Plant-insect interaction beyond folivory: chemical ecology of a non-folivore herbivore *Leucinodes orbonalis*'s interaction with its host *Solanum melongena*

A thesis

Submitted in partial fulfillment of the requirements

of the degree of

Doctor of Philosophy

By

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20162008

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Dedicated to my family

Declaration

I declare that this written submission represents my ideas in my own words and where others' ideas have been included, I have adequately cited and referenced the sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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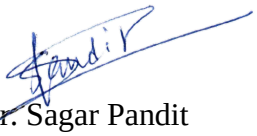

Dr. Sagar Pandit

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Synopsis

Title: Plant-insect interaction beyond folivory: chemical ecology of a non-folivore herbivore *Leucinodes orbonalis*'s interaction with its host *Solanum melongena*

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Chapter 1: Introduction

Plant-insect herbivore interactions have been researched mainly using folivore models. Leaf's responses to insect folivory and insect counter-responses have been rigorously studied. Interactions of frugivores, the fruit-feeding insects, with their host plants have not been studied. As a frugivore model, we used *Leucinodes orbonalis* (Lepidoptera), the eggplant shoot and fruit borer (ESFB). It is a specialist pest of eggplant (*Solanum melongena*, Solanaceae). Eggplant cultivation faces severe and widespread infestation (45-90 % yield loss) by this multi-insecticide-resistant pest. As this insect bores plant organs, it remains protected from many insecticides, especially non-systemic ones. Farmers tend to apply insecticides in doses much higher than recommended doses which directly hamper the health of consumers, producers, and the environment. Integrated pest management or IPM has gained attention in recent times which involves the combinatorial use of many different pest management techniques without harming the environment. For effective integrated pest management, it is necessary to understand the chemistry of this interacting plant-pest system, where chemical ecology gives us a platform. I tried understanding eggplant-ESFB interaction during two of ESFB's growth stages- moth and larva.

Chapter 2: Understanding the role of eggplant volatiles in ESFB adult's host location

Our field observations of gravid ESFB females' behavior that they (1) can locate and oviposit on solitary eggplants of susceptible varieties present under the dense and aromatic tree canopy and (2) oviposit predominantly on leaves, led us to hypothesize that ESFB follows contactless leaf olfactory cues for host location. We also observed that ESFB does

not oviposit on the Himalayan eggplant variety RC-RL-22 (RL22) in the agricultural field, where seven eggplant varieties were planted following complete randomized block design, as well as inside mesocosm. This indicated the presence of repellants in RL22s' leaf-blend. Thus, RL22 was selected for further studies to understand the basis of its resistance.

We investigated leaf-volatiles of six susceptible varieties and RL22. The solid-phase microextraction and gas chromatography combined with a mass spectrometer and flame ionization detector (SPME/ GCMS/ FID)-based volatile profile showed that geraniol was released only by RL22. Its exogenous application on susceptible varieties reduced oviposition by >90 %. To further validate geraniol's role in RL22's ESFB-deterrence, we identified eggplant's *geraniol synthase* gene and confirmed its role by heterologous expression of its protein with *in-vitro* characterization. Then we silenced RL22's *geraniol synthase* gene using virus-induced gene silencing. Geraniol biosynthesis suppression rendered RL22 ESFB-susceptible; foliar geraniol application on the *geraniol synthase*-silenced plants restored oviposition deterrence. We inferred that ESFB females use olfactory cues for host choice and prefer low-geraniol hosts.

Exploring the defense mechanisms of a resistant eggplant variety RL22 led us to identify geraniol, which is cheap and the U.S. Food and Drug Administration (FDA)-certified Generally Recognized as Safe (GRAS) food additive. Thereby, geraniol-based formulations can readily be used in eco-friendly ESFB management. Interestingly, geraniol has already been used in ESFB-deterrence, as it is emitted by many aromatic plants, traditionally used for intercropping in eggplant fields to reduce ESFB infestation. Together, this chemical ecology-based study shows that controlling the ovipositing females is a suitable alternative to controlling herbivorous larvae. Geraniol can be used in IPM to reduce hazardous pesticide load. In the future, more such studies on exploring plant defense mechanisms can help to identify compounds, with antixenosis properties, which can be incorporated into sustainable agricultural practices.

Chapter 3: Understanding eggplant's response to ESFB frugivory

We also studied plant's response to ESFB frugivory. We observed that ESFB infestation of fruits was associated with increased flowering at the apical buds of the infested-fruit bearing branches. Whether flowering was induced by ESFB infestation was investigated. Liquid chromatography-mass spectrometry-based phytohormone profiling revealed that after two days of ESFB frugivory, abscisic acid (ABA) was induced (~5-fold) in the fruit.

It also showed that ABA was induced in the sink leaf (>3-fold) after five days of frugivory. To find whether the fruit-induced ABA moved to the leaf, we infiltrated the deuterium-labeled ABA (d6-ABA) into the fruit; we observed that it was transported to the leaf, indicating a frugivory-induced fruit-to-leaf ABA signaling. Both ABA infiltration in fruit, and its application on the leaf, induced flowering at the apical bud which is similar to frugivory. We inferred that ESFB frugivory induces ABA in fruit. It acts as a long-distance signal to the leaf, where it plays role in flowering induction.

Fruit is a sink organ. The knowledge of fruit to another organ phytohormone signaling is absent. To our best knowledge, this is the first study showing that fruits, despite being sinks, are capable of generating phytohormone signals upon biotic stress. Frugivory, and ABA applications induced flowering. This knowledge can be used increasing crop yield. From the ecological perspective, such flowering induction may help compensate for the plant's fruit loss or may ensure fruits for ESFB's next generation.

