Impact of Biocontrol Agents on Biochemical Changes of Aquatic Weed Water Hyacinth, Pontederia crassipes (Mart.) Solms-Laubach

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Abstract

Invasive weeds are rapidly adapting to evolutionary changes, brought about by exposure to the plethora of plant-antagonist's interactions, which eventually leave an effect on the biological control of the weed. These interactions create an array of biochemical responses in the plant, which induce a range of defensive mechanisms to reduce the threat of injury. The present investigation reports the role of plant secondary metabolites in plant defense that may involve deterrence of antifeedant activity associated with the application of biocontrol agents against *Pontederia crassipes*. Generally, an infestation is preferred on non-challenged plants, as increased content of alkaloid, phenol, or tannin, which *Pontederia crassipes* produces when infested by its agents, deters the latter by providing a toxic unpleasant atmosphere. Variation in flavonoid level also brings about some physiochemical changes in the weed which impede the entry of phytopathogens. When a few metabolites are used to dissuade the agents, some, like glycosides, attract feeders to lay eggs and allow their population to flourish. This study concentrates on the signals that enable *P. crassipes* to recognize and respond to the attack and measure the effect in biochemical terms. Through this has, an overall outlook of the fitness costs of attack not only for the weed but over the range of trophic levels has been enlightened with more scope to understand the underlying mechanisms, before the multi-agent release of agents.

Introduction

Under the rapidly changing environmental conditions, plants are exposed to a wide range of biotic interactions, which include insect and pathogens attack. Hence plants have evolved multiple defense mechanisms by which they are able to cope or become resistant to various kinds of biotic and abiotic stress that help to retain their fitness (Ballhorn et al. 2009; Mitchell et al. 2016). Secondary metabolites (SMs) are the products formed by interactions with the environment during plant growth and development. Many plant SMs act as chemical defenses against herbivores(Fraenkel 1959) and the presence of such metabolites has facilitated coevolution between plants and herbivores (Ehrlich and Raven 1964; Berenbaum et al. 1992). Ecological analysis of the costs and benefits of chemical defenses has allowed ecologists to make predictions about the number of chemical defenses in plants. Haukioja and his co-workers proposed a model of plant defense, which suggests that these 'induced defenses' reflect the ability of meristematic tissues to compete for resources that in turn could be allocated to growth and defense (Haukioja 1991; Honkanen et al. 1994). Fast-growing plants (species or individuals) synthesize smaller amounts of SMs than slow-growing plants because the replacement of plant materials lost to herbivores is more difficult in slow-growing plants (Feeny and Bostock 1968; Janzen 1974; Rhoades and Cates 1976; Coley et al. 1985; Bazzaz et al. 1987; Herms and Mattson 1992).

Most studies aim at understanding one-to-one interaction, but plants are under the influence of multiple organisms that are constantly interacting with each other, affecting not just the plants' responses but also how other co-existing species respond to each other (Ray and Hill 2016; Dutta and Ray 2017). Associations among vascular plants, fungi, and insects have a long history (Dutta et al. 2015). Aromatic compounds accumulating in the host tissue of plants affected by parasites, like fungi, bacteria or viruses, is a widespread phenomenon (Farkas and Kiraaly 1962). Fungal infection of plants usually alters plant

chemistry, by either increasing defense-chemical levels or decreasing nutrient levels, which, in turn, can influence insect performance (Raman et al. 2012). Pathogenic fungi are associated with plants either as biotrophs or as necrotrophs. Necrotrophs extract nutrients by killing host plant tissues, whereas biotrophs extract nutrients without doing so. Both induce specific, altered physiologies in their host plants. Such changes may affect the growth and development of insects negatively (Tasin et al. 2012). Plant-feeding insects and plant-pathogenic fungi often co-occur on the same plants (Karban et al. 1987). When infected by fungi (e.g., *Botrytis cinerea* Pers., Sclerotiniaceae), *Vitis vinifera* L. (Vitaceae) leaves synthesize SMs, pathogenesis-related proteins, chitinase, and β-1, 3 glucanase (Trotel-Aziz et al. 2006). In contrast, fungal infection can suppress plants' defense responses by altering secondary-metabolic pathways and improving nutritional quality, rendering the plant amenable to insect colonization (Cardoza et al. 2003). Pathogenic fungi modify plant volatiles and their profiles (Witzgall et al. 2012). Many SM-like cyanogenic glycosides and secondary glucosinolates occur as inactive precursors and become active in response to tissue damage or pathogen attack. This activation often involves enzymes, which are released due to a breakdown in cell integrity (Osbourn 1996; Dutta 2021).

Water hyacinth (WH), *Pontederia crassipes* Mart. Solms-Laubach (Pontederiaceae), a native of tropical South America, is, one such serious aquatic weed (Holm et al. 1977), on which multiple phytopathogenic and arthropodal biocontrol agents have been released(Coetzee et al. 2011) but scanty reports portray the scenario of the biochemical changes or the mechanisms that might be occurring as a result of the release of multiple agents (Dutta and Ray 2017). Several studies have shown synergistic effects on the release of two or more biocontrol agents (Ray and Hill 2012), but contrary antagonistic results with the release of multiple agents can make the biocontrol process less effective or even riskier (McEvoy and Coombs 2000; Dutta and Ray 2017). Therefore, to understand how phytopathogens may alter the chemistry of the host plant, making it favorable for herbivore consumption or leaving a direct impact on herbivore(Dutta and Ray 2017) biochemical analysis of affected and non-affected *P. crassipes*, prior to the release of agents may be useful. With the challenge of controlling the infestation of the target weed, understanding the underlying mechanisms can enlighten us to realize the perfect combinations of the biocontrol agents to be released against the target weed.

In this study, we have focused on various secondary metabolic profiles with respect to the stress undergone by WH in response to its biocontrol agents. The experiments were conducted on different sets of WH reared separately and infested with water hyacinth weevil *Neochetina bruchi* (Hustache) (NB)(Coleoptera: Curculionidae) and mite *Orthogalumna terebrantis* Wallwork (Acarina: Galumnidae) which are arthropod agents (AD). *Fusarium oxysporum* Schlecht (FO) was used as the fungal biocontrol agent (Dutta 2021). The SMs including phenols, glycosides, tannins, and flavonoids were studied in response to the mentioned biocontrol agents and their effect was evaluated over the period of a year.

Materials and Methods

Collection and maintenance of plant

Fresh, insect and disease-free WH in their early growth stages (4–6 leaves per plant, 15–25 cm in length, from shoot to the tip of the root) were collected from the field surveys from various waterbodies of Kolkata and its outskirts. They were transferred into a big 1000 L tank in the greenhouse as the stock culture at Presidency University, Kolkata, India. All the tanks were filled with tap water and supplemented with farmyard manure and 15–3–12 N: P: K, slow-release fertilizer. The plants were acclimatized before using them for the experiment.

Fungal pathogen and insects used for the study

Cultures of fungi *Fusarium oxysporum* Schlecht (FO) were isolated from diseased leaves of WH and collected during periodical surveys of various water bodies in and around Kolkata (Dutta et al. 2023). They were selected for this study because they have an excellent potential for causing disease against water hyacinths under controlled conditions (Dutta 2021). A 14-day-old culture of the fungus grown in a Biological Oxygen Demand (BOD) incubator at 27^{o}C and 12h photoperiod was used for all the experiments.

Neochetina bruchi (Hustache) (Coleoptera: Curculionidae) (NB), the water hyacinth weevil, and *Orthogalumna terebrantis* Wallwork (Acarina: Galumnidae) (OT), the water hyacinth mite, were collected from arthropod infested WH in local water bodies. The arthropods were reared separately in several 500 L tanks under greenhouse conditions. The tanks containing weevils were covered with fine mesh nets to prevent their escape. The second set of tanks, where mite was maintained, were placed at a distance, to prevent their invasion of weevil-reared tanks. The tanks were replenished with water and fertilizers (NPK) for plant maintenance as and when required. All dead leaves, stems and daughter plants were removed from time to time to maintain a healthy culture. Adult insects were collected from these stock cultures for experimentation.

