in compactness (+68%) and carbohydrates (+30%) but little change in growth traits.

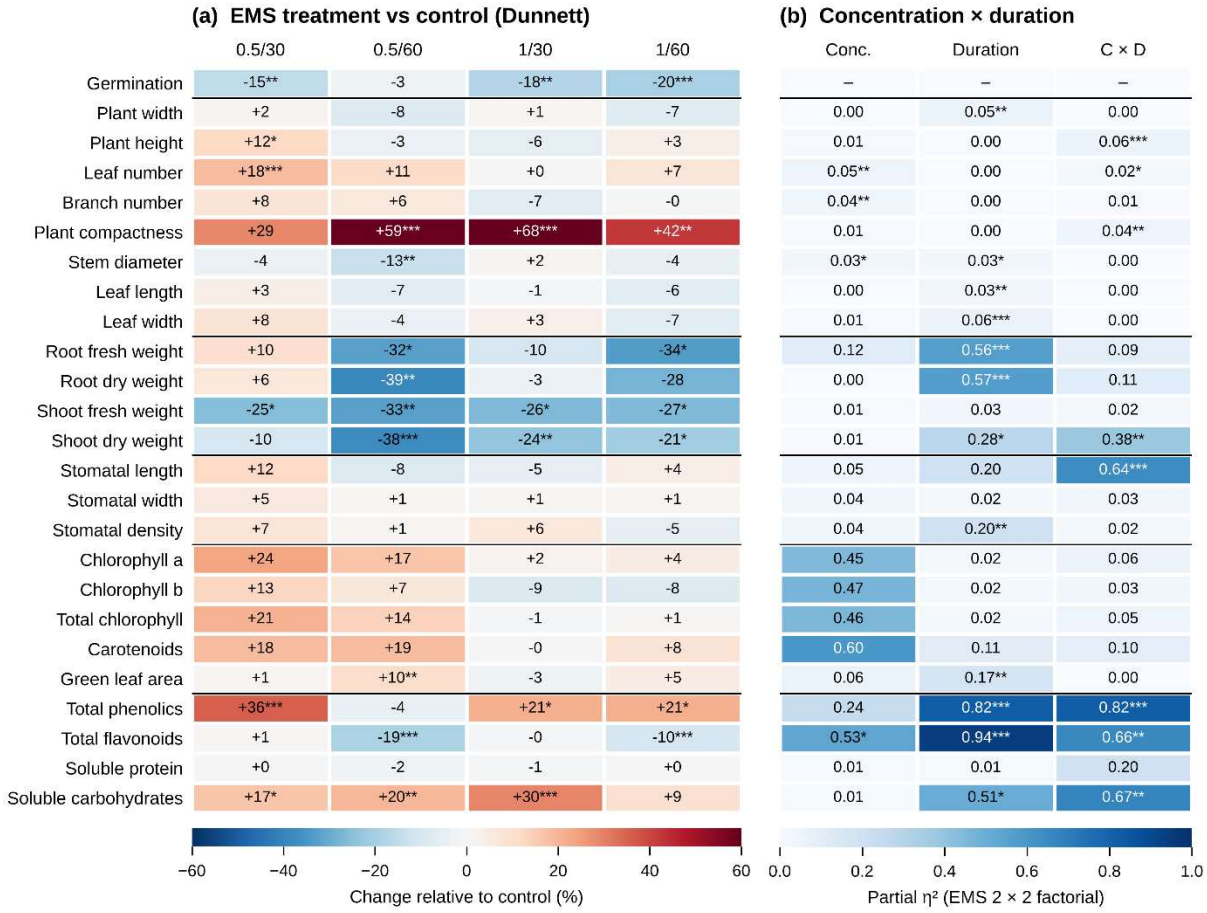

Fig. 8 Response profiles of *Hypoestes phyllostachya* to EMS seed treatments. (**a**) Percentage change of each EMS treatment relative to the untreated control for 25 traits; asterisks mark significant differences from the control (Dunnett's test for continuous traits; Holm-adjusted Fisher's exact test for germination). (**b**) Partial η^2 of concentration (Conc.), soaking duration and their interaction (C × D) in a two-way ANOVA of the four EMS treatments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Horizontal lines separate germination, architecture, biomass, stomata, pigments/leaf color and metabolites.

