Eur. J. Hortic. Sci. | ISSN 1611-4426 print, 1611-4434 online | https://doi.org/10.17660/eJHS.2022/057 | © ISHS 2022

Original article

Culture methods for high hydrangenol and phyllodulcin contents in *Hydrangea macrophylla* subsp. *serrata* (Thunb.) Makino

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Summary

***Hydrangea macrophylla*/*Hydrangea macrophylla* subsp. *serrata* cultivars are common ornamental plants, appreciated for their big and often colourful flowers. Some cultivars contain high amounts of dihydroisocoumarins (DHCs) which are of nutritional or medicinal interest. We cultured cuttings from two cultivars, which contain hydrangenol (HG) and phyllodulcin (PD), both DHCs, under greenhouse forcing conditions. Using peatmoss-based or inert substrates and drip or ebb-flow irrigation in addition with hydroponic fertilizers as well as full-spectrum, high-pressure sodium (HPS) lamps in short-day seasons, we aimed at improving biomass production and accumulation of HG and PD pre-harvest. The content of HG and PD was measured in young and old leaves after foliar application of three plant hormones, ethylene, salicylic acid and methyl jasmonate (MeJA). Repeated applications of MeJA significantly increased HG and PD contents, especially in young leaves of vegetative plants, in contrast to the other hormones. However, more than five applications of 60 µmol MeJA per plant did not lead to further enhancements. The hormone applications had no significant positive impact on leaf fresh matter biomass. The biggest difference in biomass was observed between plants in vegetative and generative growth state. Our findings show, that HG and PD contents can be increased in plants repeatedly treated with MeJA, preferably during vegetative growth without influencing biomass production.**

Keywords

dihydroisocoumarins, greenhouse cultivation, hydroponics, methyl jasmonate

Significance of this study

What is already known on this subject?

- Some *H. macrophylla* subsp. *serrata* cultivars synthesize hydrangenol and phyllodulcin, constituents of nutritional or medicinal interest but in insufficient quantities for intensive production.

What are the new findings?

- The pre-harvest content of hydrangenol and phyllodulcin in leaves can be increased by methyl jasmonate treatment.

What is the expected impact on horticulture?

- Refining cultivation methods for year-round production of strictly vegetative growing cultivars, yielding high leaf biomass containing sufficient amounts of dihydroisocoumarins for extraction at industrial scale.

Introduction

Hydrangea macrophylla subsp. *serrata*/*Hydrangea macrophylla,* named 'garden hydrangea' or 'garden hortensia', are woody plants which are cultured for the use as ornamental species. For this purpose, many cultivars have been bred with the aim to improve growth properties, cold hardiness (Adkins et al., 2003), or ornamental values (Adkins and Dirr, 2003; Khaing et al., 2016; Uemachi and Okumura, 2012). Presently, the phylogenetic relationship of *H. macrophylla* and *H. serrata* is not definitively determined (Uemachi et al., 2014). We refer to Moll et al. (2021), by addressing *H. serrata* as *H. macrophylla* subsp. *serrata*.

Some cultivars of *Hydrangea macrophylla* subsp. *serrata* contain dihydroisocoumarins (DHCs) and in particular phyllodulcin (PD) and hydrangenol (HG), both of nutritional or pharmaceutical value (Kawamura et al., 2002; Matsuda et al., 1998; Nozawa et al., 1981). Phyllodulcin is of particular interest because of its sweet taste, and suitable extraction methods are available for isolation (Jung et al., 2016). *Hydrangea serrata* varieties containing higher amounts of hydrangenol and phyllodulcin are wild growing in Japan (Ujihara et al., 1995). Consistently, agricultural practices for intensive cultivation leading systematically to higher accumulation of DHCs are not available. However, a recent study showed that shading of the plants had no effect on DHC contents (Moll et al., 2021).

Besides some general data based on the labelled precursors acetate and tyrosinate (Kindl, 1971), the exact biosynthesis of hydrangenol and phyllodulcin is presently unclear as well as the biological functions of the compounds. Since the herbivorous spider mite *Tetranychus kanzawai* avoid feeding on *Hydrangea macrophylla* (Gotoh and Gomi, 2000; Murase and Fujita, 2018), it can be speculated that the compound might contribute to defense of the plants against chewing herbivores (Pangesti et al., 2016). Plant defense reacti-

on are highly complex, and involve the hormones jasmonate and salicylic acid (Beyer et al., 2021; Croy et al., 2021; Li et al., 2019) while jasmonic acid is known to regulate plant resistance to chewing pests (Pangesti et al., 2016). Therefore, we decided to test applications of methyljasmonate (MeJA) and salicylic acid (SA) for possible enhancing the hydrangenol and phyllodulcin contents in the leaves of *H. macrophylla* subsp. *serrata*. In addition, ethylene applications released by ethephon treatment were included (Yang, 1969), as ethylene has multiple functions in plants such as influencing the regulation of secondary metabolite metabolism (Park et al., 2006).

In this study, we aimed to increase the hydrangenol and phyllodulcin accumulation in cultivars of *H. macrophylla* subsp. *serrata* plants grown in greenhouses under controlled cultivation conditions. The experiments were performed in 2019 and 2020. We tested whether plant hormone applications on foliage leaves can increase DHC contents in differently aged leaves and if so, whether hormone treatments impede biomass production. We also proved if extent pruning is of advantage for that purpose.

Materials and methods

Plant material

In spring of 2019 and 2020, *H. macrophylla* subsp. *serrata* 'Oamacha' and/or 'Amagi Amacha' cultivars were cultured in greenhouses of the University of Applied Sciences Osnabrück. Plants were received as terminal cuttings produced by Kötterheinrich-Hortensienkulturen e.K. (Lengerich, Germany). In 2020, plants were placed in 3 litre pots filled with perlite (Gramoflor, Vechta, Germany) or on rock wool (Grodan, Roermond, Netherlands) and cultured hydroponically. Watering was done in recirculating systems either automatically via drip irrigation (up to eight times per day, keeping an average drain of 30–40%) or manually on ebb-flow tables (up to three times per day). Water pH was adjusted to 6.0 using nitric acid and supplemented with a commercially available (15-7-22) N-P-K fertilizer, suited for hydroponic cultivation, from Planta Düngemittel GmbH (Regenstauf, Germany) adjusted to 1.3 mS cm^{-1}. In 2019, plants were grown in commercially available peatmoss substrate (Klasmann-Deilmann, Geeste, Germany). Here, plants were watered on ebb-flow-tables, using fertilizer-supplemented water adjusted to 1.8 mS cm^{-1} throughout the experiment.

The ambient temperature in the computer-controlled greenhouses was adjusted to 21/18°C at day/night. Aeration temperature was adjusted to 23/20°C at day/night. Shading was applied automatically when the natural illuminance exceeded 55 klx.

If not stated otherwise, plants were grown under natural illumination without additional artificial illumination. When artificial illumination was used, plants were grown under full-spectrum HPS-Lamps (Philips Master Colour CDM-TMW Elite 315W) using CDM-KE modules from DH-Licht (Wülfrath, Germany) (Figure 1).

Plant hormone applications

Methyljasmonate was obtained from Symrise AG (isomer mixture of 98.5% purity), SA (99.3% purity), ethephon (≥ 96% purity) were purchased from VWR (Germany).

Hormones were dissolved in dH_2O supplemented with 300 µL L^{-1} of a surfactant (Break Thru S 301, AlzChem Group AG) at room temperature. If not stated otherwise, control group plants received surfactant-supplemented dH_2O. Applications were conducted using a commercially available sprayer. Plants receiving the same treatment were always grouped and treated together outside the greenhouse. Each plant received 12 mL hormone solution within 3 sec, ensuring that all above-ground plant parts were sufficiently wetted. After 15 min of open-air drying, plants were checked for any leftovers of the application solutions. Only plants without leftovers were transferred back into the greenhouse according to the experimental design. Applications were always done between 10:00–11:59 am. The time between two consecutive applications was always 48 h.

Sampling for DHC quantification

Sampling was always done at noon (12:00 pm). Each sample comprised two leaves from the same node. Leaves were dried at 40°C for at least 48 h immediately after sampling. If leaves of different ages were sampled, they were always taken from the same shoot. Young leaves, named "1st leaves" (and older leaves named accordingly), were defined as fully developed, rolled out leaves of at least 30 mm length, yielding a dry sample weight for DHC quantification of at least 10 mg. The 2nd, 3rd, and 4th youngest leaves were likewise taken from the respective nodes below. Samples were taken 24 h after the last hormone application if not stated otherwise.