Application of spore suspension and arthropods individually and in combination

The fungal conidial suspension was formulated to contain about 10^5 – 10^7 spores/ mL using a hemocytometer. To this Tween 20 (Oxysorbic polyoxyethylene sorbitan monooleate) was added at the rate of 0.05 mL/ 50 mL of fungal suspension as a surfactant (Ray). Freshly prepared spore suspension of the fungus was applied to WH. Arthropod-damaged (AD) WH leaves were collected from their respective stock cultures. The conidial suspension was applied on WH in various treatments, in combination with *N bruchi* (NB) and O. *terebrantis* (OT) against the weed. Experimental control was taken, which included fresh unaffected plants, with no biocontrol agents. The selected fungi (FO) and two arthropods were applied on WH plants in different combinations of FO + WH, OT + WH, NB + WH, FO + AD + WH and WH (FO – Fungus infected; OT – mite damaged; NB - weevil damaged; AD – both mite and weevil damaged; WH – undamaged and uninfected water hyacinth). The experiment was terminated after 30 days of application of biological control agents. The plants were taken out from the experimental tanks and washed gently with water and sent to the laboratory for further studies.

Preparation of plant sample

Biochemical studies were undertaken using the treated plants (with arthropod and fungal biocontrol agents) and untreated fresh plants. The root portions were cut-off, and the remaining parts were washed thoroughly under running tap water to free them from debris. The leaves and shoot portion of the weed were chopped into small pieces and dried in shade and then in a hot air oven (at 40^{o} C). The dried plants were made into a fine powder using a mixer grinder. The leaf powders of the test weed were stored for further studies.

Preparation of plant extracts

The powdered plant parts from each treatment were separately extracted with 95% ethanol, 95% methanol and distilled water, overnight at 4^{o}C. Samples for glycoside content were extracted using chloroform and chilled overnight at 4^{o}C. The extracts were then filtered using Whatmann No. 1 filter paper (42.5 mm Circle) and the solutions were concentrated using rotary evaporator (Buchi 100v Rotavapor), following their respective temperature and pressure conditions (Table 1). The crude samples isolated were then used for performing the tests (Lalitha et al. 2012).

To achieve optimal distillation results with a rotary evaporator, it is important to consider the specific solvent being used. The following guidelines can be followed:

- Water Bath Temperature: Set the water bath temperature to 60°C. There is no need to exceed this temperature as it can potentially degrade the solvent or lead to unnecessary evaporation.
- Cooling Water Temperature: Ensure that the cooling water temperature is below 20°C. This helps to efficiently condense the evaporated solvent vapor, facilitating its collection and preventing excessive loss.
- Vacuum Adjustment: Adjust the vacuum level based on the boiling point of the solvent being distilled. The boiling point determines the required vacuum to achieve efficient evaporation. Refer to the list below for recommended vacuum levels corresponding to different solvent boiling points:

1. For solvents with a boiling point below 40°C, adjust the vacuum level accordingly to facilitate their evaporation.
2. For solvents with a boiling point at or around 40°C, ensure the vacuum is set at an appropriate level to maintain efficient distillation.
3. For solvents with a boiling point above 40°C, a lower vacuum level may be sufficient to achieve the desired distillation.

Table 1
Solvents and its vacuum boiling point maintained

	Vacuum for boiling point at 40^{o} C
Solvent	**(mbar)**
Ethanol	175
Methanol	337
Distilled Water	72

Tests for alkaloids

Dragendroff's test: The extract on treating with a few drops of Dragendroff's reagent (Millipore Sigma), formation of orange, brown precipitate confirms the presence of alkaloids (Adams and Camp 1966).

Tests for flavonoids

Sodium hydroxide (NaOH tests): 1 ml of the stock solution was taken in a test tube and a few drops of 4%NaOH solution was added to it. The appearance of an intense yellow color in the test tube, which goes colorless with the addition of a few drops of dilute acid, indicated the presence of flavonoids (Mahesh et al 2013.)

Test for glycosides

Concentrated H_2SO_4 test: In 5 ml of extract, 2 ml of glacial acetic acid, one drop of 5% Ferric chloride ($FeCl_3$) and conc. Sulphuric acid (H_2SO_4) was added. The brown ring coloration in interphase marked the presence of glycosides(Gumbinger et al. 1992).

Ferric chloride ($FeCl_3$) test: The extract was treated with 2 ml of water and 10% aqueous, freshly prepared, $FeCl_3$ solution and observed for blue or green coloration (Hirakura et al. 1986).

Tests for tannins

Lead acetate test: The ethanol extract was treated with a few drops of 1% lead acetate solution (w/v) and observed for the formation of a yellow or red precipitate (Wall et al. 1969).

Ferric chloride test: Blue or black precipitate formation on treating the ethanol extract with 2 ml of freshly prepared $FeCl_3$ solution confirms the presence of tannins (Mailoa et al. 2013).

Tests for terpenoids

The qualitative phytochemical analysis for the presence of terpenoid was determined by using 0.8 g of plant sample, taken in a test tube, and 10 mL of 95% methanol poured into it. The mixture was shaken well and filtered. 0.5 mL extract of plant sample and 2 mL of 99% chloroform were mixed, and 3 mL of concentrated H_2SO_4 (98% v/v) was then added to it. The formation of reddish-brown color indicates the presence of terpenoids in the selected plants (Rao et al. 2021).

Quantitative analysis

Estimation of alkaloids

The methanol-filtered extract was evaporated on Buchi100v Rotavapor under vacuum at a temperature of 40^{o}C to dryness. A part of this residue was dissolved in 2 N Hydrochloric acid (HCl) and then filtered. 1 mL of the solution was transferred to a separatory funnel and washed with 10 mL (about 0.34 oz) chloroform (3 times). The pH of this solution was adjusted to neutral with 0.1 N NaOH. Then 5 mL of bromocresol green

solution (BCG), was prepared (Tabasum et al. 2016]). 5 mL of phosphate buffers and 5 mL of freshly prepared BCG were added to the solution.

For the preparation of standard (Figure S1A), aliquots of caffeine standard solution (made by dissolving 1 mg pure atropine in 10 mL distilled water) were measured and transferred to a separatory funnel and 5 mL (about 0.17 oz) of pH 4.7 phosphate buffers and 5 mL of BCG were added.

The mixtures, in both cases, were shaken and the complex formed was extracted with 1, 2, 3- and 4 mL chloroform by vigorous shaking. The extracts were collected in a 10 mL volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against a blank prepared above without caffeine.

Estimation of flavonoids

Flavonoids were estimated based on the formation of the flavonoids-aluminium complex. 1 mL of the extract was mixed with 0.075 mL of 5% sodium nitrite solution and incubated at room temperature for 10 min (Zhishen et al. 1999). 10% Aluminum Chloride ($AlCl_3$) was then added and incubated at room temperature for 6 min. Later 1 N NaOH was added to it. The absorbance was read at 510 nm against a blank reagent. The absorption of standard quercetin solution (following serial dilution) was measured under the same conditions (Figure S1B)

Estimation of glycosides

10 mL of the extract and 10 mL of Baljet's reagent (5ml of 1% of picric acid solution in ethanol and 5 ml of 10% alcoholic potassium hydroxide solution). were taken and allowed to stand for an hour, then the solution was diluted with 20 mL distilled water and mixed. An orange-red color complex appeared. The intensity (absorbance) of color produced is proportional to the concentration of glycosides which is measured against the blank at 495 nm using a spectrophotometer. The difference between the test and the control was taken for the calculation.