Partitioning the variance among the four EMS treatments (Fig. 8b) showed that exposure time and its interaction with concentration explained far more variance than concentration alone for biomass, stomata and metabolites. Duration alone explained 56–57% of the variance in root biomass and 82–94% in phenolics and flavonoids. Concentration dominated only the pigment traits, which were not significant overall. The full ANOVA results, including mean squares, effect sizes and the factorial tests, are given in Additional file 1: Table S1, and all treatment means with standard errors in Additional file 1: Table S2.

Induced phenotypic variability

Besides shifting means, EMS enlarged the spread of phenotypes. The variance of plant width, height, leaf number and leaf length differed among treatments (Brown–Forsythe $P = 0.0002$–0.012; Additional file 1: Table S1). The coefficients of variation of the seven continuous growth traits were on average 1.4 times larger in EMS-treated plants than in controls, reaching 26–28% for height, leaf number and branch number (Fig. 9c). The largest relative increases were for leaf length (12% in controls vs. 19–24% after EMS) and plant height (16% vs. 21–26%).

We next measured each plant's multivariate distance from the control phenotype (Mahalanobis D^2 over seven growth traits). The median D^2 rose from 6.1 in the controls to 11.0–16.9 in the EMS treatments (Kruskal–Wallis $H = 52.4$, $P < 0.001$; Fig. 9a). Plants beyond the 97.5% quantile of the χ^2 distribution, which we term multivariate off-types, made up 2% of controls but 56%, 40%, 30% and 38% of the 0.5%/30, 0.5%/60, 1%/30 and 1%/60 populations, respectively (Fisher's exact test $P < 0.001$ for each; Fig. 9b). When each trait was considered separately, 52–66% of EMS-treated plants fell outside the control mean ± 2 SD for at least one trait, compared with 22% of controls. The 0.5%/30 min treatment therefore produced the largest proportion of off-type plants while also giving the most vigorous population.

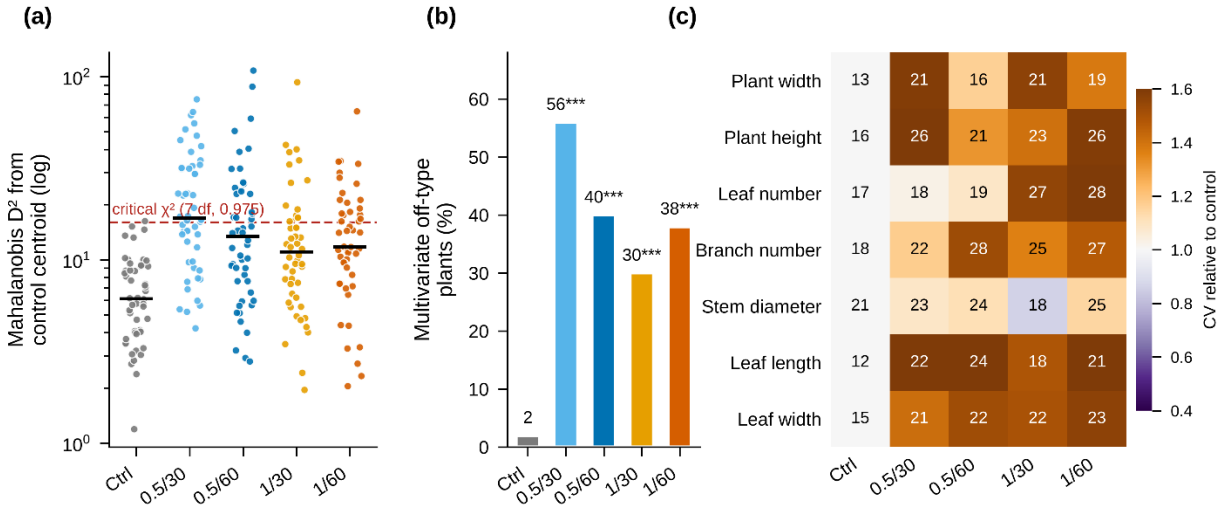

Fig. 9 Phenotypic dispersion in M_1 populations of *Hypoestes phyllostachya*. (**a**) Mahalanobis distance (D^2, log scale) of each plant from the control centroid computed over seven growth traits; horizontal bars are medians and the dashed line is the 97.5% quantile of χ^2 with 7 df. (**b**) Percentage of plants exceeding that threshold (multivariate off-types); *** $P < 0.001$ vs. control (Fisher's exact test). (**c**) Coefficients of variation (%, numbers) of growth traits, shaded by their ratio to the control CV. n = 50 plants per treatment.