Chapter 4: Summary

Our findings unfold the chemistry of fruit-frugivore interaction. Moreover, this work has agricultural applications- geraniol's oviposition deterrence can be used in ESFB management, and the knowledge of the flowering induction mechanism can be used for productivity enhancement.

Chapter 1
Introduction

1. Introduction

1.1. Plant and insect interaction

About 0.3 million plants and 1 million insects are present on earth. In an ecosystem, various plants and insects frequently interact with each other (Chen & Mao, 2020). Their interaction dynamics can generate specialized relationships that drive evolution (Hamann *et al.*, 2021). Plant-insect relationships can be mutualistic like pollinators promoting plant reproduction or antagonistic like herbivores devouring plants. Sometimes, the same insect species can have both mutualistic and antagonistic relationships with the host at the same or different stages of its life cycle (Kessler *et al.*, 2010, 2012). Out of four major stages of insect herbivores' life cycle (adults, eggs, larvae, and pupae), insects interact with host plants in two stages, adults and larvae. Adults locate, select, oviposit, or feed on hosts. They follow host olfactory cues for long-distant host location, visual and olfactory cues for short-distant host location, and gustatory cues for host selection (Thorsteinson, 1960; Meng *et al.*, 2018). Larvae predominantly feed on hosts.

Among all the plant-insect interactions, the most common one is insect herbivory on plants and plant's response to it (Gatehouse, 2002). Plants defend insect herbivory using several structural, molecular, and biochemical means. Insects counter-defend plant defense using means like avoidance, tolerance, and other biochemical adaptations. This interaction can be well understood by the analogy of the 'arms race' (Gatehouse, 2002; Després *et al.*, 2007).

I focused on understanding plant's response to insect herbivory using an agriculturally important crop-pest system, eggplant-eggplant shoot and fruit borer (ESFB), and whether and how that can be incorporated into sustainable agriculture practices. Along with that, this system served as a fantastic plant-frugivore model, which helped to understand frugivory-induced plant response, which is understudied.

In this chapter, I have summarized the current knowledge of plant's responses to insect herbivory, precisely lepidopteran folivory and the crucial role of plant metabolites in that, followed by a detailed description of the eggplant-ESFB system, the practical problems associated with ESFB management, the importance of understanding eggplant's responses to ESFB-attack and discussed the possibility of incorporating that knowledge into ESFB management.

1.2. Plant's response to insect herbivory

Plants, being sessile, are forced to withstand a multitude of biotic stresses, insect herbivory being one of the major types of stresses. Considering 350 million years of coexistence and the massive diversity of plants and insects present on earth, it is not surprising to understand that plant's response to herbivory is highly diverse. Plant's response to resist or eliminate herbivory is understood as plant defense (Gatehouse, 2002).

Plant defense mechanisms can be categorized as direct/ indirect and constitutive/ induced defenses (Kessler & Baldwin, 2002; Calatayud *et al.*, 2016). The plant traits that themselves affect herbivore performance, belong to the direct defense category. These include certain physical features, like spines, thorns, trichomes, hardened leaves, etc., and chemical constituents, like repellants, antifeedants, toxicants, etc., which themselves protect plants from attackers (Hanley *et al.*, 2007; Howe & Jander, 2008). These chemicals include secondary metabolites, phenylpropanoids, small peptides, proteinase inhibitors, several enzymes, etc. These function by poisoning or deterring non-adapted herbivores or by restricting the resource of adapted herbivores (Maffei, 2007). Since, these metabolites are used by the first trophic level i.e., plants, as direct defense to control the upper trophic level i.e., herbivores. Therefore, they are commonly termed as a 'bottom-up' control' of herbivores. On the other hand, indirect defense includes the production of molecules that attract and retain natural enemies of herbivores (Díaz-Castelazo *et al.*, 2004; War *et al.*, 2012; Wang *et al.*, 2017). Plants emit volatiles that attracts insect-parasitoids, secrete nectar, from extrafloral nectaries, which serve as food for insect-killing predators like ants and provide shelter to natural enemies (Hawkins *et al.*, 1997; Kessler & Baldwin, 2002; Díaz-Castelazo *et al.*, 2004; Ode, 2006). Since, these metabolites are used by the plants as indirect defenses; they attract the natural enemies (the third trophic level) of the herbivores (second trophic level), and thus, they mediate the herbivore control. Therefore, the indirect defenses are surmised to facilitate the 'top-down' control' of herbivores.

Defense metabolite production and maintenance are expensive. That is why many defense metabolites are not constitutively present. Instead, these are induced only after herbivory recognition (Pichersky & Gershenzon, 2002; Kessler & Baldwin, 2002; Dudareva *et al.*, 2004; Holopainen, 2004; War *et al.*, 2011; Holopainen & Blande, 2012; Pierik *et al.*, 2014). The success of induced defense depends on accurate herbivore recognition and effective defense mounting. This process is time-demanding, and before the defense is mounted, some plant parts are already consumed by herbivores, which is why, maintaining

defense constitutively is also essential (Maffei *et al.*, 2007a; Mithöfer & Boland, 2012). Constitutive defense is mostly direct, and induced defense can be direct, or indirect (Maffei *et al.*, 2007b).

1.3 Role of plant metabolites in plant's response

Plants are known to produce as high as 200,000 or even more specialized metabolites, which participate in ecological challenges (Pichersky & Lewinsohn, 2011). Plant chemistry influences the insect community interacting with it. Conversely, herbivory is a strong selective force of plant defense evolution. Plants have evolved to produce a plethora of metabolites to deal with herbivory (Ruiz *et al.*, 2002). Plants' volatile/ non-volatile metabolites and their influence on herbivores have intrigued scientists for several decades.