Concentration (%) =(Absorbance*100)/17 g %

For standardization, 0.02% digitoxin was prepared in chloroform: methanol (1:1) and the graph was plotted using various concentrations (Figure S1C.).

Estimation of total phenols

Total phenols were estimated using Folin - Ciocalteau method (Bray and Thorpe 1954). 1 mL of the extracted solution was transferred into a test tube, to which 0.5 mL of 2 N Folin - Ciocalteau reagent was added and incubated at room temperature for 3 min. Further 1.5 mL of 20% Sodium Carbonate (Na_2CO_3) was added after 3 min and the volume was made up to 8 mL with distilled water, followed by vigorous shaking and incubating in a boiling water bath for 1 min. Rapidly cooled and the absorbance was read at 650 nm against blank reagent. The data were used to estimate total phenols using a standard calibration curve obtained from various diluted concentrations of gallic acid, measured at 765 nm (Figure S1D), after which the results were expressed as mg/g sample.

Estimation of tannins

500 mg of the sample was weighed into a 200 mL plastic bottle. 50 mL of distilled water was added and mixed thoroughly for 1 h in a shaker. This was filtered into a 50 mL volumetric flask and made up to the mark. Then 5 mL of the filter was pipetted out into a test tube and mixed with 2 mL of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide (C FeK N). The absorbance was read at 120 nm within 10 mins(Van Buren and Robinson 1969) The standard calibration was obtained using different concentrations of gallic acid, measured at 765 nm, following which the optical density (OD) values measured, were expressed in mg/mL (Van Buren and Robinson 1969). (Figure S1D).

Estimation of terpenoids

The previously prepared sample for qualitative analysis was transferred from the assay tube to a colorimetric cuvette [95% (v/v)], methanol to be used as blank to read absorbance at 538 nm. For the standard curve, 200 µl of previously prepared linalool solution in methanol was added to 1.5 mL of chloroform and serial dilution was done, where volume make-up was done by the addition of 95% (v/v) methanol (Figure S1E).

Statistical Analysis

Each set of treatments was replicated six times. Statistical analysis was conducted with R version 4.2.2 in platform aarch64-apple-darwin20 (64-bit). Shapiro-Wilk test and histogram confirmed ($P < 0.05$) rejection of the data normality null hypothesis among all samples. Kruskal-Wallis Rank Sum test non-parametric method to evaluate overall significant differences ($P < 0.05$) among treatments (Control, Field, Fungus, Fungus + Insect and Insect). Dunn's test (1964) pairwise multiple comparisons with no P-value correction as a post hoc method to identify significant differences ($P < 0.05$, $P < 0.01$, $P < 0.001$) between treatments. Correlation between treatment solvents was quantified through Spearman's rank correlation, only strong positive and/or negative relationships ($-0.7 > R > 0.7$) were used for supplementary visualizations.

Results

Qualitative analysis

Phytochemical screening of methanol extracts of *P. crassipes* showed the presence of all tested active constituents, whereas ethanol and aqueous extract selectively detected their presence (Table 2). Ethanol extract showed the presence of all tested metabolites (alkaloids, tannins, phenol, glycosides, flavonoids and terpenoids), except tannin, whereas aqueous extract could not confirm the presence of alkaloids and flavonoids. The presence of glycosides was detected in the chloroform extract of the study plant samples.

Table 2
Qualitative analysis of metabolites in ethanol, methanol, aqueous and chloroform extract of P. crassipes. (+) shows positive yield in the solvent (-) shows negative yield in the solvent. (NA) -Not applicable shows the solvent not used for the extraction.

Compound	Ethanol (E) (Solv)	Methanol (M) (Solv)	Aqueous (A) (Solv)	Chloroform (C) (Solv)
Phenol	+	+	+	NA
Alkaloid	+	+	-	NA
Flavonoid	+	+	-	+
Terpenoid	+	+	+	NA
Glycosides	+	+	+	+
Tannin	-	+	+	NA

Quantitative analysis

Table 3 displays the optimal quantitative yields of metabolites using four distinct solvents: ethanol (E), methanol (M), chloroform (C), and aqueous (A). Notably, ethanol exhibited the highest yields for phenol alkaloids, flavonoids, and terpenoids. For tannin, quantitative analysis was exclusively conducted using aqueous solvent. Remarkably, glycoside was the only metabolite that yielded measurable results in all four solvents utilized in the study.The quantitative analysis showed variable results (Fig. 1) among the SMs identified from water hyacinth in various solvents like methanol (M), ethanol (E), chloroform (C) and aqueous (A). The phenolic content in weevil-fed water hyacinth (NB + WH) was evidently higher than in comparison to fungus (FO + WH) infected (M = 54.248 mg/mL; E = 57.408 mg/mL; A = 56.16 mg/mL) and FO + AD + WH (fungi-arthropod infested water hyacinth) (M = 40.387 mg/mL E = 37.195 mg/mL A = 43.975 mg/mL), as observed from both the alcoholic (M = 77.401 mg/mL, E = 78.213 mg/mL) and aqueous extraction (75.717 mg/mL) (Fig. 1a).

Ethanol extract of water hyacinth showed the highest alkaloid activity, over methanol and aqueous extracts. From the ethanolic (E) and aqueous (A) extract, the alkaloid content was observed to be maximum, with 0.214 mg/mL and 0.175 mg/mL respectively, when infested with FO + AD + WH, than in undamaged (WH) or FO + WH (E = 0.193 mg/mL; A = 0.124 mg/mL) or NB + WH (E = 0.159 mg/mL; A = 0.162 mg/mL) treated leaves separately. But the methanol (M) extract showed a decline in the alkaloid content with an increase in damage to the weed with both FO + WH (0.104 mg/mL) and FO + AD + WH (0.109 mg/mL) showing a higher drop than just NB + WH (0.128 mg/mL) damaged leaves (Fig. 1b).

Damaged leaves showed higher flavonoid content than control leaves (WH = 0.199 µg/mL) of WH. The methanol extract showed NB + WH leaves (0.209 µg/mL) have more flavonoid content than FO + WH (0.201 µg/mL), and the ethanol extract showed the same trend as methanol (FO + WH = 0.203 µg/mL, FO + AD + WH = 0.202 µg/mL) (Fig. 1c).

The alcohols proved better (0.048mg/mL), in extracting terpenoids from water hyacinth as compared to aqueous extract (0.047 mg/mL) (Fig. 1d).

The glycosidic content increased in NB + WH leaves (M = 0.586 mg/mL; E = 0.565 mg/mL) than in WH (fresh unaffected leaves) (M = 0.493 mg/mL; E = 0.481 mg/mL), followed by FO + WH (M = 0.489 mg/mL; E = 0.474 mg/mL) and FO + AD + WH (M = 0.390 mg/mL; E = 0.325 mg/mL) treated water hyacinth (Fig. 1e).

Tannin did not show a noticeable change when extracted in methanol, though its presence was detected in the qualitative test. The quantitative value was estimated in water (A) for tannin. The level of tannin content increased in damaged leaves of WH, maximising in FO + AD + WH treatments with 1.931 mg/mL of tannin, and FO + WH (1.572 mg/mL) respectively (Fig. 1f).

The best yield of the different metabolites in the different sets of treatments (WH, OT + WH, FO + WH, NB + WH and FO + AD + WH) are highlighted using boxplots (Fig. 2) showing the statistically significant differences among the different solvents methanol, ethanol, aqueous, chloroform for the SMs' extraction. No significant differences between the solvents and the treatment groups were observed (Fig. 2b and Fig. 2d). There are significant differences noticed in phenol, flavonoid, glycoside and tannin. The density histogram (or densigram) shows the highest concentration of each solvent (Fig. 2a, Fig. 2c, Fig. 2e and Fig. 2f).