For the assessment of HG and PD contents in flowering buds of generative 'Oamacha' cultivars, 16 composite samples from 288 flowering plants were taken (i.e., all flowers/inflorescences from18 plants per sample).

Experimental setup

1. Impact of ethylene, methyl jasmonate and salicylic acid on DHC accumulation in 1st leaves (2019). Each plant hormone was investigated separately. The experiments were conducted consecutively in the same greenhouse starting with the application of ethephon, which was applied only once, MeJA and SA were applied three and two times respectively.

The hormones were applied in five different concentrations, including a concentration of 0 mM for the control groups. Plants were arranged in four blocks in the same greenhouse. Treatment groups (i.e., the different hormone concentrations) were randomly assigned to plants within one block. The total number of plants receiving the same concentration of a particular hormone (n) was 24. The following amounts were applied:

- *Ethephon*: 0/0.173/0.867/1.734/3.468 mmol $plant^{-1}$;
- *MeJA*: 0/12/36/72/108 µmol $plant^{-1}$;
- *SA*: 0/0.0166/0.166/1.66/16.6 mmol $plant^{-1}$.

The 1st (youngest) leaves were sampled after the last hormone application for DHC quantification.

2. Impact of MeJA applications on DHC accumulation in vegetative and generative *H. macrophylla* subsp. *serrata* (2020a). From a total of 300 plants, two evenly sized groups (N=90) were selected. Plants were assigned to these groups depending on their visually identical developmental stages. The first group of plants being in the generative growth phase encompasses plants with one or more flowers or flowering buds at more than 50% of their shoots. The second group encompasses plants in vegetative growth phase, where more than 50% of all shoots had no flowers or flowering buds (see Figure 1, picture 1 and 2 for both groups). All plants were positioned on five parallel channels (36 plants per channel, see Figure 1, picture 3) in the same greenhouse in a fully randomised design and treatments were randomly assigned.



Figure 1. Vegetative (1) and generative (2) growing plants arranged in a 4×4 square before hormone application. Growth states are defined in the method section (2020a). (3) Plants cultured hydroponically using perlite.

Treatment assignment was done separately for both, vegetative and generative plants.

Treatment groups were as follows:

- *Water control*: 4× dH_2O without surfactant;
- *Surfactant (control)*: 4× dH_2O including surfactant;
- *1× MeJA*: 3× dH_2O, 1× 60 µmol MeJA $plant^{-1}$;
- *2× MeJA*: 2× dH_2O, 2× 60 µmol MeJA $plant^{-1}$;
- *3× MeJA*: 1× dH_2O, 3× 60 µmol MeJA $plant^{-1}$;
- *4× MeJA*: 4× 60 µmol MeJA $plant^{-1}$.

The number of plants receiving the same treatment (*n*) was 15. Hormone application(s) took place during the 9th of June and the 15th of June, sampling on the 16th of June, as indicated in Figure 2(A).

The 1st, 2nd, 3rd, and 4th youngest leaves were sampled for DHC quantification.

To assess potential long-term effects of MeJA-applications on HG and PD contents, a second sampling was done one month after the last application, again sampling the 1st to 4th youngest leaves on the 13th of July.

3. Impact of the number of MeJA applications on DHC accumulation (2020b). The effect of more than four MeJA applications was examined using the same plants, which were already used in spring (see above, 2020a). To ensure homogenous vegetative growth, plants were pruned on the 31st of August and artificial illumination was turned on before sunrise and after sunset to ensure long-day conditions (18 h of light) up until the end of experiment.

Plants were randomly assigned to either control (receiving 9× dH_2O) or treatment group (receiving 9× 60 µmol MeJA $plant^{-1}$). The MeJA applications and samplings were conducted during the 5th and the 22nd of October.

Starting one day after the first application, 18 (*n*) composite samples from both groups (control and treatment) were taken every fourth day, as outlined in Figure 2B. For this, MeJA receiving plants were randomly assigned to one of 18 groups, each containing five individual plants for composite sampling of 1st leaves. Control plants were grouped in the same manner. The allocation of plants to these groups was kept constant throughout the experiment (T1 to T7).

After the ninth application, 1st, 2nd, and 3rd youngest leaves were taken (T9 sampling) from every single plant for individual DHC quantification.

Throughout this experiment, each shoot was sampled only once.

4. Impact of MeJA applications on DHC accumulation in 'Amagi Amacha' cultivar (2020c). A total of 100 vegetative

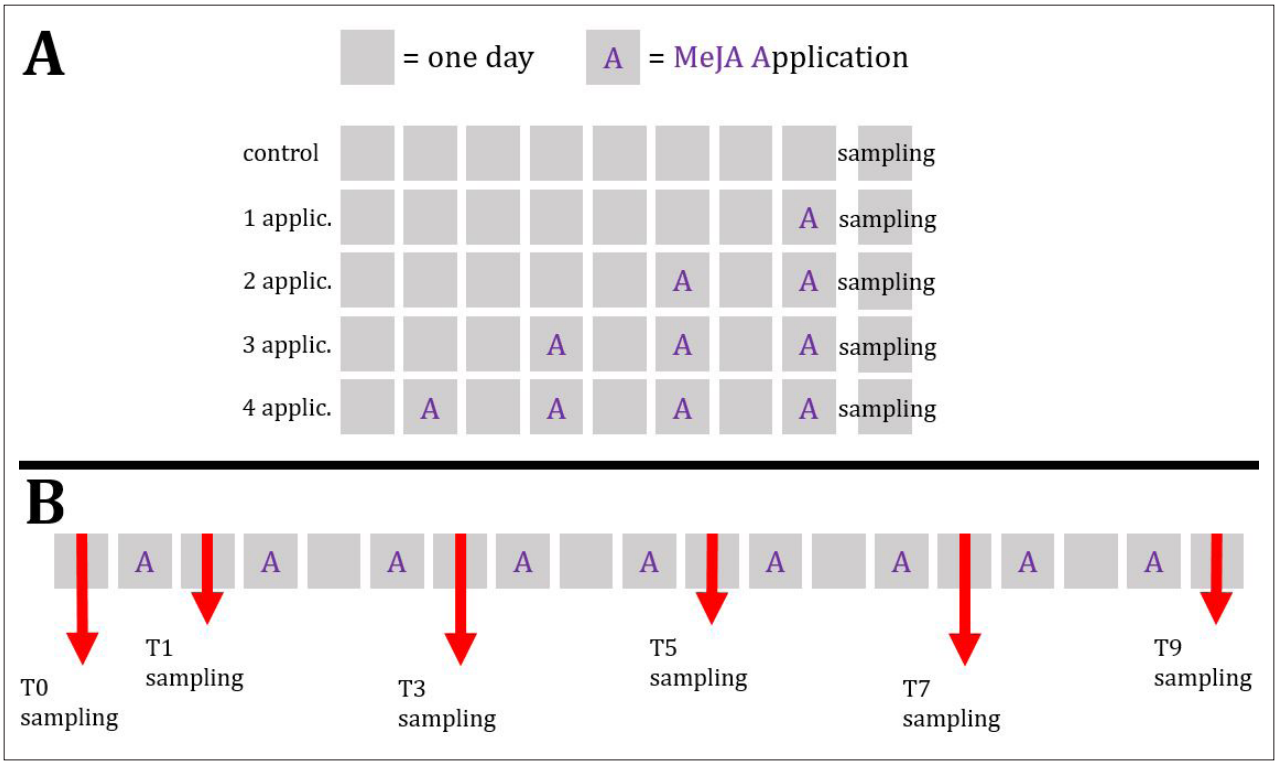


FIGURE 2. Experimental timelines, denoting the duration of the experiments in days (grey squares), the dates of hormone application (grey squares containing an 'A'), and sampling dates (red arrows) for DHC quantification in the experiments 2020a (A) and 2020b (B).