Plant VOCs, often mentioned as byproducts of metabolism or metabolic overflow (Kant *et al.*, 2009), are known to play a crucial role in protection (Pierik *et al.*, 2014; Hammerbacher *et al.*, 2019). Plant VOCs majorly belong to chemical classes which are fatty acid derivatives, benzene derivatives, and terpenoids (Pichersky & Gershenzon, 2002; Dudareva *et al.*, 2004). VOC composition varies across plant organs, species, and abiotic or biotic factors. (Clavijo McCormick *et al.*, 2012). Many studies have documented the role of plant VOCs in direct or indirect plant defense (Holopainen, 2004; Holopainen & Blande, 2012). These compounds can directly serve as insect repellants, toxins, etc., or indirectly attract natural enemies and/ or alarm neighboring plants (Zhou & Jander, 2022). Upon herbivory, the plant VOC blend composition can change. Induced VOCs are herbivore-induced plant volatiles (HIPV) (Holopainen, 2004; Dicke & Baldwin, 2010; Pierik *et al.*, 2014). HIPVs often attract natural enemies of herbivores and are referred to as the 'cry for help' (Dicke & Baldwin, 2010; Stam *et al.*, 2014).

Several secondary metabolites like phenolics, flavonoids, terpenoids, alkaloids, tannins, cyanogenic glycosides, etc., play a role in plant protection (Mithöfer & Boland, 2012; War *et al.*, 2012). Herbivory activates the transcription of a diverse group of enzymes like polyphenol oxidase, peroxidase, lipoxygenase, superoxide dismutase, ascorbate peroxidase, and many others, ultimately leads to various defense mechanism activation. Plants produce compounds like silica, latex, quinones, proteinase inhibitors, lectins, and defensins which inhibit insects' digestion (Mithöfer & Boland, 2012; War *et al.*, 2012). These proteins and metabolites participate in membrane disruption, inhibition of

metabolism, absorption, and transport of nutrients, ions, and hormonal control dysregulation and disruption of other physiological processes (Mithöfer & Boland, 2012).

The success of herbivore elimination depends on accurate herbivore recognition, efficient signal transduction, and effective defense mounting (Maffei *et al*., 2007a, b; Mithöfer & Boland, 2012). The process of herbivore recognition by plants is highly fine-tuned. Based on herbivore type and extent of herbivory, plant's response varies. Immediately after recognition, a cascade of plant signaling initiates at the wound. The signal transmits to other parts systemically. It triggers the transcriptional and metabolic reconfiguration to mount the defense. This is termed herbivory-associated molecular pattern-triggered immunity (Erb *et al.*, 2012). This herbivory-mediated plant response is observed both at the local herbivory site and distal plant parts.

1.4. Plant's response to lepidopteran herbivory

Lepidoptera is the second-largest insect order (Perveen & Khan, 2017). A major section of agriculturally harmful insect pests belongs to the order Lepidoptera. Lepidopteran herbivores can be classified into many types based on their feeding nature. These are folivores (leaf-eating herbivores), rhizovores (root-eating ones), frugivores (fruit-eating ones), granivores (seed-eating ones), etc. (Dethier, 1941). Plant's response is well documented for lepidopteran folivores (Ehrlich & Raven, 1964).

1.4.1. Plant's response to lepidopteran folivory

The two mobile stages of the folivore-insects' life cycle are, adults and larvae, which predominantly interact with hosts. The adults benefit plants as they pollinate plants while feeding on nectar. Females also locate hosts and oviposit. Plant metabolites play a major role in insects' host choice. Plant chemistry helps insects to differentiate between hosts and non-hosts in complex vegetation. Females follow olfactory, visual, and gustatory cues for host choice before oviposition (Holopainen, 2004; Pierik *et al.*, 2014). Sometimes eggs release certain compounds that can interact with plant physiological processes and suppress plant immunity. In turn, the plants can recognize egg deposition and trigger defense well before herbivory begins (Hilker & Meiners, 2011). The eggs hatch and larvae start feeding on leaves. Feeding induces plant response (Fig. 1.1).

1.4.1.1. Recognition

Lepidopteran insects are chewing insects. While chewing leaves, larvae deposit saliva or oral secretion (OS) on that wound. OS contains some compounds, also known as herbivory-associated molecular patterns or HAMPs. Some examples of HAMPs are fatty acid-amino acid conjugates (FACs), sulfur-containing fatty acids or calciferins, peptides, digested plant proteins, lipases, etc. (Erb *et al.*, 2012; Moran, 2018). These compounds are perceived by plant receptors present at the wounded site. Consequently, a signaling cascade begins (Snyder *et al.*, 2006).

1.4.1.2. Signaling

Intracellular calcium ion and reactive oxygen species (ROS) signaling belong to the early events of signaling, which further initiates various molecular and biochemical events. Soon, biosynthesis of phytohormone, jasmonic acid (JA), begins. Insect chewing causes chloroplast membrane degradation that releases free fatty acids, on which the 13-lipoxygenase (LOX) enzyme works to produce 13-hydroperoxy octadecatrienoic acid, an intermediate of JA biosynthesis. JA conjugates with the amino acid isoleucine (Ile) to form JA-Ile, which is the bioactive form of JA (Erb *et al.*, 2012; Wang *et al.*, 2019). JA and its derivatives, together known as jasmonates, play the role of 'master regulators' in mediating direct and indirect plant response upon folivores' attack.

Other phytohormones like salicylic acid (SA), abscisic acid (ABA), ethylene (ET), auxins (majorly indole acetic acid- IAA and indole butyric acid- IBA), gibberellins (GAs, majorly GA_3), cytokinins (CKs, majorly kinetin- K, zeatin riboside- ZR, 6-amino benzyl purine- 6BAP), brassinosteroids (BRs) and strigolactones may join the interplay. Such phytohormone interactions vary from one system (plant-folivore interacting pair) to another qualitatively and quantitatively (Maffei *et al*., 2007a, b; Bari & Jones, 2009; Erb *et al*., 2012a).