The correlation analysis showed that the extraction of methanol and ethanol in phenol and glycoside are closely related and have a similarity of 0.87 and 0.85. Also in glycoside, methanol and ethanol have 0.74 and 0.80 co-efficient with aqueous similarity respectively indicating the yield to be similar (Figure S2A, S2B, S2C, S2D). The correlation analysis gives us a vivid idea about the yield of metabolites irrespective of the different kinds of solvents used for extraction in different groups of treatments.

Table 3
Quantitative yield of metabolites in ethanol, methanol, aqueous and chloroform extract from *P. crassipes*

Compound	Ethanol (Solv)	Methanol (Solv)	Aqueous (Solv)	Chloroform (Solv)
Phenol	+++	+++	++	-
Alkaloid	+++	+	+	-
Flavonoid	+++	+	+	-
Terpenoid	+++	+++	+	-
Glycosides	+	+	+	+
Tannin	-	-	+++	-

Solv: solvent, +++: Highest amount of yield, ++: Moderate amount of yield, +: Lowest amount of yield, -: Lack of yield.

Discussion

Plants face various biotic and abiotic challenges such as diseases, herbivores, and water and nutrient deficiencies that impact their overall growth significantly (Misra et al. 2023). In response to such biotic and abiotic stresses, multilayered defense strategies have been incorporated during this magnanimous evolutionary time scale (Ahmad et al. 2022). Classical weed biocontrol also depends a lot on ecological interactions and the evolutionary history of specialist herbivores and their host plants to manage invasive plants in their introduced range (Dutta et al. 2023). The secondary biochemistry of plants plays a significant role in the evolution of plant-arthropods-fungi relationships, along with the shared history of co-evolution and complementation which leads to co-existence and host-specificity (Dethier 1954).

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure (Ellof 1998). Plant SMs are compounds that have no fundamental role in the maintenance of life processes in plants (Hartmann 1991), but they are important for the plant to interact with its environment for adaptation and defense (Misra et al. 2023). These defenses can be either 'constitutive', with high levels of the metabolite naturally maintained within the plant, or 'induced', where the metabolite is changed in abundance following herbivore or pathogen attack (Bezemer and Van Dam 2005). In higher plants, a wide variety of SMs are synthesized from primary metabolites (e.g., carbohydrates, lipids and amino acids). A variety of factors (toxins, cutinases, chitinases, cellulases, and phytoalexin demethylases) have been implicated as causal components of fungal plant disease; however, the mode of action of a fungus in pathogenicity is largely unknown (Ciuffetti et al. 1983; Kolattukudy 1985; Johal and Briggs 1992). In most cases, the interaction between fungi and plants is very intimate and the association occurs over a longer period. Under varying circumstances, distinct structures and barriers develop in the host as a response to infection, which was evident in the role of phenols in resistance to various fungi or arthropods (Harborne 1988). Fungi utilize the carbon content in the plants and convert it into their own biomass for their growth and proliferation (Huaer and Lamberti 2017).Thus, plants under low light or nutrient stress (low carbon supply) contain three folds lower concentrations of phenol than in any control or nitrogen-fed plants (Larsson et al 1986.), which hints at the drop in the phenol content level for FO + AD + WH and FO + WH treatments. Plants show proanthocyanins and even small amounts of dihydroquercetin production, involved in the defense against *Fusarium* species, very likely for the mentioned treatments (Skadhauge et al. 1997). Our findings thus reveal that under stress produced by any fungal infection, plants rapidly accumulate phenols at the site of infection (Matern et al. 1988), as the initial step of the defense mechanism, which penetrates microorganisms to cause considerable damage to cell metabolisms. The phenolic content for OT + WH (M = 76.991 mg/mL; E = 77.202 mg/mL) and NB + WH (M = 77.401 mg/mL; E = 78.213 mg/mL) treatments did not show a significant change as compared to undamaged treatment (WH) (Fig. 2a), though its rise in aqueous extract for NB + WH (75.717 mg/mL) was observed (Fig. 1a). This phenol resistance of water hyacinths may be one of the likely reasons for unchanged phenolic content in arthropod-treated water hyacinths, since idioblasts situated in the palisade layer contain phenolic acids which are implicated in the control of infection or attack (Galbraith 1987). Alcoholic extractions showed overall better efficacy in metabolite detection (Table 1 or 2). Ethanolic extracts for FO + WH (0.193 mg/mL) and FO + AD + WH (0.214 mg/mL) treatments showed an elevated alkaloid content, portraying an increase in endophytic colonization on the plants, which acts as an antifeedant and is toxic for other herbivores (Johnson et al. 1985). The surge in the alkaloid content of the ethanol and aqueous extract FO + AD + WH treatments, with

respect to untreated and undamaged (Fig. 1b), proves how the slow introduction of multiple agents and herbivore resistance, helps the coevolution of the host plant and specialist or non-specialist herbivores (Harborne 1988).

The defense-related flavonoids can be divided into two groups: “preformed” and induced” compounds (Treutter 2005). Flavonoids induced after injury by pathogens or arthropods are a well-known phenomenon (Barry et al. 2002) tallying with the consequences that methanol extracts of affected water hyacinths. Beckman pointed out that modulating the effect on the action of Indole Acetic Acid by flavonoids might lead to changes in tissue differentiation and promotion of the formation of callus and tylose, leading to closing vessels and locking out aggressive endophytes like FO (Beckman 2000). Contrarily, ethanol extract portraying lower flavonoid content for NB + WH treatments than FO-infested ones might be a result of the sensitivity of several insects to the preformed flavonoid content or deterred by it (Widstrom et al. 2001; Haribal and Feeny 2003, Chen et al 2004) (Fig. 1c).

Terpenes are related plant SMs reported to be important factors in resistance to several insect pests and pathogens. The insecticidal activity of the terpenes is either due to their action as antifeedants (or deterrents), toxins, or modifiers of insect development (sterols such as the phyto ecdysones). Study showed terpenoid, limonene, deters *Atta cephalotes* L. (a leaf-cutting ant) from citrus plants (Cherrett 1972). Chemical analysis showed that terpenoid emission was inhibited more strongly in infested lima bean plants than in Brussels sprout plants after fosmidomycin treatments, showing the variation of the same terpenoid effect on different sets of plants (Mumm et al. 2008). Though the change in terpenoid content has been insignificant, alcohol proved better in extracting terpenoids (Fig. 2d). Phytoalexins (sometimes in the form of diterpenes and sesquiterpenes) are low-molecular-weight compounds that are produced as part of the plant defense system, which makes it difficult to be traced.

High glycosides concentration in AD + WH treatments was observed with higher extraction values for the alcohols (Fig. 1e, 2e). It is expected as high concentrations of plant toxins deter generalist feeders while attracting specialist biocontrol agents that either use them as a cue to locate or to accept the host plant for laying eggs and/or feeding or use them for their defense (Van der Meijden 1996; Renwick 2002). Iridoid glycosides are toxic to many generalist herbivores. Although, Nieminen with colleagues that individual plants of *Plantago lanceolate* L. with high iridoid glycoside concentrations in the field, favored oviposition by the specialist fritillary butterfly *Melitaea cinxia* L. significantly more than those plants with low concentrations (Nieminen et al 2003). Plants are cyanogenic and can form hydrocyanic acid (HCN) in response to tissue damage (D’Mello et al. 1991), associated with defense against pathogens and herbivores (Davis 1991), breaking down the cyanogenic glycosides. Healthy control plants have no detectable HCN suggesting that the cyanogenic glycosides, normally separated from the enzymes, catalyze HCN release (Osbourn 1996). Though little correlation is drawn between glycoside level and resistance to fungal pathogens reports show that high glycosidic plants are more susceptible to endophytic attack (Osbourn 1996). However, the breakdown of HCN to formamide by cyanide hydratase enzyme (Wang et al. 1992.; Fry et al. 1977) may be one reason for the low glycosidic content in FO + WH treatments, in comparison to arthropod-treated (NB + WH and OT + WH treatments) and untreated plants.