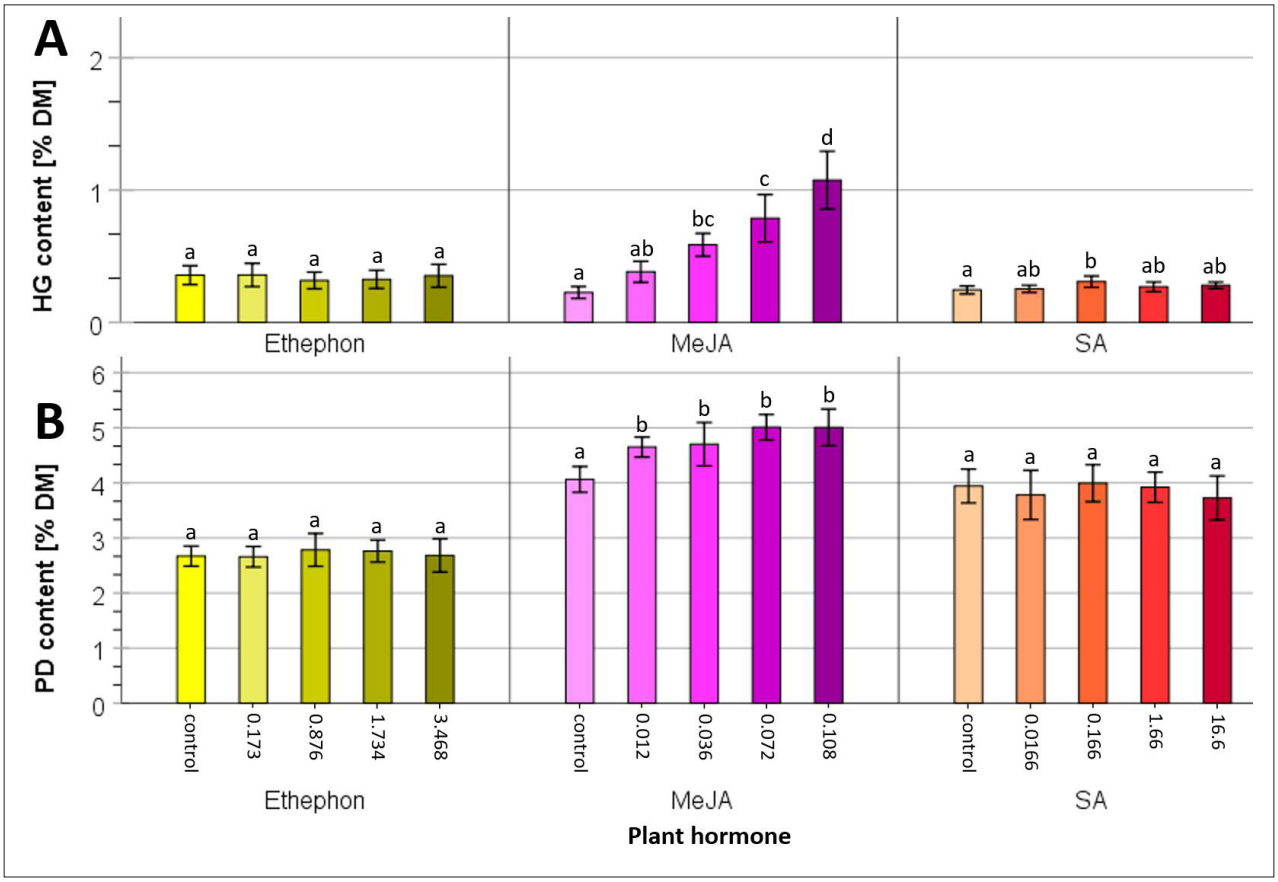


FIGURE 3. Average content (%) of hydrangenol (A) and phyllodulcin (B) in leaf dry matter of *H. macrophylla* subsp. *serrata* cultivar 'Oamacha' in 1st (i.e., youngest) leaf samples depending on the applied amounts of hormone (mmol plant^{-1}). Different hormone concentrations are indicated with light (control group) to dark (highest hormone concentration) colour shades. Significant differences between concentrations within one group of plants receiving the same hormone are indicated by different letters (Bonferroni, $n = 8$, $\alpha = 0.05$). Errors bars indicate 95% confidence intervals.

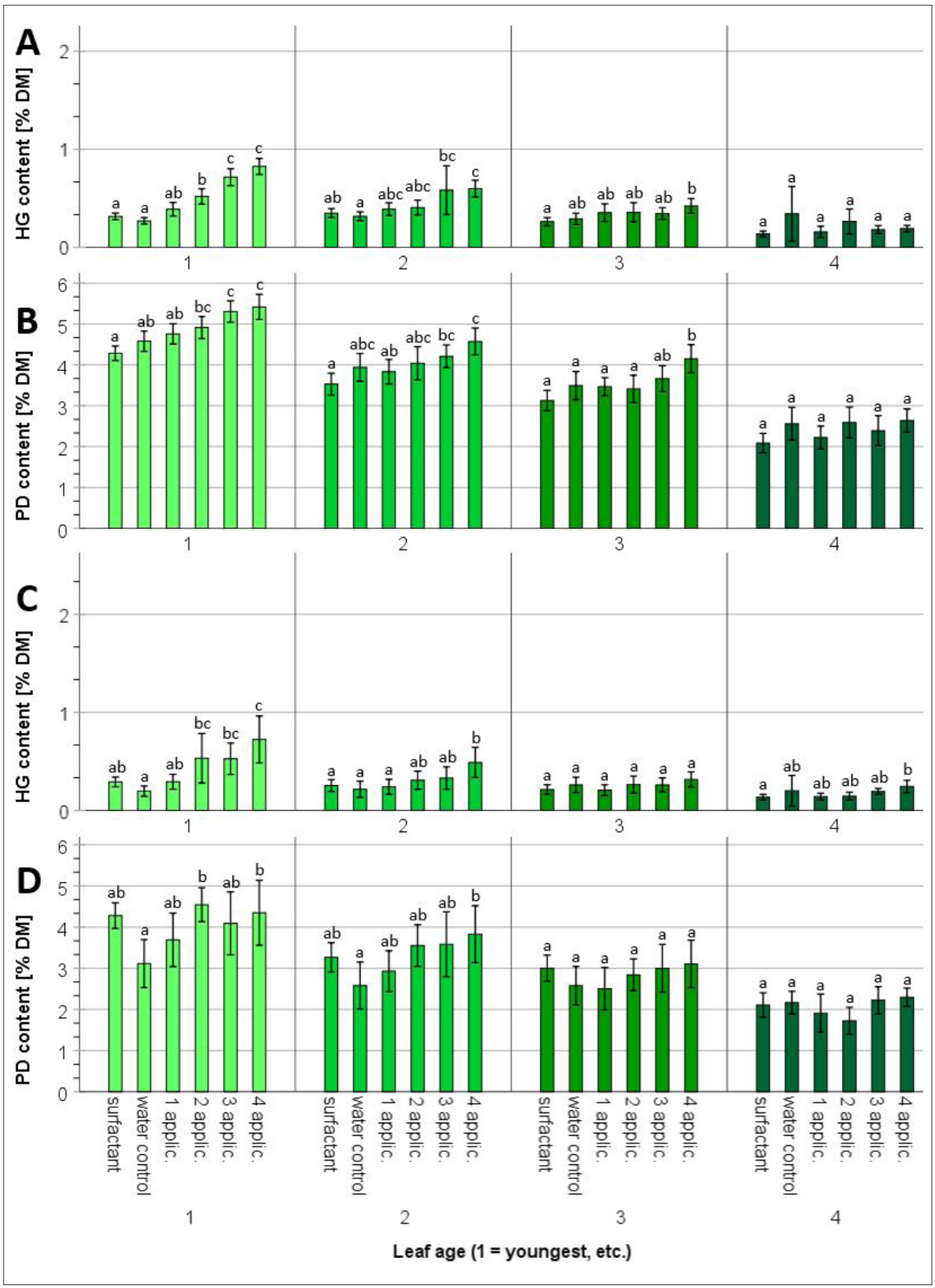


FIGURE 4. Average content (%) of hydrangenol (A, C) and phyllodulcin (B, D) in 1st to 4th youngest leaves (light to dark green: 1st to 4th youngest leaf) in vegetative (A, B) and generative (C, D) 'Oamacha' cultivars depending on the number of MeJA applications. Significant differences between the no. of applications within leaves of the same age are indicated with different letters (Bonferroni, $n = 15$, with the exception of the 4th youngest leaf (A, B), where $n = 14$ and (C, D), where $n = 8$; $\alpha = 0.05$). Errors bars indicate 95% confidence intervals. See also Supplemental Information – Figure S1.

plants were pruned on three different dates (9th, 13th, and 16th of October) and randomly assigned to treatment (receiving 4× 60 µmol MeJA plant^{-1}) and control group (receiving 4× dH_2O) thereafter. The first application was done 26, 29, 33 days after pruning, respectively. One day after the last application, sampling of 1st leaves for DHC quantification ($n = 50$) was done in the same way as already described.