1.4.1.3. Response

JA-dependent phytohormonal signaling leads to massive transcriptional reprogramming (Armbruster & Baldwin, 1998; Devoto *et al*., 2005; Pauwels *et al*., 2008; Staswick, 2008; Zhang & Turner, 2008). It includes the synthesis of several proteins with antinutritive properties like proteinase inhibitors and threonine deaminases, synthesis of secondary metabolites like alkaloids, terpenoids, aliphatic glucosinolates, isoflavonoids and

phenylpropanoids, formation of physical barriers like trichomes, all of which mount direct defense (Wang *et al.*, 2019). Along with that, HIPVs are emitted (Holopainen, 2004). These VOCs deter insects as a mode of direct defense or attract folivores' natural enemies as a mode of indirect defense (Dicke & Baldwin, 2010; Stam *et al.*, 2014). Another function of these VOCs is their ability to prime neighboring plants, and consequently, those start mounting a defense well before being attacked. JA can induce extrafloral nectar production to attract, retain and increase the performance of natural enemies as another way of indirect defense (Bezerra *et al.*, 2021).

1.4.2. Plant's response to lepidopteran non-folivory

All of the above information is primarily known from the research on folivores. Recently, in the last two decades, some studies have documented plants' responses to rhizovory (Soler *et al.*, 2013). Apart from folivory and rhizovory, there are other kinds of interactions. Plant-shoot and fruit borer (SFB) interaction is one of them (Janz & Nylin, 2008; Winkler & Mitter, 2008). Borers make cavities inside shoots, fruits, etc., and remain in constant physical and chemical contact with wounded plant parts. Both their OS and excreta remain inside the plant part, which make them unique from other herbivores. There are very few studies on the salivary components of borers (Bichanga *et al.*, 2017; Xue *et al.*, 2023). To the best of our knowledge, the detailed chemical analyses of borers' saliva, and more important, frugivores' saliva and excreta and how the plant responds to those are absent.

Devising techniques to manage SFB infestations is a major challenge, as they remain protected inside the plants from many challenges like insecticides, natural enemies, and harsh weather conditions. SFB infestation can reach as high as 100 % in certain seasons. For instance, 90- 100 % infestation in eggplant by the insect eggplant SFB (ESFB) *Leucinodes orbonalis* (Oommen, 2004; Meena, 2014; Stommel *et al*., 2015; Nayak *et al*., 2021), 80- 100 % infestation in tomato by *Helicoverpa armigera* (Hanafy & El-sayed, 2013) and 40- 50 % in okra by *Earias vittella* (Shukla *et al.*, 1997) are reported. How eggplants perceive and respond to the borer's herbivory is understudied. In this project, I tried to understand this interaction. I used eggplant, the native Indian Solanaceae crop, and its lepidopteran pest ESFB as a model system.