Selected M_1 variants

Visual screening of the M_1 populations identified ten variants with distinctive habit or foliage (Fig. 10; Table 2). From the 0.5%/30 min population, A70 had a trailing habit with a greener leaf surface (Fig. 10a) and A64 had an altered spotting pattern (Fig. 10b). The 0.5%/60 min population yielded the largest number of variants: B29 and B65 were compact with modified leaf shape and color (Fig. 10c,d), B26 carried one green and one red main branch, which indicates a sectorial chimera (Fig. 10e), and B43 was small and profusely branched, with wrinkled leaves (Fig. 10f). Variants from the 1% treatments included a plant with greener leaves (D26; Fig. 10g), plants with elongated, deep-red leaves on a small frame (D53, E27; Fig. 10h,i) and a compact plant with pink-white spotted leaves (E44; Fig. 10j).

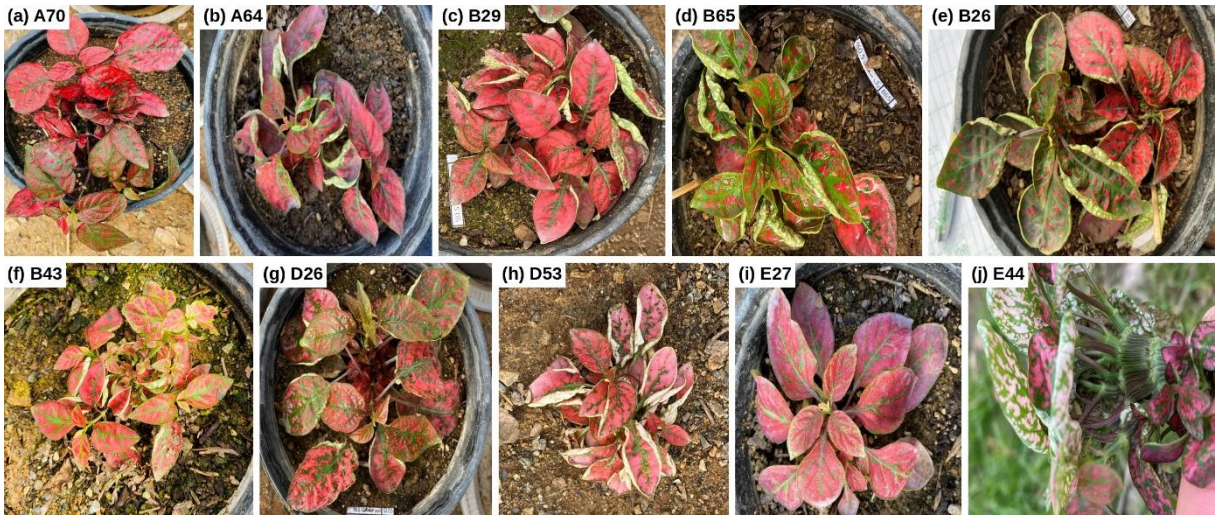

Fig. 10 Selected M_1 variants of *Hypoestes phyllostachya* 'Confetti Compact Red' obtained after EMS seed treatment. (**a**) A70, trailing habit; (**b**) A64, altered spotting; (**c**) B29 and (**d**) B65, compact habit with modified leaf shape; (**e**) B26, sectorial chimera with green and red branches; (**f**) B43, small, profusely branched plant with wrinkled leaves; (**g**) D26, greener leaves; (**h**) D53 and (**i**) E27, elongated deep-red leaves; (**j**) E44, compact habit. Line codes and treatments are given in Table 2.

Table 2 Description of M_1 variants of *Hypoestes phyllostachya* selected after EMS seed treatment.