Fresh biomass

Fresh biomass was determined as described by Cornelissen et al. (2003). In short, three days after the second sampling in 2020a and five days after the last sampling in 2020b all plants were cut above-ground (i.e., above the lowest nodes to facilitate regrowth) and fresh weight was immediately determined using a scale (Sartorius BCE6202–1S).

DHC quantification

Phyllodulcin and hydrangenol were quantified after full hydrolysis of the glycosidic precursors via endogenous glucosidases during fermentation. Leave samples were dried as described above and milled in a mortar prior to analyses. From the milled dried leaves two technical replicates were weighed in (about 15–25 mg), fermented, extracted with methanol and analyzed via UPLC against phyllodulcin and hydrangenol standards as described in Moll et al. (2021). Values were calculated as mass percent based on dry matter (% DM).

Statistical analysis

Data were collected and sorted using Microsoft Excel 2016. Statistical calculations were done using IBM SPSS 25.1 software. For normally distributed (Kolgomorov-Smirnoff) and homoscedastic (Levene) data, one-way analysis of variance (ANOVA) was calculated to reveal significant experimental factors by comparing means. If not stated otherwise, Bonferroni adjustment was used as a post-hoc test procedure to determine homogenous subgroups within treatment factors at a p-value of ≤ 0.05.

Results

All displayed DHCs contents consider the dry matter percentage (% DM) of the specific DHC found in the respective leaves. The presentation of the results starts with the data obtained after application of ethylene, SA and MeJA on HG and PD accumulation in 2019, followed by the responsiveness of vegetative and generative plants to MeJA depending on leaf age and the number of applications, studied in 2020a and b. The effects of repetitive MeJA applications on 'Amagi Amacha' cultivars, experimented in 2020, are presented in (c). Finally, impacts of hormone treatment on the fresh biomass of the 'Odoriko' cultivar, determined in 2020, are shown.

Impact of ethylene, methyl jasmonate and salicylic acid on DHC accumulation in 1st leaves (2019)

The applications of ethylene in form of ethephon did not

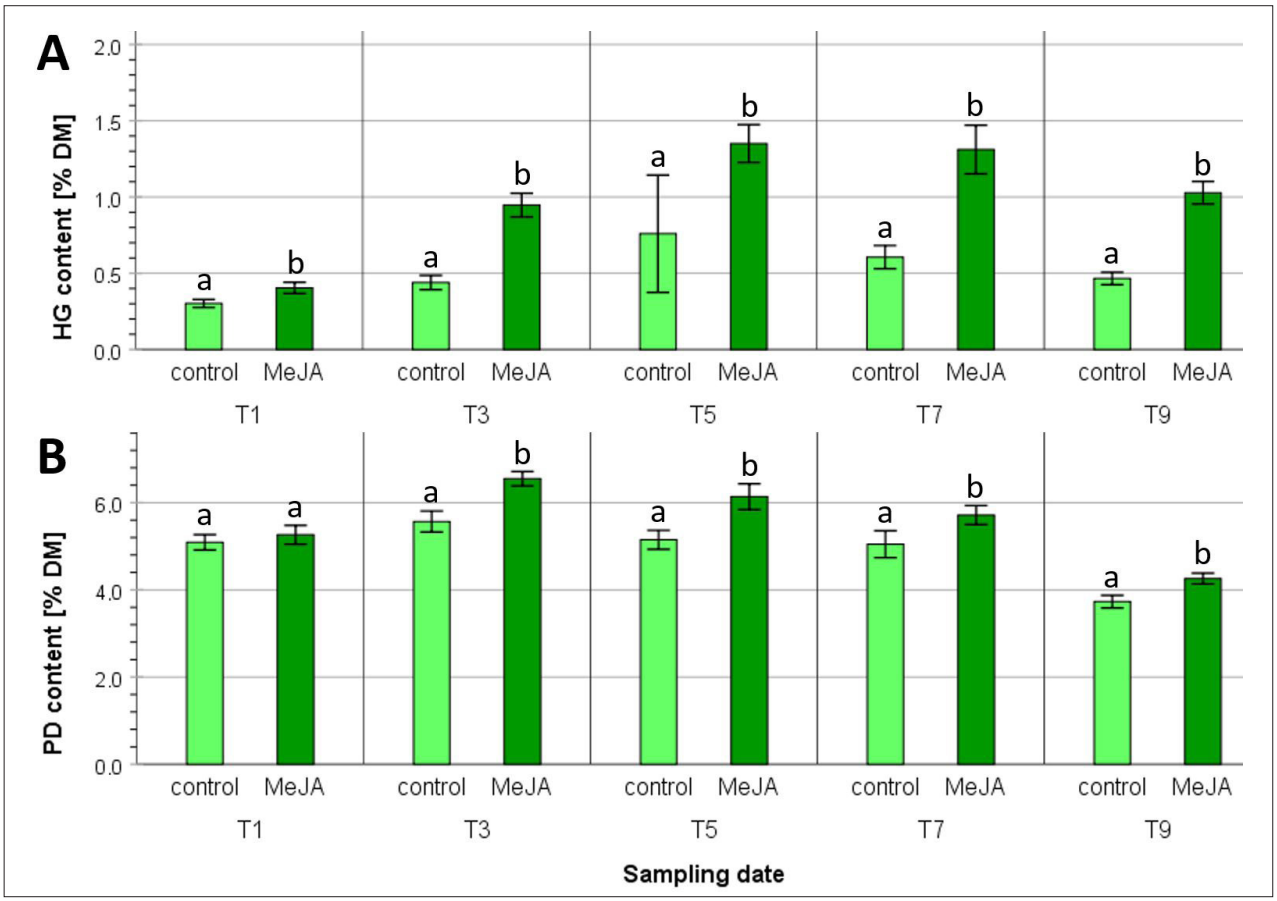


Figure 5. Average HG (A) and PD (B) content in young leaves of 'Oamacha' cultivars after 1, 3, 5, 7, and 9 applications (T1–T9) of either water (control) or 60 µmol MeJA per plant. Significant differences between control and MeJA-treated groups calculated for each sampling date (ANOVA, $n = 18$ with the exception of T9 where $n = 90$, $\alpha = 0.05$) are indicated with letters. Errors bars indicate 95% confidence intervals.

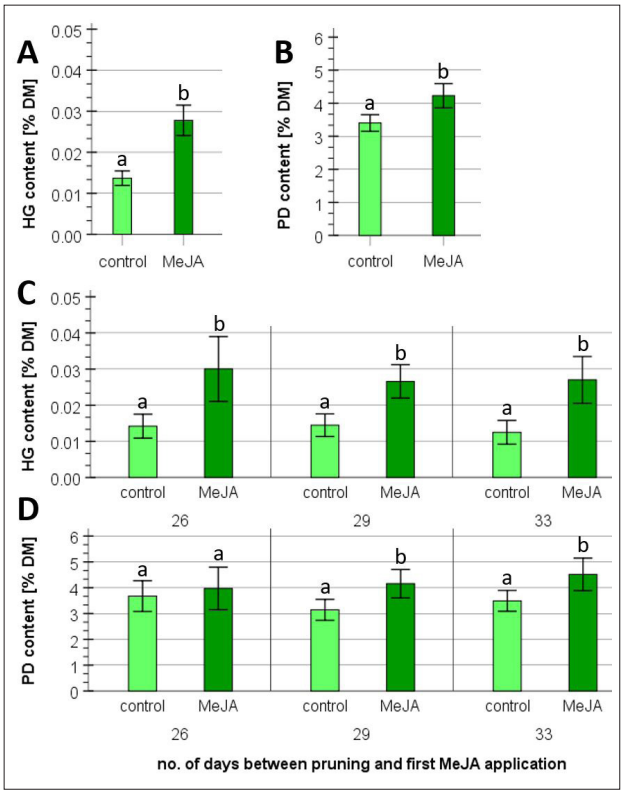


Figure 6. Average HG (A, C) and PD (B, D) contents in 1st (youngest) leaf dry matter of 'Amagi Amacha' cultivars treated four times with either water (control) or 60 µmol MeJA per plant (A, B = aggregated; C, D = contrasting the no. of days between pruning and first application). Significant differences between control and MeJA-treated groups calculated (separately for each subgroup in C and D) by ANOVA (A, B: $n = 50$ and C, D: $n = 13$, 16, 17 for subgroups 26, 29, and 33, respectively, $\alpha = 0.05$) are indicated with different letters. Please note that, compared to the other Figures, a different scale for HG contents had to be used. Errors bars indicate 95% confidence intervals.

increase hydrangenol contents in young leaves ($F = 0.360$, $p = 0.835$) as shown in Figure 3A. SA treatment had an impact on HG accumulation ($F = 3.109$, $p = 0.027$) but only in plants treated with 165.7 µmol SA. However, as revealed by post-hoc testing, the significant increase of HG content was averaged only to 25%, compared to control (A).