Another responsible metabolite, tannins, can defend leaves against insect herbivores by deterrence and/or toxicity. Tannins are general toxins that significantly reduce the growth and survivorship of many herbivores, reflected by the increase in the content of the metabolite in weevil) or fungi attacked water hyacinth or in their combination, thus repelling the arthropods (Fig. 1f). The defensive properties of tannins are attributed to their ability to their protein binding properties (Mazid et al. 2011).

Among all the metabolites studied, phenolics have received special attention in the study of plant-herbivore interaction, due to their presence and differentiation in large concentrations, in comparison to others. They are widely considered deterrents, antifeedants and toxins that can change the nutritional quality of plant tissues for herbivores (Lambers 1993). Although field studies suggest that the defense mechanism is overemphasized (Coley 1983), our data revealed that a. significant variation in alkaloids, glycosides, tannins, and phenols resulted in control of the weed via the biocontrol agents (arthropods and pathogens) (Fig. 1). These notable changes in metabolites and combined effect of both the pathogen and arthropods have successfully helped to control the targeted weed and instigated us for further analysis of bimolecular interaction for future designing of integrated biological control.

Conclusions

About 100,000 known SMs are involved in plant chemical defense systems. These phytochemicals have been shaped through the millions of years during which plants have co-evolved with their antagonists and mutualists (Wink 2008). With thousands of metabolites interplaying, their pathways and environmental interactions are difficult to understand. Although higher concentrations of SMs might result in a more resistant plant, the production of SMs is considered costly and reduces plant growth and reproduction (Thaler and Karban 1997; Siemens et al. 2002). The cost of defense has been invoked to explain why plants have evolved induced defense, where concentrations generally increase only in stressful situations (Tollrian and Harvell 1999).

Declarations

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office) and is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Authors.

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Availability of data and material

Relevant data applicable to this research are within the paper. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests:

The authors declare no conflict of interest.

Author Contributions:

conceptualization, D.M., W.D., and P.R.; writing – original draft, D.M., W.D., P.R.; writing – reviewing and editing, methodology, D.M.; W.D.; D.M.; data curation and data analysis, J.C.G.P.; All authors have read and agreed to the published version of the manuscript.

References

1. Adams HR, Camp BJ (1966) `The isolation and identification of three alkaloids from *Acacia Berlandieri*. Toxicon 4:85–90. https://doi.org/10.1016/0041-0101(66)90002-X
2. Ahmad I, del Mar Jiménez-Gasco M, Luthe DS, Barbercheck ME (2022) Endophytic *Metarhizium robertsii* suppresses the phytopathogen, *Cochliobolus heterostrophus* and modulates maize defenses. PLoS One 17: e0272944. https://doi.org/10.1371/JOURNAL.PONE.0272944
3. Ballhorn DJ, Kautz S, Heil M, Hegeman AD (2009) Cyanogenesis of Wild Lima Bean (*Phaseolus lunatus* L.) Is an Efficient Direct Defence in Nature PLoS One 4:e5450. https://doi.org/10.1371/JOURNAL.PONE.0005450
4. Barry K, Davies N, Pathology CM-F, 2002 undefined (2002) Effect of season and different fungi on phenolics in response to xylem wounding and inoculation in *Eucalyptus nitens*. Wiley Online Library 32:163–178. https://doi.org/10.1046/j.1439-0329.2002.00281.x
5. Bazzaz FA, Chiariello NR, Coley PD, Pitelka LF (1987) Allocating Resources to Reproduction and Defense. Bioscience 37:58–67. https://doi.org/10.2307/1310178
6. Beckman CH (2000) Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? Physiol Mol Plant Pathol 57:101–110. https://doi.org/10.1006/PMPP.2000.0287
7. Berenbaum MR, Bowers MD, Butler LG, et al (1992) Herbivores: Their interactions with secondary plant metabolites, volume 2, ecological and evolutionary processes. 31:4043
8. Bezemer TM, Van Dam NM (2005) Linking aboveground and belowground interactions via induced plant defenses. Trends in Ecol. Evol. 20: 617–624.
9. Bray HG, Thorpe WV (1954) Analysis of Phenolic Compounds of Interest in Metabolism. Methods Biochem Anal 1:27–52. https://doi.org/10.1002/9780470110171.CH2
10. Cardoza YJ, Lait CG, Schmelz EA, et al (2003) Fungus-Induced Biochemical Changes in Peanut Plants and Their Effect on Development of Beet Armyworm, *Spodoptera Exigua Hübner* (Lepidoptera: Noctuidae). academic.oup.com 32:220–228

11. Chen K, Ohmura W, Doi S, Aoyama M (2004) Termite feeding deterrent from Japanese larch wood. Biores. Tech. 95:129–134.
12. Cherrett JM (1972) Chemical aspects of plant attack by leaf-cutting ants. In: Harborne JB, ed. Phytochem. Ecol. London: Academic Press 13-24.
13. Ciuffetti LM, Pope MR, Dunkle LD, et al (1983) Isolation and Structure of an Inactive Product Derived from the Host-Specific Toxin Produced by *Helminthosporium carbonum*. Biochemistry 22:3507–3510. https://doi.org/10.1021/BI00283A031
14. Coetzee J, Hill M, Byrne M, et al (2011) A review of the biological control programmes on *Eichhornia crassipes* (C. mart.) solms (Pontederiaceae), *Salvinia molesta* DS Mitch. (Salviniaceae), *Pistia stratiotes* L. journals.co.za. https://doi.org/10.4001/003.019.0202
15. Coley PD (1983) Herbivory and Defensive Characteristics of Tree Species in a Lowland Tropical Forest. Ecol Monogr 53:209–234. https://doi.org/10.2307/1942495
16. Coley PD, Bryant JP, Chapin FS (1985) Resource Availability and Plant Antiherbivore Defense. Science (1979) 230:895–899. https://doi.org/10.1126/SCIENCE.230.4728.895
17. Davis RH (1991) Glucosinolates. In Toxic Substances in Crop Plants, J.P. DMello, C.M. Duffus, and J.H. Duffus, eds (Cambridge, UK: Royal Society of Chemistry), pp. 202-225.
18. Dethier VG (1954) Evolution of Feeding Preferences in Phytophagous Insects. Evolution (N Y) 8:33. https://doi.org/10.2307/2405664
19. D'Mello, J.F., Duffus, C.M. and Duffus, J.H. eds., 1991. Toxic substances in crop plants. Woodhead Publishing.
20. Dutta W (2021) Exploring the possibilities of enhancing enzymatic activities of phytopathogenic fungi for their utility in biological control of waterhyacinth. Presidency University
21. Dutta W, Misra D, Chowdhury P, et al (2023) The mycobiota associated with the weed water hyacinth *Pontederia crassipes* in Kolkata, India, with emphasis on biological control of the macrophyte. https://doi.org/101080/0958315720232191298. https://doi.org/10.1080/09583157.2023.2191298
22. Dutta W, Ray D, Ray P (2015) Molecular characterization and host range studies of indigenous fungus as prospective mycoherbicidal agent of water hyacinth. Indian Journal of Weed Science 47:59–65
23. Dutta W, Ray P (2017) A glimpse into the compatibilities and conflicts between arthropods and fungal biological control agents of aquatic weed waterhyacinth. Phytoparasitica 45:429–437. https://doi.org/10.1007/S12600-017-0605-Y/METRICS
24. Ehrlich PR, Raven PH (1964) Butterflies and Plants: A Study in Coevolution. Evolution (N Y) 18:586. https://doi.org/10.2307/2406212
25. Eloff JN (1988) Which extracts and should be used for the screening and isolation of antimicrobial components from plants. J. Ethnopharmacol.60: 1- 8.
26. Farkas GL, Kiraaly Z (1962) Role of Phenolic Compounds in the Physiology of Plant Diseases and Disease Resistance. Journal of Phytopathology 44:105–150. https://doi.org/10.1111/J.1439-0434.1962.TB02005.X