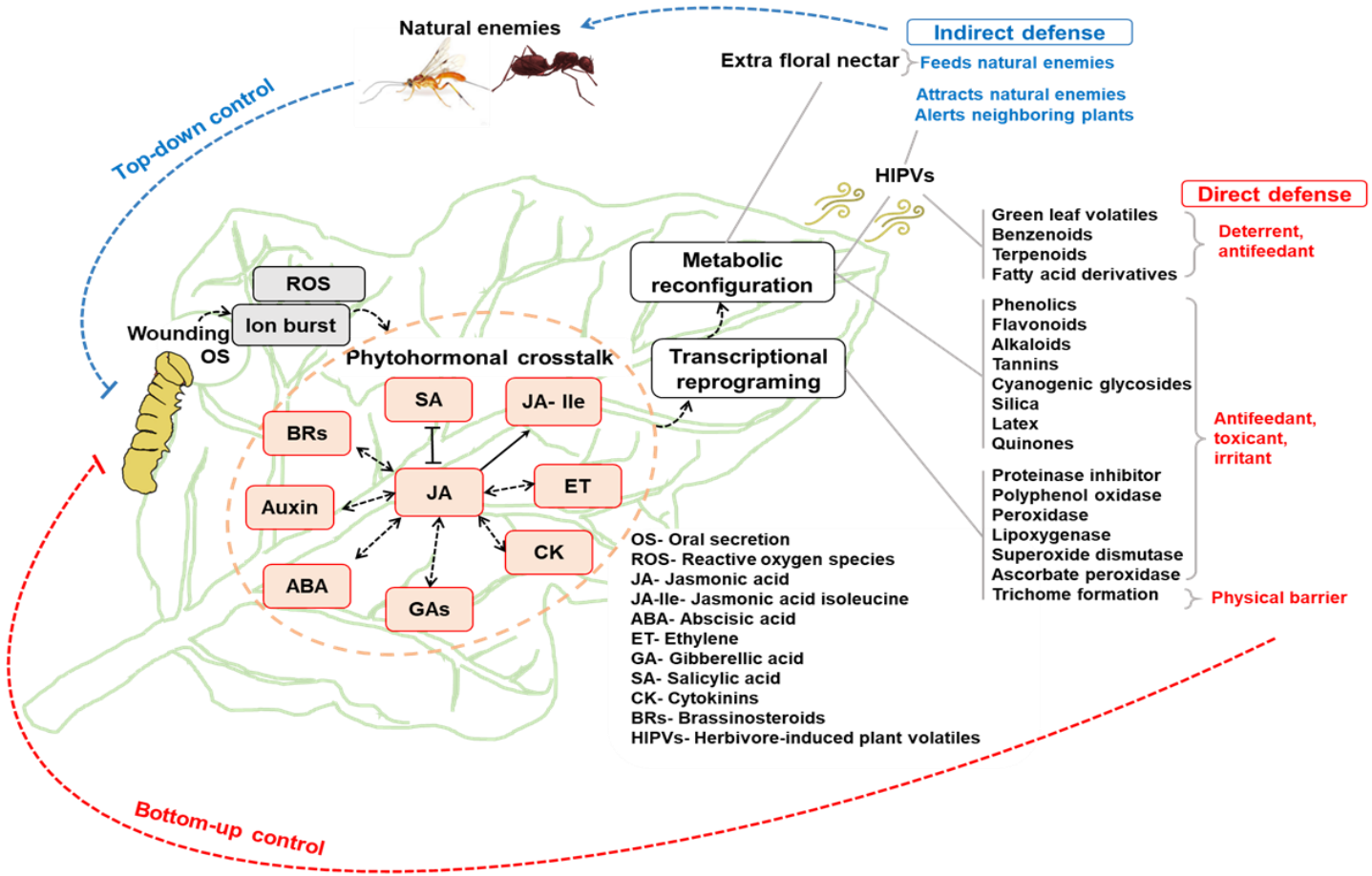

Fig. 1.1 Induced plant response to lepidopteran folivory. Wounding and OS deposition by lepidopteran folivore induces plant responses which includes folivory recognition, signal transduction, and mounting direct and indirect defenses. Highly fine-tuned crosstalk between the 'master-regulator' jasmonates/s (JA and its derivatives) and other phytohormones initiates transcriptional reprograming followed by metabolic reconfiguration and ultimately establishes direct and indirect defenses.

1.5. Eggplant-ESFB interaction

1.5.1. The host: eggplant

Eggplant (*Solanum melongena* Linn.) ranks fifth globally in crop vegetable production after potato, tomato, pepper, and tobacco (Taher *et al.*, 2017; FAOSTAT, 2022). It is commonly called eggplant in North America and Australia; brinjal in Asia and Africa; aubergine, guinea squash, melongene, and garden egg in different parts of Europe, respectively (Lalita *et al.*, 2020a). Eggplant is predominantly cultivated in the tropics and subtropics in India, Pakistan, China, Philippines, Bangladesh, Egypt, France, Italy, the Middle East, the Far East, and the U.S.A (Nayak *et al.*, 2021). India stands second after China in terms of its production worldwide. India produces 2.826 million tons of eggplants annually, with 17.5 million tons/ ha average annual production, with an area cover of 736000 ha (Lalita *et al.*, 2020a; Sharma & Singh, 2020). Eggplant is cultivated year-wide in India; in both Kharif (June to September) and Rabi (November to February) seasons (Sharma & Singh, 2020). It is India's second most consumed vegetable after potato (Choudhary & Gaur, 2009).

Eggplant is often called the ‘king of vegetables' due to its vast diversity in shapes, sizes, colors and wide use in different cuisines (Lalita *et al.*, 2020a). Eggplant is also called the ‘poor man’s crop’ because of its easy and cheap maintenance, profitability in small-scale production and highly nutritious nature (Sidhu *et al.*, 2004; Kolady & Lesser, 2008; Huda *et al.*, 2009). Eggplant fruits are low in calories and contain ample amounts of vitamins [ascorbic acid (C), phylloquinone (K), folic acid (B9), niacin (B3), pyridoxine (B6), pantothenic acid (B5)], minerals (potassium, iron, magnesium, manganese, phosphorus and copper), dietary fibers, polyunsaturated fatty acids and fewer carbohydrates (Sathe *et al.*, 2016). Including eggplant into the daily diet is essential because of its remarkable phenolic content, and phenolics are well known for their various medicinal properties (Sharma & Singh, 2020). Eggplant ranks within the top ten vegetables in terms of its oxygen radical scavenging properties (Lalita *et al.*, 2020a), and here phenolics of eggplant play the primary role (Latha *et al.*, 2018). Eggplant fruits have analgesic, antipyretic, antioxidant, anti-inflammatory, anti-asthmatic, hypolipidemic, hypotensive, antiplatelet, intraocular pressure reducing, anti-cholestremic, antidiabetic, anticarcinogenic, central nervous system depressant and anaphylactic reaction inhibitory activities (Stommel *et al.*, 2015; Solanke M.S.B., 2019). Eggplant is also mentioned in Ayurvedic medicines for being useful in preventing and treating diabetes, cardiovascular diseases, and liver

problems (Naik *et al.*, 2015; Sathe *et al.*, 2016). Besides, it is known for being a good appetizer, aphrodisiac, cardiac tonic, laxative, and reliever of inflammation (Latha *et al.*, 2018).

Out of 53 insect pests that infest eggplant, ESFB, *Leucinodes orbonalis* Guen. (Lepidoptera: Pyralidae) is the most severe pest of eggplant (EFSA Panel on Plant Health *et al.*, 2021). Eggplant is native to India and southeast Asia. ESFB co-occurs with eggplant in all these regions. The highest ESFB occurrence is reported from South Asian countries like India, Bangladesh, Pakistan, China, Philippines followed by countries of middle Africa, North Australia, Mexico, and north of South America (EFSA Panel on Plant Health *et al.*, 2021). There are more than hundred eggplant varieties present worldwide (Lit *et al.*, 2002; Manaday *et al.*, 2014). ESFB infestation depend on the seasons and it vary across varieties. The highest ESFB infestation is observed in humid and warm rainy seasons (Degri, 2014; Meena, 2014). ESFB is responsible for 45- 100 % of crop loss every year, sometimes even more, in south and south-east Asia (Srinivasan, 2008; Divya et al., 2019, Meena, 2014; Nayak et al., 2021; Stommel et al., 2015). The ESFB infestation varies with plants' physicochemical properties; calyx and fruit diameter, protein, sugar, chlorophyll, water and lignin content are positively correlated to the infestation whereas, phenol content, poly phenol oxidase, phenylalanine ammonium lyase expressions are negatively correlated (Dadmal *et al.*, 2004; Devi *et al.*, 2015; Kumar, 2017; Lalita *et al.*, 2020)