The application of MeJA led to significant changes in the accumulation of HG ($F = 32.710$, $p = 0.000$) in young leaves (Figure 3A). The increase was averaged to 375% when 108 µmol MeJA were applied. Higher amounts of MeJA led as well to a low increase of PD ($F = 10.248$, $p = 0.000$). Phyllodulcin was increased in tendency (Figure 3B) when applying more than 36 µmol MeJA. However, even according to less conservative post-hoc procedures, e.g., Tukey HSD, the tested amounts of MeJA did not affect PD contents differently. For the applications of ethylene and SA, no significant effect on PD accumulation was found ($F = 0.321$, $p = 0.862$ and $F = 0.563$, $p = 0.691$, respectively).

Impact of MeJA applications on DHC accumulation in vegetative and generative *H. macrophylla* subsp. *serrata* (2020a)

Repeated application of 60 µmol MeJA caused significant increases in HG ($F = 50.967$, $p = 0.000$) and PD ($F = 13.093$, $p = 0.000$) contents in young leaves of vegetative plants (Figure 4A+B). Up to three applications are necessary to elicit an increase of HG and PD contents, respectively, as revealed by post-hoc testing. Four applications yielded the highest increases in HG (206%) and PD (18%) accumulation. Application of MeJA is almost inefficient in older leaves and effects disappeared completely in the oldest ones.

With generative plants, MeJA treatment was only significant in 1st and 2nd youngest leaves (Figure 4C+D). Here, HG in 1st ($F = 6.945$, $p = 0.000$) and 2nd leaves ($F = 4.383$, $p = 0.001$) were significantly increased after 2 and 4 MeJA applications, respectively. The PD contents in 1st ($F = 3.409$, $p = 0.009$) and 2nd leaves ($F = 2.892$, $p = 0.019$) were not significantly different in comparison to at least one of both control groups (i.e., surfactant or water).

Based on dry leaf matter, neither hydrangenol nor phyllodulcin contents were still elevated one month after the last application, regardless of the number of repetitions and of leaf age (Supplemental Information – Figure S1A–D). However, leaves from vegetative plants still contain higher contents. By summing up HG and PD contents of all (1st – 4th youngest) leaves from both control groups of generative and vegetative plants, it is obvious that HG and PD contents are significantly higher in vegetative plants one day ($F = 9.138$, $p = 0.003$ and $F = 17.364$, $p = 0.000$, respectively) and one month ($F = 9.233$, $p = 0.003$ and $F = 6.143$, $p = 0.014$, respectively) after the last application (see Supplemental Information – Figure S2A–D). In flowers, averaged HG and PD contents are 0.2 and 1.6% DM (Supplemental Information – Figure S3).

Impact of the number of MeJA applications on DHC accumulation (2020b)

Data gathered in 2020b evidenced no considerable further enhancement of HG contents in young leaves after more than three MeJA applications, compared to untreated plants (Figure 5A). Averaged increase determined one day after the first application (T1) was 34.0% and at T3 115.6%, while staying in this range after further applications (T7: 116.5%, T9: 120.7%). Hence, by repeating MeJA applications nine (T9) instead of three (T3) times resulted only in additional 5.1% net-gain. All averaged increases were significantly different from control with $p \leq 0.000$. The PD contents (Figure 5B) increased significantly after three applications (17.7%). Then, differences between control and MeJA-treated group, although remaining significant, declined.

Impact of MeJA applications on DHC accumulation in 'Amagi Amacha' cultivar (2020c)

HG levels in 'Amagi Amacha' cultivars were much lower than in 'Odoriko' plants. MeJA applications lead to significant increases of HG ($F = 47.409$, $p = 0.000$) and PD ($F = 13.909$, $p = 0.001$) contents in young leaves (Figure 6A+B). The DHC increase averaged to 102.9% for HG (A) and to 24.3% for PD (B). HG contents increased significantly ($F = 17.177$, $p = 0.000$), in contrast to PD contents ($F = 0.346$, $p = 0.56$) in plants pruned 26 days before hormone treatment (Figure 6C+D). When treated 29 days after pruning, concomitant with a higher biomass increment, the effect of hormone application was significant for both DHCs. This finding is in accordance with the results from 2020b, where 'Odoriko' plants were pruned 36 days before the first MeJA application.

In 2020a, data of fresh biomass yield (in gram) were stratified according to the developmental states (i.e., vegetative and generative plants). Statistical analysis reveals, that the developmental state significantly affects yield ($F = 66.475$,