27. Feeny PP, Bostock H (1968) Seasonal changes in the tannin content of oak leaves. Phytochemistry 7:871–880. https://doi.org/10.1016/S0031-9422(00)84845-1
28. Fraenkel GS (1959) The raison d'etre of secondary plant substances. Science (Washington D.C.) 129:1466-1470.
29. Fry WE, Evans PH (1977) Association of formamide hydrolyase with fungal pathogenicity to cyanogenic plants. Phytopathology 67:1001-1006.
30. Li Z, Xiong K, et al (2023) Functional Endophytes Regulating Plant Secondary Metabolism: Current Status, Prospects and Applications. International Journal of Molecular Sciences 2023, Vol 24, Page 1153 24:1153. https://doi.org/10.3390/IJMS24021153
31. Galbraith JC (1987) The pathogenicity of an Australian isolate of *Acremonium zonatum* to water hyacinth, and its relationship with the biological control agent, *Neochetina eichhorniae*. Aust J Agric Res 38:219–229. https://doi.org/10.1071/AR9870219
32. Geoffrey A. Cordell. View on ScienceDirect Chemistry and Biology, Volume 52 1st Edition. 391
33. Gumbinger HG, Winterhoff H, Wylde R, Sosa A (1992) On the influence of the sugar moiety on the antigonadotrophic activity of luteoline glycosides. Planta Med 58:49–50. https://doi.org/10.1055/S-2006-961388
34. Harborne JB (1988) Introduction to ecological chemistry. London: Academic Press.
35. Haribal M, Feeny P (2003) Combined roles of contact stimulant and deterrents in assessment of host-plant quality by ovipositing zebra swallowtail butterflies. J Chem Ecol 29:653–670. https://doi.org/10.1023/A:1022820719946
36. Hauer FR, and Lamberti G (Eds.) (2017). Methods in stream ecology: Volume 1: Ecosystem structure. Academic Press.
37. Haukioja E (1991) Induction of defenses in trees. Annu Rev Entomol 36:25–42. https://doi.org/10.1146/ANNUREV.EN.36.010191.000325
38. Heredity MH-, 1991 undefined (1991) The cyanogenic polymorphism in *Trifolium repens L*. (white clover). nature.com 105–115
39. Herms DA, Mattson WJ (1992) The Dilemma of Plants: To Grow or Defend. https://doi.org/101086/417659 67:283–335. https://doi.org/10.1086/417659
40. Hirakura K, Fujimoto Y, Fukai T, Nomura T (1986) Two Phenolic Glycosides from the Root Bark of the Cultivated Mulberry Tree (Morus Lhou). J Nat Prod 49:218–224. https://doi.org/10.1021/NP50044A004
41. Holm LG, Plucknett DL, Pancho JV, Herberger JP (1977) The world's worst weeds. Distribution and biology. The world's worst weeds Distribution and biology
42. Honkanen T, Haukioja E, Suomela J (1994) Effects of Simulated Defoliation and Debudding on Needle and Shoot Growth in Scots Pine (*Pinus sylvestris*): Implications of Plant Source/Sink Relationships for Plant-Herbivore Studies. Funct Ecol 8:631. https://doi.org/10.2307/2389926
43. Janzen DH (1974) Tropical Blackwater Rivers, Animals, and Mast Fruiting by the *Dipterocarpaceae*. Biotropica 6:69. https://doi.org/10.2307/2989823

44. Johal GS, Briggs SP (1992) Reductase activity encoded by the HM1 disease resistance gene in maize. Science (1979) 258:985–987. https://doi.org/10.1126/SCIENCE.1359642
45. John B, Sulaiman CT, George S, Reddy VRK (2014) Spectrophotometric estimation of alkaloids in selected *Justicia* species. Int. J. Pharm. and Pharmc. Scie. 6 (5):647-648
46. Johnson MC, Dahlman DL, Siegel MR, et al (1985) Insect Feeding Deterrents in Endophyte-Infected Tall Fescue. Appl Environ Microbiol 49:568–571. https://doi.org/10.1128/AEM.49.3.568-571.1985
47. Karban R, Adamchak R, Schnathorst WC (1987) Induced resistance and interspecific competition between spider mites and a vascular wilt fungus. Science (1979) 235:678–680. https://doi.org/10.1126/SCIENCE.235.4789.678
48. Kolattukudy PE (1985) Enzymatic Penetration of the Plant Cuticle by Fungal Pathogens. Annu Rev Phytopathol 23:223–250. https://doi.org/10.1146/ANNUREV.PY.23.090185.001255
49. Lalitha P, Sripathi SK, Jayanthi P (2012) Secondary metabolites of *Eichhornia crassipes* (Waterhyacinth): A review (1949 to 2011). Nat Prod Commun 7:1249–1256. https://doi.org/10.1177/1934578X1200700939
50. Lambers H (1993) Rising CO2, secondary plant metabolism, plant-herbivore interactions and litter decomposition. CO2 and biosphere 263–271. https://doi.org/10.1007/978-94-011-1797-5_17
51. Larsson S, Wiren A, Lundgren L, Ericsson T (1986) Effects of light and nutrient stress on leaf phenolic chemistry in *Salix dasyclados* and susceptibility to *Galerucella lineola* (Coleoptera). Oikos 47:205-210.
52. Mahesh AR, Ranganath MK, & Harish Kumar DR (2013) Enrichment of flavonoids from the methanolic extract of *Boerhaavia diffusa* roots by partitioning technique. *Research Journal of Chemical Sciences*
53. Mailoa MN, Mahendradatta, M, Laga, A, & Djide, N (2013) Tannin extract of guava leaves (*Psidium guajava* L) variation with concentration organic solvents. International Journal of Scientific and Technology Research, 2(9), 106-110.
54. Matern U and Kneusal RE. (1988) Phenolic compounds in plant disease resistance. Phytopathology. 16:153-170.
55. Mazid M, Khan TA, Mohammad F (2011) Role of secondary metabolites in defense mechanisms of plants. Biol. Med. 3 (2):232-249.
56. McEvoy PB, Coombs EM (2000) Why Things Bite Back: Unintended Consequences of Biological Weed Control. Nontarget Effects of Biological Control 167–194. https://doi.org/10.1007/978-1-4615-4577-4_11
57. Misra D, Dutta W, Jha G, Ray P (2023) Interactions and Regulatory Functions of Phenolics in Soil-Plant-Climate Nexus. Agronomy 2023, Vol 13, Page 280 13:280. https://doi.org/10.3390/AGRONOMY13020280
58. Mitchell C, Brennan RM, Graham J, Karley AJ (2016) Plant defense against herbivorous pests: Exploiting resistance and tolerance traits for sustainable crop protection. Front Plant Sci 7:1132. https://doi.org/10.3389/FPLS.2016.01132/BIBTEX
59. Mumm R, Posthumus MA, Dicke M (2008) Significance of terpenoids in induced indirect plant defense against herbivorous arthropods. Plant Cell Environ 31:575–585. https://doi.org/10.1111/J.1365-3040.2008.01783.X