1.5.2. The frugivore: ESFB

ESFB is present in tropical regions of Asia, Africa, and North and South America (the UK, 1976; EFSA Panel on Plant Health *et al*., 2021). It is considered to be the limiting factor of eggplant cultivation (Adiroubane & Raghuraman, 2008). ESFB alone causes 45- 100 %, or sometimes even more, fruit loss in the south and south-east Asia (Srinivasan, 2008; Divya *et al.*, 2019). In a humid warm climate, the fruit infestation can be as high as 90 % to even 95 % (Oommen, 2004; Meena, 2014; Stommel *et al*., 2015; Nayak *et al*., 2021). Even though ESFB is a Solanaceae specialist, it predominantly feeds on eggplant (Devi *et al.*, 2015; Taher *et al.*, 2017; Kassi *et al.*, 2019; Reshma *et al.*, 2019). Other minor hosts include *Solanum tuberosum*, *S. lycopersicum*, *S. nigrum*, *S. indicum*, *S. torvum*, *S. myriacanthum*, and *S. xanthocarpum* (Ardez *et al.*, 2009; Dash, 2020; EFSA Panel on Plant Health *et al.*, 2021).

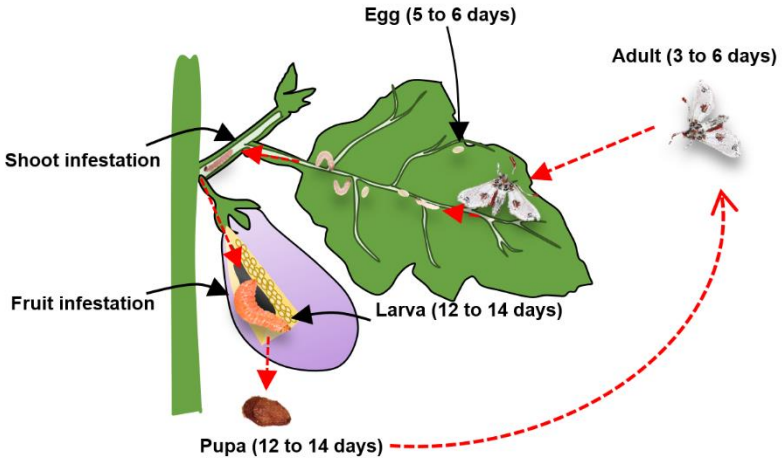

Fig. 1.2 The Life cycle of the eggplant shoot and fruit borer (ESFB). Adult locates hostplant at night and oviposits 100- 250 eggs on leaves, shoots and fruits. Eggs hatch. Larvae bores into leaf-veins, petioles, shoots and fruits. After completion of larval period of five instars, larvae pupate. Pupae emerge as adults and the life-cycle continues.

ESFB's lifecycle contains four stages- eggs, larvae, pupae, and moths (Fig. 1.2). Gravid females lay eggs on eggplant leaves, tender shoots, and crowns. One female lays around 100 to 250 eggs, mostly singly, in its lifetime of 3 to 6 days. The eggs hatch in 5- 6 days, and newly hatched larvae (neonates) start boring into plant parts (Adiroubane & Raghuraman, 2008). Leaf and shoot infestations cause wilting and drooping of shoots. Larvae majorly feed on shoot-piths and form 'dead hearts' (Challa *et al.*, 2021). Once, the larvae reach fruits, it bores inside the fruit and seals the hole using excreta. The larval stage consists of five instars- first (1- 2 days; tiny, creamy white with a dark brown head and microscopic three pairs of thoracic and five pairs of prolegs), second (1- 2 days; similar looking, and slightly bigger than 1st instar), third (1 -2 days; light pink to light brown with distinct marking in the prothoracic shield, dark brown thoracic legs), fourth (2- 3 days; pinkish, and similar to the third instar), and fifth (4- 5 days; three distinct thoracic segments, and developed five pairs of prolegs). Third and fourth instar larvae are voracious feeders. The late fifth instar is dark pink and less mobile (Jat *et al.*, 2004; Wankhede & Kale, 2009; Onekutu *et al.*, 2013). One larva, in its larval span of 12- 14 days, can infest 4- 7 different fruits (Lalita *et al.*, 2020a; AL-Amin *et al.*, 2022). In humid seasons, one fruit can contain up to 20 larvae (Lalita *et al.*, 2020b). Larvae form pupae (12- 14 days; oval with dark brown to gray) in the soil, or on different plant parts (Nayak *et al.*, 2021). Adults (3- 6 days; creamy white wings with brown spots on the forewings) emerge from pupae, mate, and female adults oviposit (Mehto *et al.*, 1983; Jat *et al.*, 2004; Wankhede & Kale, 2009; Onekutu *et al.*, 2013). Adults feed on nectar. The larval stage is the devastating stage in terms of agriculture. ESFB can attack plants of both juvenile and reproductive

stages (Singla *et al.*, 2018; Reshma *et al.*, 2019; FAOSTAT, 2022). With the onset of fruit infestations, shoot infestations drop dramatically (AL-Amin *et al.*, 2022). Researchers and farmers have developed different strategies to reduce ESFB infestation. The commonly used ESFB management techniques are discussed in the following sections.