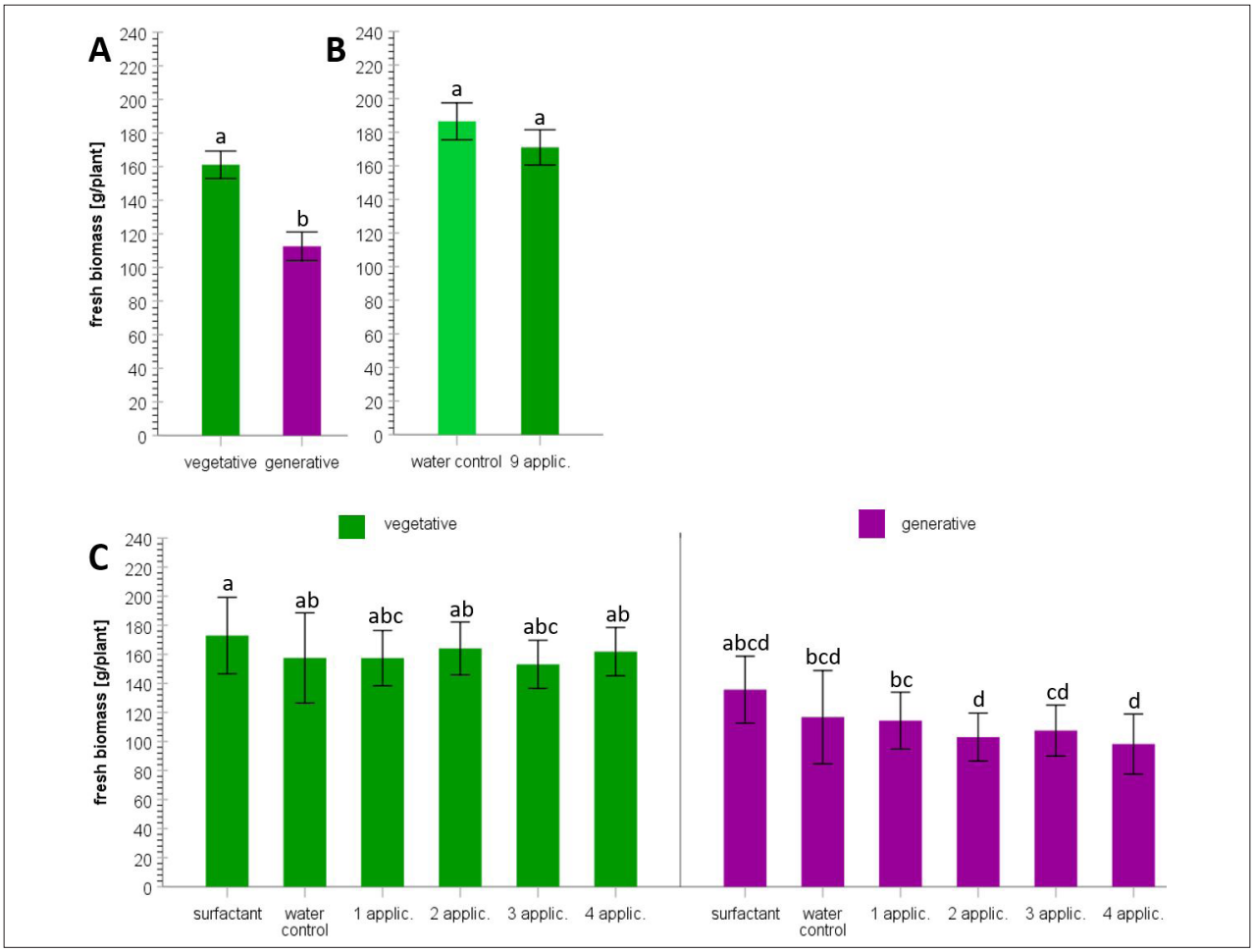


FIGURE 7. Fresh biomass per plant after 2020a (A = aggregated; C = contrasting treatments) and 2020b (B). Letters above the bars indicate significant differences (α = 0.05) according to ANOVA (A, B, C, where $n = 90$ for A and B; $n = 15$ for C). Errors bars indicate 95% confidence intervals.

p = 0.000) as shown in Figure 7A. Compared to generative plants, averaged biomass yield from vegetative plants is 43.1% higher. Hormone treatment does not affect biomass within the groups of vegetative (F = 0.454, p = 0.809) and generative (F = 1.639, p = 0.159) plants. However, post-hoc testing with all twelve subgroups confirmed that subgroups within the respective strata are not significantly different from their corresponding controls (C).

In 2020b (Figure 7B), MeJA applications did not affect fresh biomass yield significantly (F = 2.152, p = 0.144).

Discussion

In this study, neither SA nor ethylene application had a considerable effect on hydrangenol and phyllodulcin accumulation in *H. macrophylla* subsp. *serrata* 'Oamacha' cultivars. We did not observe any changes in DHC accumulation after application of moderate to high concentrations of ethephon and thus we conclude, that ethylene related signal transduction has no function in promoting hydrangenol or phyllodulcin biosynthesis. As described by Defilippi et al. (2004) for *Malus domestica*, the effects of ethylene on secondary metabolite biosynthesis can differ dependent on compounds nature or are even ethylene-independent. According to our data, HG biosynthesis is not responsive to ethylene.

Only MeJA led to significant increases in both DHCs when applied more than once. MeJA application has been recognized as a method to enhance secondary metabolite production, for instance *in vitro* culture systems (Ho et al., 2020). The elicitation mechanism leads to the induction of pathogenesis related proteins and release of reactive oxygen- and nitrogen species (ROS and RNS) and further signalling molecules including JA from the precursor α-linolenic acid in the plasma membrane. Subsequently, transcription factors involved in secondary metabolite biosynthesis are activated or de novo synthesized, leading to the initiation of secondary metabolite production (Ho et al., 2020). In SA/jasmonic acid (JA) crosstalk signalling, SA can suppress certain JA induced responses. These antagonistic effects are well documented, as well as the Janus-head nature of SA acting as a pro-oxidant and an antioxidant molecule (Caarls et al., 2015; Saleem et al., 2021). The pro-oxidant properties will increase ROS accumulation, leading to the release of JA from the precursor α-linolenic acid. It is therefore concluded, that SA at the applied concentrations suppresses JA responses and down regulates hereby the JA dependent HG synthesis, explaining why 165.7 µmol SA application, although enhancing ROS, results only to 25% increase in HG accumulation.

General differences in DHC contents measured in 2019 and 2020, where peatmoss or perlite was used, could result from the respective substrates, as recent studies with *Echinacea purpurea* L. show that substrate composition in hydroponic systems affects not only plant growth but also accumulation of secondary metabolites, such as caffeic acid (Ahmadi et al., 2021a, b).

We demonstrated for 'Odoriko' cultivars, that MeJA application increases HG and PD contents depending on (1) the applied amount (2019) and (2) on the number of applications (2020a, b). For 'Amagi Amachi' cultivars, DHC contents increased by using the same method, hence, MeJA application constitute a suitable method to enhance HG and PD containing *H. macrophylla* subsp. *serrata*.

We also showed, that MeJA increases HG and PD contents in 1st, 2nd and 3rd but not in 4th youngest leaves. We thus conclude that during leave development, genes controlling HG/PD synthesis and/or transport are turned off and in consequence remain inactive despite hormone applications.

In the youngest sampled leaves, HG contents doubled on average and even tripled in some samples after at least three applications. We also observed an immediate, significant increase in HG contents after one MeJA application. Therefore, we assume that the biosynthesis of hydrangenol could be under control of JA, in contrast to the one of phyllodulcin and seemingly, the biosynthesis and/or transport mechanisms are preferentially active in the youngest leaves. For defined secondary metabolites, a down regulation or silencing of the biosynthetic pathway in older plant tissues is a known phenomenon, for instance described for benzoxazinones and sorgoleone (Baerson et al., 2008; Jonczyk et al., 2008).

We also found that MeJA applications, which had no impact on fresh biomass accumulation, had no consistent effect in generative plants. Here, HG contents were significantly enhanced after four applications in the 1st and 2nd youngest leaves sampled. However, PD contents were not. This substantiates the assumption, that MeJA-triggered pathways primarily control HG biosynthesis and/or accumulation, essentially in young tissue.

Since MeJA treatment leads to transient increases of HG and PD, as DHC contents in leaves start to level out within days after the last application, gaining a DHC surplus from biomass extraction requires that MeJA is applied throughout the last days of cultivation up until harvest.

Our findings do not confirm previous-reports of highest PD contents in *H. macrophylla* subsp. *serrata* during flowering (Ujihara et al., 1995). Although grown under identical conditions for more than two months, vegetative control group plants (surfactant and water control) showed a remarkably higher DHC content than generative control group plants in two samplings (in June and July). Additionally, as illustrated in Supplemental Information – Figure S3, the average content of PD in flowers is considerably lower than extractable contents in foliage.

Flowering is also unfavourable in terms of an intensive, year-round production of high DHC-containing *H. macrophylla* subsp. *serrata*, as the annual cycle of all *Hydrangea* species encompasses a period of dormancy. *H. macrophylla* subsp. *serrata* cultivars were dormant after flowering in autumn and needed a cold treatment of around 1,000 hours at 2.5°C to resume growth (Preusche et al., 2022). Afterwards, the plants still developed new flowers and continued generative growth to a certain degree. Thus, maintaining suitable forcing conditions in the greenhouse in combination with regular pruning to prevent flowering and dormancy is essential for year-round production, especially when MeJA applications are used to maximize DHC contents pre-harvest.

Acknowledgments

We thank Gerhard Krammer (Symrise AG) for valuable comments and critical reading of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

References

Adkins, J.A., and Dirr, M.A. (2003). Remontant flowering potential of ten *Hydrangea macrophylla* (Thunb.) Ser. cultivars. HortSci *38*, 1337–1340. https://doi.org/10.21273/HORTSCI.38.7.1337.

Adkins, J.A., Dirr, M.A., and Lindstrom, O.M. (2003). Cold hardiness estimates for ten hydrangea taxa. Acta Hortic. *618,* 163–168. https://doi.org/10.17660/ActaHortic.2003.618.17.

Ahmadi, F., Samadi, A., Sepehr, E., Rahimi, A., and Shabala, S. (2021a). Increasing medicinal and phytochemical compounds of coneflower (*Echinacea purpurea* L.) as affected by NO_3^-/NH_4^+ ratio and perlite particle size in hydroponics. Sci. Rep. *11*, 15202. https://doi.org/10.1038/s41598-021-94589-4.

Ahmadi, F., Samadi, A., Sepehr, E., Rahimi, A., and Shabala, S. (2021b). Perlite particle size and NO_3^-/NH_4^+ ratio affect growth and chemical composition of purple coneflower (*Echinacea purpurea* L.) in hydroponics. Ind. Crops Prod. *162*, 113285. https://doi.org/10.1016/j.indcrop.2021.113285.