60. Nieminen M, Suomi J, Van Nouhuys S, Sauri P, Riekkola ML (2003) Effect of iridoid glycoside content on oviposition host plant choice and parasitism in a specialist herbivore. J. Chem. Ecol. 29:823–844.
61. Osbourn AE (1996) Preformed Antimicrobial Compounds and Plant Defense against Fungal Attack. Plant Cell 8:1821. https://doi.org/10.1105/TPC.8.10.1821
62. Raman A, Wheatley W, Popay A (2012) Endophytic Fungus-Vascular Plant-Insect Interactions. Environ Entomol 41:433–447. https://doi.org/10.1603/EN11317
63. Rao MJ, Ahmed U, Ahmed MH, et al (2021) Comparison and Quantification of Metabolites and Their Antioxidant Activities in Young and Mature Leaves of Sugarcane. ACS Food Science and Technology 1:362–373. https://doi.org/10.1021/ACSFOODSCITECH.0C00133/ASSET/IMAGES/MEDIUM/FS0C00133_M005.GIF
64. Ray P, Sushilkumar and A.K. Pandey (2008). Efficacy of pathogens of waterhyacinth (*Eichhornia crassipes*), singly and in combinations for its biological control. Journal of Biological Control, 22 (1): 173-177.
65. Ray P, Hill MP (2016) More is not necessarily better: the interaction between insect population density and culture age of fungus on the control of invasive weed water hyacinth. Hydrobiologia 766:189–200. https://doi.org/10.1007/S10750-015-2454-3/METRICS
66. Ray P, Hill MP (2012) Impact of feeding by *Neochetina* weevils on pathogenicity of fungi associated with water hyacinth in South Africa. J. Aq. Pl. Manag. 50: 79-84.
67. Renwick JAA (2002) The chemical world of crucivores: lures, treats and traps. Proceedings of the 11th International Symposium on Insect-Plant Relationships 35–42. https://doi.org/10.1007/978-94-017-2776-1_4
68. Rhoades DF, Cates RG (1976) Toward a General Theory of Plant Antiherbivore Chemistry. Biochemical Interaction Between Plants and Insects 168–213. https://doi.org/10.1007/978-1-4684-2646-5_4
69. Siemens DH, Garner SH, Mitchell-Olds T, Callaway RM (2002) Cost of defense in the context of plant competition: *Brassica rapa* may grow and defend. Ecology 83(2):505–517.
70. Skadhauge B, Kristian Thomsen K, Von Wettstein D, von D (1997) The Role of the Barley Testa Layer and its Flavonoid Content in Resistance to *Fusarium* Infections. Wiley Online Library 126:147–160. https://doi.org/10.1111/j.1601-5223.1997.00147.x
71. Tasin M, Knudsen GK, Pertot I (2012) Smelling a diseased host: grapevine moth responses to healthy and fungus-infected grapes. Anim Behav 83:555–562. https://doi.org/10.1016/J.ANBEHAV.2011.12.003
72. Treutter D (2005) Significance of Flavonoids in Plant Resistance and Enhancement of Their Biosynthesis. Plant Biol. 7:581–591.
73. Tabasum S, Khare S, & Jain K (2016) Spectrophotometric quantification of total phenolic, flavonoid, and alkaloid contents of *Abrus precatorius* L. seeds. Asian J Pharm Clin Res, 9(2), 371-374.
74. Thaler JS, Karban R (1997) A phylogenetic reconstruction of constitutive and induced resistance in *Gossypium*. American Naturalist 149:1139–1146. https://doi.org/10.1086/286042
75. Thoison O, Sévenet T, Niemeyer H, et al Insect antifeedant compounds from *Nothofagus dombeyi* and *N. pumilio*. Elsevier

76. Tollrian R, Harvell C (1999) The ecology and evolution of inducible defenses
77. Trotel-Aziz P, Couderchet M, Vernet G, Aziz A (2006) Chitosan stimulates defense reactions in grapevine leaves and inhibits development of *Botrytis cinerea*. Eur J Plant Pathol 114:405–413. https://doi.org/10.1007/S10658-006-0005-5/METRICS
78. Van Buren JP, Robinson WB (1969) Formation of Complexes between Protein and Tannic Acid. J Agric Food Chem 17:772–777. https://doi.org/10.1021/JF60164A003
79. Van der Meijden E (1996) Plant defense, an evolutionary dilemma: contrasting effects of (specialist and generalist) herbivores and natural enemies. Proceedings of the 9th International Symposium on Insect-Plant Relationships 307–310. https://doi.org/10.1007/978-94-009-1720-0_69
80. Wall ME, Taylor H, Ambrosio L, Davis K (1969) Plant antitumor agents III: A convenient separation of tannins from other plant constituents. J Pharm Sci 58:839–841. https://doi.org/10.1002/JPS.2600580709
81. Wang P, Matthews D, and HV-A of biochemistry, 1992 undefined Purification and characterization of cyanide hydratase from the phytopathogenic fungus *Gloeocercospora sorghi*. Elsevier
82. Widstrom N, Breeding MS-P, (2001) Recurrent selection for maysin, a compound in maize silks, antibiotic to earworm. Wiley Online Library 120:357–359. https://doi.org/10.1046/j.1439-0523.2001.00610.x
83. Wink M (2008) Plant secondary metabolism: Diversity, function and its evolution. Nat Prod Commun 3:1205–1216. https://doi.org/10.1177/1934578X0800300801
84. Witzgall P, Proffit M, Rozpedowska E, et al (2012) “This is not an apple”-yeast mutualism in codling moth. J Chem Ecol 38:949–957. https://doi.org/10.1007/S10886-012-0158-Y
85. Zhishen J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 64:555–559. https://doi.org/10.1016/S0308-8146(98)00102-2