1.6. ESFB management practices

1.6.1. Synthetic chemical application

Using synthetic chemicals such as insecticides, repellants, and irritants has become a cheap and easy technique for insect elimination. >30 % of eggplant production cost is invested in synthetic chemical applications (Alam *et al.*, 2006; Lalita *et al.*, 2020a). Chemicals frequently used for ESFB control, belong to many different insecticide classes based on their mode of action (MoA) as determined by the Insecticide Resistance Action Committee (IRAC) (Sparks & Nauen, 2015). These include acetylcholinesterase inhibitors [carbamates- thiodicarb (Journal *et al.*, 2014; Sahu *et al.*, 2018), carbofuran (Sahu *et al.*, 2018), carbosulfan (Mamum *et al.*, 2014); organophosphates- triazophos (Sahu *et al.*, 2018), monocrotophos (Deshmukh & Bhamare, 2006), profenofos (Pooja & Kumar, 2022), disulfoton (Satpathy, 1974)], GABA-gated chloride channel blockers [organochlorines- endosulfan (Sharma, 2010); phenylpyrazoles- fipronil (Sahu *et al.*, 2018)], sodium channel modulators [Pyrethroids- cypermethrin (Deshmukh & Bhamare, 2006; Kalawate & Dethe, 2012; Pooja & Kumar, 2022), bifenthrin (Yousafi *et al.*, 2015), cyhalothrin (Sharma, 2010), deltamethrin (Sharma, 2010)], nicotinic acetylcholine receptor (nAChR) competitive modulators [neonicotinoids- imidacloprid (Mamum *et al.*, 2014; Pooja & Kumar, 2022), thiamethoxam (Reshma *et al.*, 2019)], nAChR allosteric modulators- site I [spinosyns- spinetoram (Muthukrishnan *et al.*, 2013; Yousafi *et al.*, 2015)], nAChR channel blockers [nereistoxin analogues- cartap hydrochloride (Deshmukh & Bhamare, 2006; Sahu *et al.*, 2018; Reshma *et al.*, 2019)], ryanodine receptor modulators [diamides- chlorantraniliprole (Reshma *et al.*, 2019; Pooja & Kumar, 2022), flubendiamide (Reshma *et al.*, 2019; Pooja & Kumar, 2022)], voltage-dependent sodium channel blockers [oxadiazines- indoxacarb (Patra *et al.*, 2009; Shirale *et al.*, 2012)], ecdysone receptor agonists [diacylhydrazines- methoxyfenozide (Patra *et al.*, 2009)], uncouplers of oxidative phosphorylation via disruption of the proton gradient [pyrroles- chlorfenapyr (Shirale *et al.*, 2012)], and inhibitors of chitin biosynthesis affecting chitin synthase 1 (CHS1) [benzoylureas- novaluron (Sharma, 2010)].

ESFB larvae remain concealed inside the shoots or fruits. Most non-systemic insecticides fail to reach the pest or reach it in low/ sublethal doses (Choudhary & Gaur, 2009). Consequently, framers tend to use insecticides many-fold higher (as high as 180-times in India and Bangladesh; (Islam *et al.*, 2019; Lalita *et al.*, 2020a) than their recommended dosages (Lalita *et al.*, 2020a) because they subjectively assess the pest load based on the visual presence of insects or insect-associated phenotypes like the presence of holes, excreta and shoot wilting (Kumar, 2017a). Indiscriminate synthetic chemical usage harms human health and the environment (Islam *et al.*, 2019). Pesticide residues cause allergy, stomach and gut problems, and other long-lasting effects on human health (McLeod *et al.*, 2002; Saimandir *et al.*, 2009; Alam *et al.*, 2011; Bharati, 2013; Muthukrishnan *et al.*, 2013; Bharati *et al.*, 2015; Sharma *et al.*, 2018). Chemical mixing with soil and water degrades the fertility of agricultural lands and pollutes water bodies and groundwater (Kumar, 2017a). Overuse of these chemicals kills the natural enemies of the pest. Sometimes it leads to secondary pest outbreaks (Kumar, 2017a). To that, repeated use of sublethal doses of pesticides cannot kill the pest but imparts stress on the pest. Stress enhances mutation rates and further leads to pesticide resistance (Ghosh *et al.*, 2023; Gressel, 2011; Guedes *et al.*, 2017; Tehranchian *et al.*, 2017; Bantz *et al.*, 2018; Rahman, 2009; Murali *et al.*, 2017; Shirale *et al.*, 2017; Kariyanna *et al.*, 2020; Botre B. S., Salunke P. B., Munje S. S., 2014; Kaur *et al.*, 2014; Kodandaram *et al.*, 2017; Jahan *et al.*, 2018; Munje S. S.).

1.6.2. Eggplant germplasm characterization

Other than synthetic chemical application, eggplant germplasm screening for biophysical and chemical resistance factors has been practiced by researchers extensively. Any morphological or biochemical properties of plants that can interfere with insect pests' oviposition, hatchability of eggs, larval growth, development, movement, pupation, pupal eclosion, health, and mating of adults can be used as resistance factors (Wagh, 2012; Wagh *et al.*, 2012).

These factors include morphological features (fruit length, diameter, shape, epicarp thickness, spines, trichome density, leaf hair density, shoot thickness, etc.) and chemical constituents (chlorophyll, protein, fiber, fat, silica, sugar, ash, moisture, lignin, ascorbic acid, phenol, anthocyanin, inorganic ions like potassium, zinc, glycoalkaloid, enzymes like polyphenol oxidase, peroxidase, etc.) (Khorsheduzzaman *et al.*, 1970; Elanchezhyan *et al.*, 2008; Wagh, 2012; Devi *et al.*, 2015; Kumar, 2017b; Kumar *et al.*, 2017)]. Using varieties/ cultivars inherently resistant or tolerant to ESFB can be a suitable, economically sound,

and environment-friendly method. Even varieties with low tolerance levels sometimes can reduce the pesticide load dramatically as Wagh (2012) described.