Line	EMS treatment	Habit	Leaf traits	Figure
A70	0.5%, 30 min	Trailing (prostrate) growth	Greener leaf surface	10a
A64	0.5%, 30 min	Similar to control	Altered spotting pattern and leaf color	10b
B29	0.5%, 60 min	Compact	Altered leaf shape and color	10c
B65	0.5%, 60 min	Compact	Altered leaf shape; greener leaf surface	10d
B26	0.5%, 60 min	Two main branches, one green and one red (sectorial chimera)	Greener leaf surface	10e
B43	0.5%, 60 min	Small, profusely branched	Wrinkled leaves	10f
D26	1%, 30 min	Similar to control	Greener leaf surface	10g
D53	1%, 30 min	Small	Elongated, deep-red leaves	10h
E27	1%, 60 min	Small	Elongated, deep-red leaves	10i
E44	1%, 60 min	Compact	Pink-white spotted leaves	10j

Line codes give the treatment (A, 0.5%/30 min; B, 0.5%/60 min; D, 1%/30 min; E, 1%/60 min) and plant number. All descriptions refer to the M_1 generation; heritability has not yet been tested.

Multivariate structure

A principal component analysis of the eight architectural traits measured on all 250 plants extracted a first component (40.9% of variance) that described overall vigour, with high loadings for plant width, leaf number, height and branch number (0.76–0.82). The second component (18.2%) contrasted leaf size (leaf width 0.73, leaf length 0.62) with leaf and branch number (−0.43 to −0.46). Compactness was nearly independent of both components and of the other traits ($|\rho| \leq 0.19$) and loaded on the third component (0.88; 13.9%). The treatment centroids differed significantly but overlapped widely (PERMANOVA $R^2 = 0.065$, $P = 0.001$), with the 0.5%/30 min population displaced towards higher vigour (Fig. 11a).

Leaf and branch number were the most closely associated traits (ρ = 0.83), followed by leaf length and width (ρ = 0.66), height and width (ρ = 0.65) and height and branch number (ρ = 0.61) (Fig. 11b). Stem diameter was only weakly related to the other traits (ρ ≤ 0.31). Ward clustering of the standardised treatment means across all 25 traits placed the 0.5%/30 min treatment on its own branch, characterised by high values for growth, stomatal size and phenolics. The two 60-min treatments formed a second group with low biomass and flavonoids, and the control grouped with 1%/30 min (Fig. 11c).