Figures

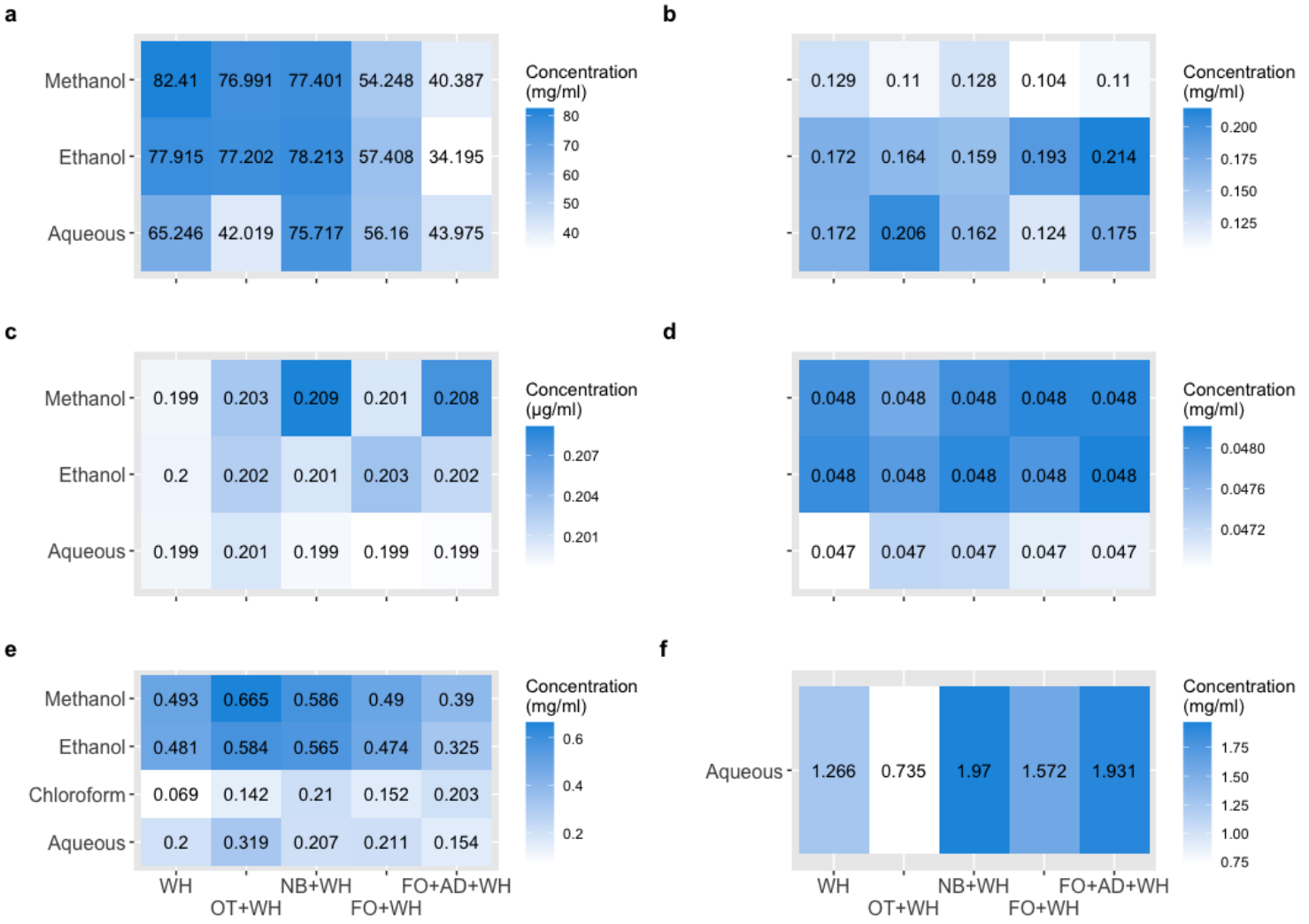

Figure 1

Average yield measurements heatmap of “treatment” (x-axis [n = 5]) ~ solvent (y-axis [n = 5]) for each metabolite. **(a)** Phenol demonstrated highest yield average for the “NB+WH” treatment, followed by “WH”. The lowest yield was achieved in the “FO+AD+WH” treatment. Overall highest yield was measured in “WH” ~ Methanol. **(b)** Alkaloid demonstrated the lowest yield average overall measurements using Methanol solvent, and the highest yield by Ethanol. Considering treatments, “FO+AD+WH” ~ Ethanol showed the highest overall yield average. **(c)** Flavonoids demonstrated the highest overall yield average measurement through “NB+WH” + Methanol and lowest was seen overall in “WH” treatment along with the Aqueous solvent. **(d)** Terpenoids demonstrated no difference between “Treatment” and/or solvent use, having identical yield measurements overall. **(e)** Glycoside achieved the highest overall average with “OT+WH” ~ Methanol, and the overall highest yield through the “OT+WH” treatment. **(f)** Tannin demonstrated the highest yield value through the “NB+WH” treatment, followed by the “FO+AD+WH” treatment; all treatments were used along the aqueous solvent. (Control: “WH”, Field: “OT+WH”, Fungus: “FO+WH”, Insect: “NB+WH”, Fungus + Insect: “FO+AD+WH”)

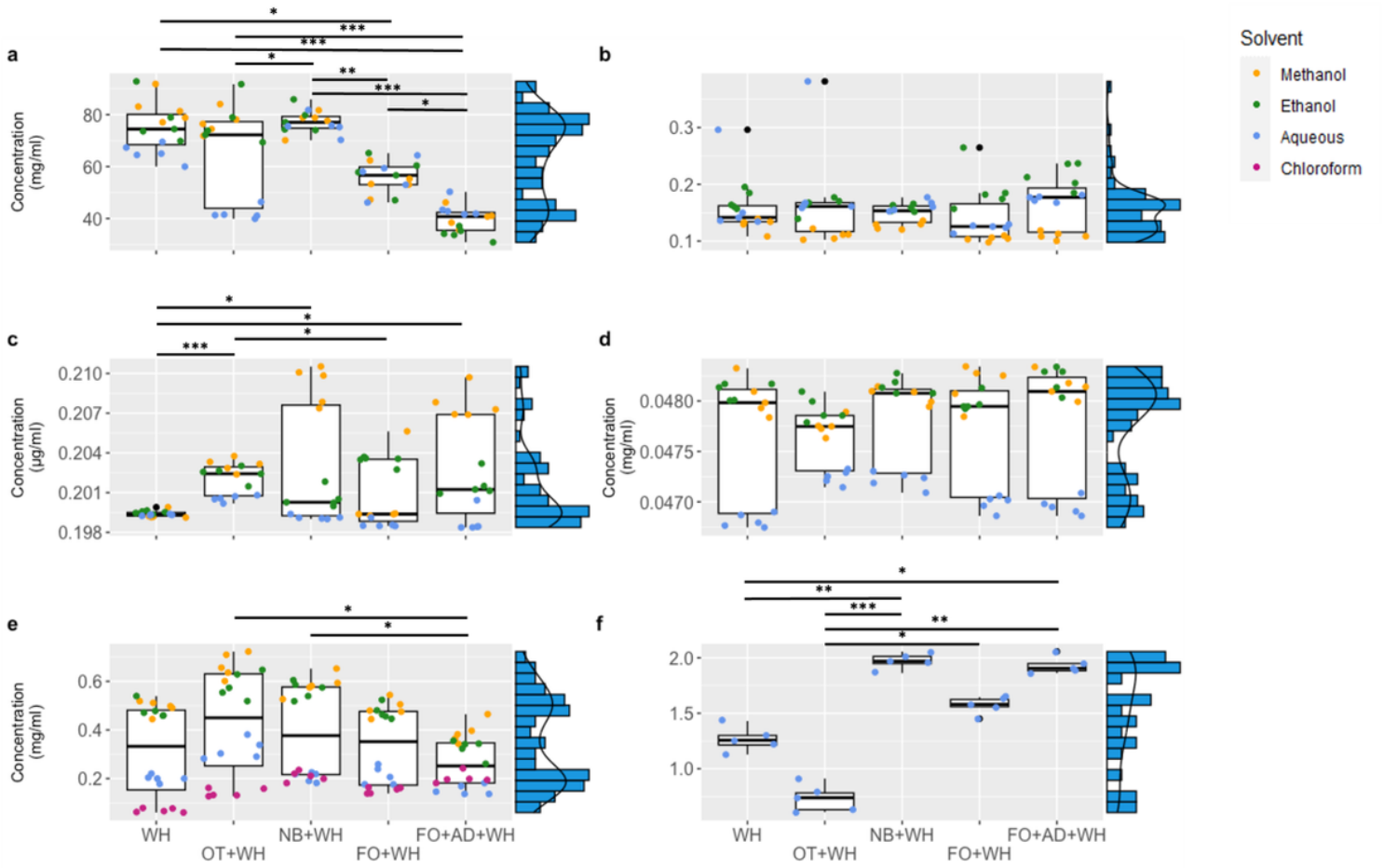

Figure 2

Boxplot with jitter and density histogram “treatment” (x-axis [n = 5]) ~ solvent (y-axis [n = 5]) for each metabolite differentiating solvents depicted by assigned colors. **(a)** Phenol has an overall high distribution cluster within the 70-80 mg/ml yield, with an overall scattered distribution of solvents. **(b)** Alkaloid has a distribution clustered in the bottom (0.2-0.1 mg/ml) of the diagram, with Methanol in the lowest distribution along 0.1 mg/ml through all “treatments”. **(c)** Flavonoids has a distribution clustered along the bottom of the diagram (0.198-0.201 µg/ml). Methanol has an overall high clustering along the highest measurements of yield within “FO+AD+WD” and “NB+WH”. **(d)** Terpenoids has a high concentration of measurements clustered along the top of the diagram (>0.04775 mg/ml) for all treatments and an overall low yield measurement using the Aqueous solvent. **(e)** Glycoside has the strongest distributions in the bottom of the diagram (0.1-0.2 mg/ml) through all “treatments” with clustering observed between Chloroform and Aqueous solvents, and a clustering at the top of the diagram (0.4-0.6 mg/ml) between Methanol and Ethanol solvents. **(f)** Tannin has an overall distribution scattered for all “treatments” and solvents. The highest yield was observed using “FO+AD+WH” and “NB+WH”. (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, Control: “WH”, Field: “OT+WH”, Fungus: “FO+WH”, Insect: “NB+WH”, Fungus + Insect: “FO+AD+WH”)).

Supplementary Files

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