Baerson, S.R., Dayan, F.E., Rimando, A.M., Nanayakkara, N.P.D., Liu, C.-J., Schröder, J., Fishbein, M., Pan, Z., Kagan, I.A., Pratt, L.H., et al. (2008). A functional genomics investigation of allelochemical biosynthesis in *Sorghum bicolor* root hairs. J. Biol. Chem. *283*, 3231–3247. https://doi.org/10.1074/jbc.M706587200.

Beyer, S.F., Bel, P.S., Flors, V., Schultheiss, H., Conrath, U., and Langenbach, C.J.G. (2021). Disclosure of salicylic acid and jasmonic acid-responsive genes provides a molecular tool for deciphering stress responses in soybean. Sci. Rep. *11*, 20600. https://doi.org/10.1038/s41598-021-00209-6.

Caarls, L., Pieterse, C.M., and Van Wees, S.C. (2015). How salicylic acid takes transcriptional control over jasmonic acid signaling. Front Plant Sci. *6*, 170. https://doi.org/10.3389/fpls.2015.00170.

Cornelissen, J.H.C., Lavorel, S., Garnier, E., Díaz, S., Buchmann, N., Gurvich, D.E., Reich, P.B., ter Steege, H., Morgan, H.D., van der Heijden, M.G.A., et al. (2003). A handbook of protocols for standardised and easy measurement of plant functional traits worldwide. Aust. J. Bot. *51*, 335. https://doi.org/10.1071/BT02124.

Croy, J.R., Carvajal Acosta, N., and Mooney, K.A. (2021). Regulating plant herbivore defense pathways in the face of attacker diversity. New Phytol. *231*, 2110–2112. https://doi.org/10.1111/nph.17509.

Defilippi, B.G., Dandekar, A.M., and Kader, A.A. (2004). Impact of suppression of ethylene action or biosynthesis on flavor metabolites in apple (*Malus domestica* Borkh.) fruits. J. Agric. Food Chem. *52*, 5694–5701. https://doi.org/10.1021/jf049504x.

Gotoh, T., and Gomi, K. (2000). Population dynamics of *Tetranychus kanzawai* (Acari: Tetranychidae) on hydrangea. Ex. Appl. Acarol. *24*, 337–350. https://doi.org/10.1023/A:1006428503702.

Ho, T.-T., Murthy, H.N., and Park, S.-Y. (2020). Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. Int. J. Mol. Sci. *21*, 716. https://doi.org/10.3390/ijms21030716.

Jonczyk, R., Schmidt, H., Osterrieder, A., Fiesselmann, A., Schullehner, K., Haslbeck, M., Sicker, D., Hofmann, D., Yalpani, N., Simmons, C., et al. (2008). Elucidation of the final reactions of DIMBOA-glucoside biosynthesis in maize: Characterization of *Bx6* and *Bx7*. Plant Physiol. *146*, 1053–1063. https://doi.org/10.1104/pp.107.111237.

Jung, C.-H., Kim, Y., Kim, M.-S., Lee, S., and Yoo, S.-H. (2016). The establishment of efficient bioconversion, extraction, and isolation processes for the production of phyllodulcin, a potential high intensity sweetener, from sweet hydrangea leaves (*Hydrangea*

macrophylla Thunbergii). Phytochem. Anal. *27*, 140–147. https://doi.org/10.1002/pca.2609.

Kawamura, M., Kagata, M., Masaki, E., and Nishi, H. (2002). Phyllodulcin, a constituent of "Amacha", inhibits phosphodiesterase in bovine adrenocortical cells. Regul. Toxicol. Pharmacol. *90*, 106–106. https://doi.org/10.1034/j.1600-0773.2002.900209.x.

Khaing, M.T., Jung, H.-J., and Han, T.-H. (2016). Trend of hydrangea cultivar development. Trends Agric. Life Sci. *53*, 63–68. https://doi.org/10.29335/tals.2016.53.63.

Kindl, H. (1971). Formation of a stilbene in isolated chloroplasts. Hoppe-Seyler's Z. Physiol. Chem. *352*(5), 767–768. PMID: 5579984.

Li, N., Han, X., Feng, D., Yuan, D., and Huang, L.-J. (2019). Signaling crosstalk between salicylic acid and ethylene/jasmonate in plant defense: Do we understand what they are whispering? Int. J. Mol. Sci. *20*, 671. https://doi.org/10.3390/ijms20030671.

Matsuda, H., Shimoda, H., Yamahara, J., and Yoshikawa, M. (1998). Immunomodulatory activity of thunberginol a and related compounds isolated from hydrangeae dulcis folium on splenocyte proliferation activated by mitogens. Bioorganic Med. Chem. Lett. *8*, 215–220. https://doi.org/10.1016/S0960-894X(97)10221-9.

Moll, M.D., Vieregge, A.S., Wiesbaum, C., Blings, M., Vana, F., Hillebrand, S., Ley, J., Kraska, T., and Pude, R. (2021). Dihydroisocoumarin content and phenotyping of *Hydrangea macrophylla* subsp. *serrata* cultivars under different shading regimes. Agronomy *11*, 1743. https://doi.org/10.3390/agronomy11091743.

Murase, A., and Fujita, K. (2018). Predator experience changes spider mites' habitat choice even without current threat. Sci. Rep. *8*, 8388. https://doi.org/10.1038/s41598-018-26757-y.

Nozawa, K., Yamada, M., Tsuda, Y., Kawai, K., and Nakajima, S. (1981). Antifungal activity of oosponol, oospolactone, phyllodulcin, hydrangenol, and some other related compounds. Chem. Pharm. Bull. *29*, 2689–2691. https://doi.org/10.1248/cpb.29.2689.

Pangesti, N., Reichelt, M., van de Mortel, J.E., Kapsomenou, E., Gershenzon, J., van Loon, J.J.A., Dicke, M., and Pineda, A. (2016). Jasmonic acid and ethylene signaling pathways regulate glucosinolate levels in plants during rhizobacteria-induced systemic resistance against a leaf-chewing herbivore. J. Chem. Ecol. *42*, 1212–1225. https://doi.org/10.1007/s10886-016-0787-7.

Park, Y.-S., Jung, S.-T., Kang, S.-G., Drzewiecki, J., Namiesnik, J., Haruenkit, R., Barasch, D., Trakhtenberg, S., and Gorinstein, S. (2006). In vitro studies of polyphenols, antioxidants and other dietary indices in kiwifruit (*Actinidia deliciosa*). Int. J. Food Sci. Nutr. *57*, 107–122. https://doi.org/10.1080/09637480600658385.

Preusche, M., Ley, J., and Ulbrich, A. (2022). Einfluss unterschiedlich langer Kühlphasen auf die Dormanz eines *Hydrangea serrata* Genotyps. DGG-Proc. *10*(1), 1–7. https://doi.org/10.5288/dgg-pr-10-01-mp-2021.

Saleem, M., Fariduddin, Q., and Castroverde, C.D.M. (2021). Salicylic acid: A key regulator of redox signalling and plant immunity. Plant Physiol. Biochem. *168*, 381–397. https://doi.org/10.1016/j.plaphy.2021.10.011.

Uemachi, T., and Okumura, A. (2012). The inheritance of inflorescence types in *Hydrangea macrophylla*. J. Japan. Soc. Hortic. Sci. *81*, 263–268. https://doi.org/10.2503/jjshs1.CH-092.

Uemachi, T., Mizuhara, Y., Deguchi, K., Shinjo, Y., Kajino, E., and Ohba, H. (2014). Phylogenetic relationship of *Hydrangea macrophylla* (Thunb.) Ser. and *H. serrata* (Thunb.) Ser. evaluated using RAPD markers and plastid DNA sequences. J. Japan. Soc. Hortic. Sci. *83*, 163–171. https://doi.org/10.2503/jjshs1.81.263.

Ujihara, M., Shinozaki, M., and Kato, M. (1995). Accumulation of phyllodulcin in sweet-leaf plants of *Hydrangea serrata* and its neutrality in the defence against a specialist leafmining herbivore. Res. Popul. Ecol. *37*, 249–257. https://doi.org/10.1007/BF02515827.