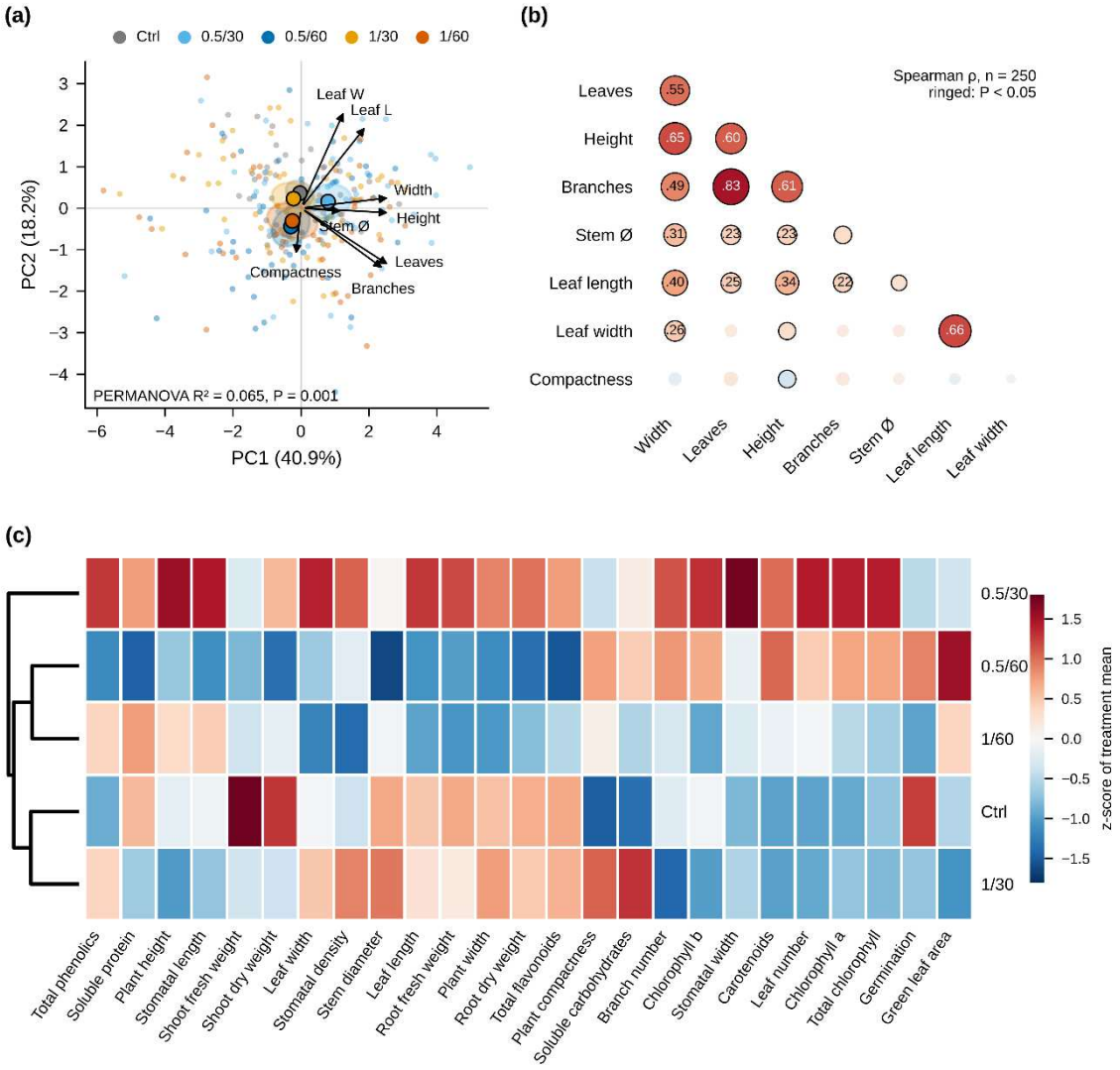

Fig. 11 Multivariate structure of the EMS response in *Hypoestes phyllostachya*. (**a**) Principal component biplot of eight architectural traits (n = 250 plants); small points are individual plants, large circles are treatment centroids with 95% confidence ellipses, and arrows are trait loadings. (**b**) Spearman correlations among architectural traits; circle size and color scale with ρ, and ringed circles are significant (P < 0.05). (**c**) Heat map of z-scored treatment means for 25 traits, with treatments ordered by Ward's hierarchical clustering.

Discussion

This study gives the first account of chemical mutagenesis in *Hypoestes*. Its main message is that a short, low-concentration EMS soak is enough to generate substantial, selectable variation in this small-seeded foliage plant, at a modest physiological cost. Below we discuss the germination response, the changes in architecture, the evidence for induced variability, and the stomatal, pigment and metabolic responses, and we then consider the limitations of an M_1 study.

Germination response

Reduced and delayed germination are classical indicators of mutagen-induced physiological damage in the M_1 generation [9,10]. In *H. phyllostachya* the delay was the dominant effect. The germination index of every EMS treatment was about half that of the control, and the hazard ratios showed that treated seeds germinated at roughly one-third of the control rate, whereas final germination remained at 78–94%. A similar pattern, in which speed is affected before final percentage, has been reported for EMS-treated chrysanthemum seeds, where germination fell only at the highest doses [14], and for carnation seeds treated with EMS for several hours [15]. It is consistent with time being needed to repair alkylation damage before cell division resumes in the embryo. Analysing the full time course with survival methods [36,37] captured this delay, which an analysis of final percentages alone would have largely missed, and it also revealed that germination of treated seeds was less synchronous.

Germination was never reduced to 50% in our dose range, so the conventional LD_{50} lies above 1% EMS for 60 min. Doses near the LD_{30}–LD_{50} are often recommended because they balance mutation frequency against survival [4,9]. However, the strong effects of our moderate doses on seedling vigour and biomass argue against simply raising the dose, and growth reduction may be a more sensitive criterion than germination for this species. The interaction between concentration and exposure time was unexpected: a longer soak improved germination at 0.5% but not at 1%. One possible explanation is that the 60-min soak in phosphate buffer completed imbibition and early repair at the low concentration, whereas at 1% the additional exposure added damage. Because each treatment was represented by a single 100-seed lot, this interaction should be confirmed with replicated seed lots before it is used to set protocols.

Plant architecture

The clearest morphological response was a move towards compact plants. Compactness is a primary breeding target in polka-dot plant: the cultivar used here belongs to a series selected for it, and pot growers otherwise rely on light management or growth regulators to prevent leggy growth [25,26]. Growth regulators can shift plant form and biochemical profiles together, as shown recently in fennel [46], but they add cost and must be reapplied in every crop cycle,

whereas a heritable compact habit would not. In crops where EMS-induced compact or semi-dwarf mutants have been characterised genetically, the causal lesions often affect brassinosteroid biosynthesis or signalling and shorten internodes without loss of fertility [47–49]. In the M_1 generation, however, compactness may also reflect transient damage to the shoot apex. The fact that compactness was almost uncorrelated with plant size in our data (Fig. 11b) suggests that it is a distinct architectural trait rather than a by-product of stunting, but only M_2 segregation can show whether it is heritable.

Low-dose stimulation of growth, as seen for leaf number and height after 0.5%/30 min, is frequently reported after mild mutagen treatment and has been attributed to stress-induced hormonal and metabolic adjustments rather than to mutation [15,17]. In pepper, for example, EMS-treated M_1 plants grew differently from the wild type before any heritable mutation could be identified [17]. The opposite response to longer soaks, with thinner stems and smaller roots and shoots, fits the view that the biological dose of EMS integrates concentration and time [9]. In our factorial analysis, exposure time and its interaction with concentration explained more variance than concentration for biomass, stomata and metabolites. Exposure time is therefore at least as important a protocol variable as concentration for small seeds such as those of *Hypoestes*, which hydrate and take up the mutagen quickly.

Induced variability

A mutagenesis protocol aims to generate variation, so treatment means alone are a poor guide to its success. EMS increased the dispersion of nearly all growth traits, and between 30% and 56% of treated plants were multivariate off-types compared with 2% of controls. Comparable increases in phenotypic variance and in the number of distinct phenotypic classes have been used to judge EMS treatments in cowpea and chrysanthemum [14,21], and PCA and cluster analyses have helped to describe the structure of such variation in mutant populations [21]. We suggest that the Mahalanobis-distance approach used here is a simple, reproducible way to quantify dispersion and to rank treatments when no molecular data are yet available. By this criterion, together with its high plant vigour and maintained root biomass, 0.5% EMS for 30 min was the most efficient treatment.

Part of the M_1 dispersion will be non-heritable, because it arises from physiological damage, and part will be heritable but chimeric. In seed mutagenesis the embryo shoot apex contains several initial cells, so mutations arise in individual cell lineages and are expressed as sectors [9,10]. The B26 plant, with one green and one red main branch (Fig. 10e), and the sectored leaves of the 1% treatments (Fig. 7d) are visible examples of such chimeras. Commercial *Hypoestes* cultivars are propagated by cuttings as well as by seed, and a patented cultivar arose

from a single mutant branch [27,50]. Desirable sectors identified in our M_1 plants could therefore be fixed by vegetative propagation, in parallel with selfing to obtain M_2 seed. *In vitro* culture of mutant sectors, as developed for chrysanthemum [16], would be a further option.

Stomatal traits

Stomatal size scales with nuclear DNA content and cell size, and across species it is inversely related to stomatal density [51,52]. The 0.5%/30 min treatment, however, combined the longest stomata with the highest density, and stomatal width was unchanged. This argues against a change in ploidy and instead points to altered epidermal development or leaf expansion in that population, in line with its larger and more numerous leaves. The opposite combination after 1%/60 min, with relatively long but sparse stomata, fits the reduced growth of that treatment. Because stomatal size and density together set the maximum leaf conductance [52], these changes may affect water use and growth rate of the variants, which should be tested in later generations. Flow cytometry would settle whether any variant has an altered genome size.