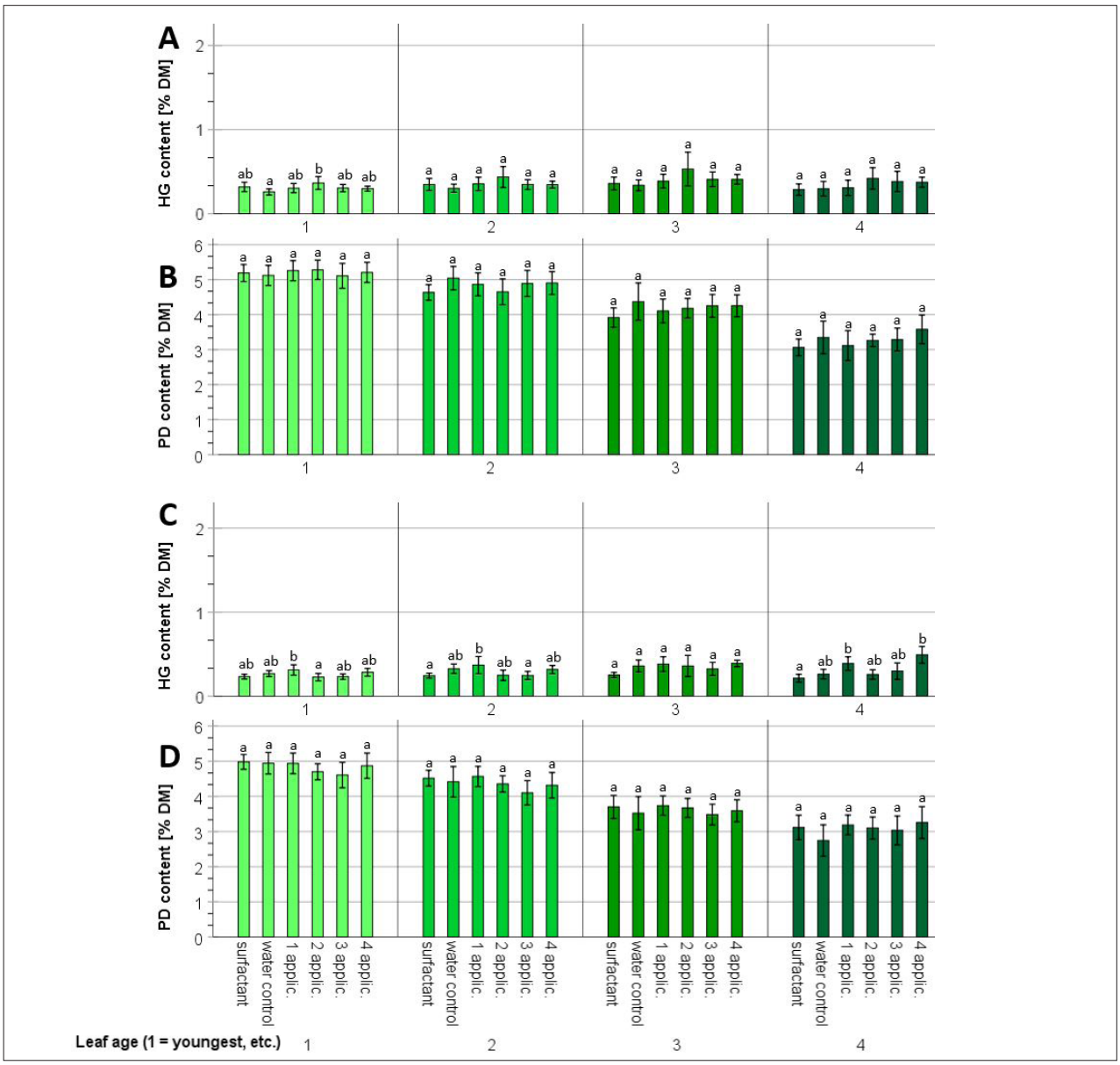
Yang, S.F. (1969). Ethylene evolution from 2-chloroethylphosphonic acid. Plant Physiol. *44*, 1203–1204. https://doi.org/10.1104/pp.44.8.1203.

Received: Mar. 10, 2022
Accepted: Jul. 22, 2022

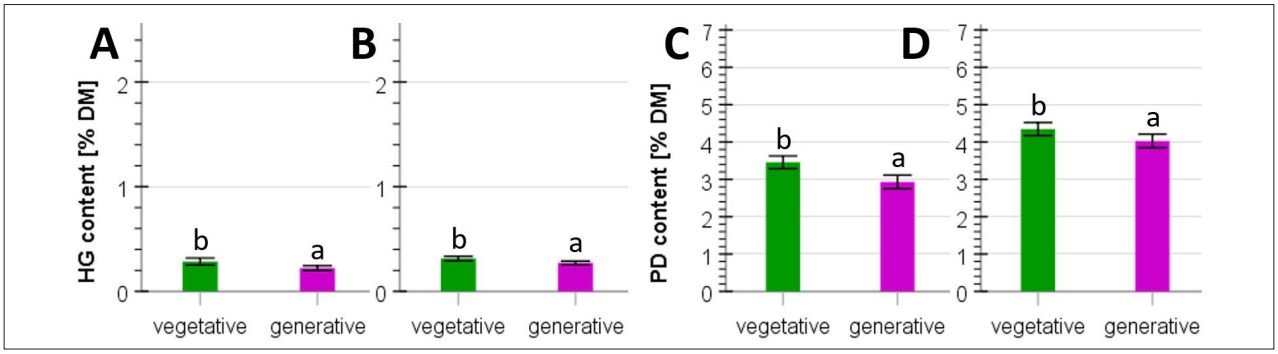
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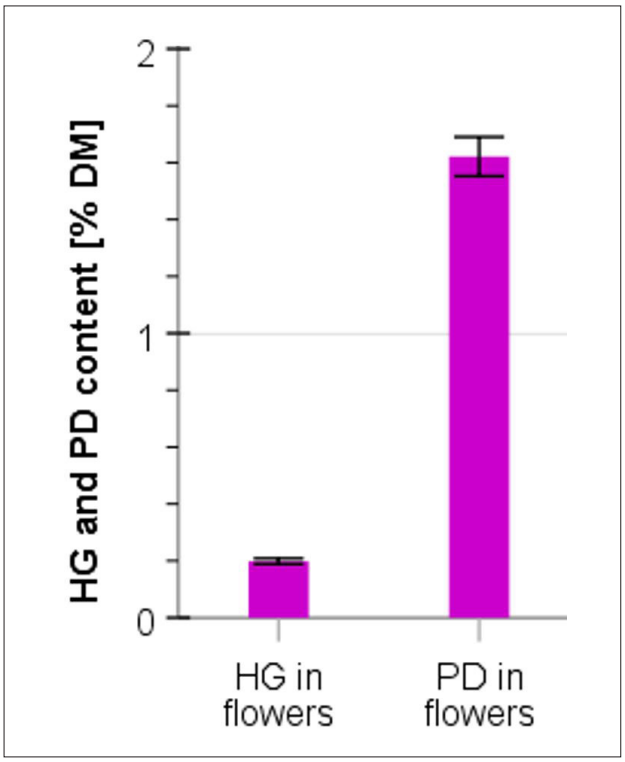
Supplemental Information



Supplemental Information – Figure S1, related to Figure 4; Average content (%) of hydrangenol (A, C) and phyllodulcin (B, D) in leaves (light to dark green: 1st to 4th youngest leaf) sampled from different shoots of the same vegetative (A, B) and generative (C, D) plants as in Figure 4 (see above), but one month after the last application. Significant differences between the no. of applications within leaves of the same age are indicated with different letters (Bonferroni, $n = 15$, with the exception of the 4th youngest leaf (A–D), where $n = 13$; $\alpha = 0.05$). Errors bars indicate 95% confidence intervals.



Supplemental Information – Figure S2, related to Figure 4; Average HG (A, C) and PD (B, D) content (%) in leaf dry matter of 1st to 4th youngest leaves of control (surfactant and water) plants depending on developmental state (i.e., vegetative or generative). Contents one day (A, C) and one month (B, D) after the last MeJA application are shown. Significant differences (ANOVA, $n = 113$ for A, C; $n = 116$ for B, D; $\alpha = 0.05$) are indicated with letters. Errors bars indicate 95% confidence intervals.



Supplemental Information – Figure S3, related to Figure 4; Average HG and PD contents of 'Oamacha' cultivars on a scale from 0–2% dry matter ($n = 16$, composite samples). Error bars indicate 95% confidence intervals.