Leaf pigmentation

Chlorophyll and carotenoid concentrations were not altered, which agrees with the stable pigment ratios and suggests that the photosynthetic apparatus of most M_1 plants was intact. Stress-induced changes in chlorophyll are usually accompanied by oxidative damage and altered pigment ratios [53], neither of which was observed here. The green fraction of the leaf surface increased after 0.5%/60 min, and several variants had greener or deeper-red leaves (Figs. 7 and 10). In polka-dot plant the pink-red spotting depends on anthocyanin accumulation and fades under low light [25], and leaf anthocyanins act mainly as photoprotective and antioxidant screens [54]. Changes in spotting therefore most likely reflect altered anthocyanin deposition in epidermal sectors rather than changes in chloroplast pigments. Measuring anthocyanins directly, and quantifying color in a calibrated color space, will be essential when the color variants are advanced.

Leaf metabolites

Total phenolics rose after three of the four treatments, while flavonoids declined after the 60-min soaks. Induction of the phenylpropanoid pathway, which starts with phenylalanine ammonia lyase, is a common response to oxidative and genotoxic stress [19,20], and increased phenolic and flavonoid contents have been reported in EMS-derived M_1 plants of the ornamental *Celosia cristata* [18]. The divergence between phenolics and flavonoids suggests that stronger treatments redirect flux away from flavonoid branches, which would also be consistent with the greener leaf surface seen after 0.5%/60 min, since anthocyanins are synthesised through the flavonoid branch [54]. Higher phenolic content may also add value to

the variants, because phenolics contribute to stress tolerance and to the antioxidant capacity of plant tissues [19,55].

The accumulation of soluble carbohydrates in treated plants with smaller shoots points to reduced sink demand for growth rather than to increased assimilation, as chlorophyll was unchanged. Soluble sugars, together with proline, are also classical osmolytes that accumulate in stressed plants [35,56], so part of the increase may reflect a lasting stress response in the M_1 plants. Soluble protein was remarkably stable, which suggests that general protein metabolism was not disturbed by the treatments.

Conclusions

Seed treatment with EMS is a practical way to create new variation in *Hypoestes phyllostachya*. Within the range tested, EMS delayed germination more than it reduced it, shifted plants towards a compact habit, reduced shoot biomass and changed leaf color and phenolic metabolism. Most importantly, it enlarged phenotypic dispersion: off-type plants were up to 28 times more frequent than in the control. Treatment with 0.5% EMS for 30 min gave the best balance of high plant vigour, the largest proportion of off-types and favourable changes in leaf number and phenolics, and we recommend it as a starting point for building mutant populations. The ten M_1 variants identified here, including compact, trailing, wrinkled-leaf and color variants, now need to be advanced to M_2 and propagated vegetatively to test whether these traits are heritable and stable.

Supplementary Information

Additional file 1: Table S1. Analysis of variance of morphological, physiological and biochemical traits across the five treatments and two-way ANOVA (concentration × duration) within the four EMS treatments. **Table S2.** Means ± SE of all traits for the five treatments (DOCX).

Abbreviations

ANOVA: Analysis of variance

CV: Coefficient of variation

CVG: Coefficient of velocity of germination

DW: Dry weight

EMS: Ethyl methanesulfonate

FW: Fresh weight

GAE: Gallic acid equivalents

GI: Germination index

GP: Germination percentage

GV: Germination value

HR: Hazard ratio

HSD: Honestly significant difference

M_1, M_2: First and second generations after mutagen treatment

MGT: Mean germination time

PCA: Principal component analysis

PERMANOVA: Permutational multivariate analysis of variance

QE: Quercetin equivalents

T_{50}: Time to 50% of final germination

U: Synchronisation index (uncertainty)

Declarations

Ethics approval and consent to participate

Not applicable. Seeds of the commercial cultivar 'Confetti Compact Red' were purchased from Sakata Seed Corporation (Yokohama, Japan); no permissions or licences were required for their use. All experimental research on plants complied with relevant institutional, national and international guidelines and legislation.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Summary statistics for all traits are provided in Additional file 1.

Competing interests

The authors declare no competing interests.

Funding

Not applicable. This research received no external funding.

Authors' contributions

FN and YV conceived and designed the study and supervised the work. HMH carried out the experiments and collected the data. YV analysed the data and prepared the figures. FN and YV wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Clinical trial number

Not applicable.

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