The major limitation of resistant plants is that the crop yield is sometimes meager. The hybridization of resistant and high-yield varieties has been a popular method to deal with this situation. However, these processes are labor-, time- and cost-intensive. Most of the studies determining plant resistance factors are based on correlation studies, and therefore many times, they lack experimental validation. There is also a lack of clear understanding of the genetics involved in resistant traits. Finding a qualitative trait that can be easily incorporated into breeding programs also can be difficult.

1.6.3. Biorational compounds

Insecticides, which are highly specific to target organisms, effective in lower concentrations, safe for non-target organisms, particularly mammals, mostly non-synthetic, environment-friendly, and biodegradable, are known as biorational insecticides (Rosell *et al.*, 2008). These include botanicals (Rosell *et al.*, 2008; Iqbal *et al.*, 2022), microbial insecticides (Isman, 1997; Gelernter & Trumble, 1999; Rosell *et al.*, 2008), and semiochemicals (Tripathi *et al.*, 2009; Nusra *et al.*, 2020; AL-Amin *et al.*, 2022).

Botanicals are plant-derived bioactive compounds, which have gained much attention in the past decade as a part of sustainable agriculture. Several botanicals have been practiced in ESFB management. Among them, the most commonly used botanical is azadirachtin, the bioactive compound of *Azadirachta indica*. Some of the azadirachtin formulations which have been popularly in use in India are Neem Seed Kernal Extract (NSKE; Mandal *et al*., 2010; Singh *et al*., 2021), Nimbo Bas (Saimandir & Gopal, 2012), Neemarin (Gautam *et al.*, 2008), Achook (Gautam *et al.*, 2008), Bioneem (Gautam *et al.*, 2008), neem oil (Rahman *et al.*, 2009)], etc. Other common botanicals serving the same purpose are pyrethrum from *Tanacetum cinerariifolium* (Owosu, 2012), nicotine from *Nicotiana tabacum* (Srivastava *et al.*, 2020), oxymatrine from *Sophora flavescens* (Adiroubane & Raghuraman, 2008) and rotenone from *Lonchocarpus* spp. (Owosu, 2012). Extracts of *Annona muricata* (Owosu, 2012), *Calotropis gigantea* (Murugesan & Murugesh, 2009), *Lantana camera* (Murugesan & Murugesh, 2009), *Millettia pinnata* (Rahman *et al.*, 2009; Yadav *et al.*, 2015), *Madhuca longifolia* (Yadav *et al.*, 2015), *Zingiber officinale* Roscoe (Malsawmzuali *et al.*, 2013), *Allium sativum* (Malsawmzuali *et al.*, 2013), *Nicotiana tabacum* (Malsawmzuali *et al.*, 2013) and *Acorus calamus* (Mannarakoth *et al.*, 2019) are

reported to be effective in ESFB management. Botanicals are used as insecticides, repellants, irritants, and antifeedants (Srivastava *et al.*, 2020; Prakash *et al.*, 2022).

Among the microbial insecticides sporulating *Bacillus* spp. is the most popular and effective (Mainali, 2014). *Bacillus thuringiensis* (*Bt*)- based commercial products are the most successful microbial insecticides (Dubey *et al.*, 2010; Mandal *et al.*, 2010; Chatterjee & Mondal, 2012; Saimandir & Gopal, 2012; Hautea *et al.*, 2016; Jouzani *et al.*, 2017). Many studies showed that the bio-efficacy of *Bt* formulations was lower than synthetic insecticides (Saimandir & Gopal, 2012). Other than *Bt*, spinosad from *Saccharopolyspora spinosa* is well-documented in ESFB management (Deshmukh & Bhamare, 2006; Patra *et al.*, 2009; Kalawate & Dethe, 2012; Mamum *et al.*, 2014). However, ESFB has gained resistance against Spinosad (Table. 1.1). Field applications of *Trichoderma longibrachiatum, Beauveria bassiana, and Verticillium lecanii* fungal solutions are found to have antagonistic effects on ESFB (Mathur *et al.*, 2012; Ghosh & Pal, 2016). Notably, their efficiency is considerably lower than synthetic chemicals (Mathur *et al.*, 2012).

Besides applying bacterial formulations, scientists have also generated *Bt*-toxin-producing genetically modified (GM) crops (Jouzani *et al.*, 2017). GM crops are restricted due to various socio-ethical and ecological concerns (Choudhary & Gaur, 2009; Herring & Chandrasekhara Rao, 2012).

Semiochemicals are mostly volatile compounds that act as signals in intraspecific or interspecific communications (Norin, 2007; Abd El-Ghany, 2019). Pheromones serve various purposes in intraspecific communications. Several studies have documented the efficacy of insect- and plant-based semiochemicals in ESFB management. ESFB females' sex pheromones, a mixture of (*E*)-11-hexadecenyl acetate and (*E*)-11-hexadecen-1-ol are often used as lures in traps and baits (Cork *et al.*, 2001; Rani *et al.*, 2017; van Vang *et al.*, 2018; Nusra *et al.*, 2020). These traps only target males. A recent study has shown the potential for using eggplant volatiles in traps or baits as ESFB (male and female adults) attractants (Nusra *et al.*, 2021).

Even though bioinsecticides are eco-friendly and sometimes can bring sufficient pest control, farmers often do not prefer the synthetic insecticides due to their high cost, limited pest range, and less stability (Gelernter & Trumble, 1999).

1.6.4. Predators and parasitoids

Usage of chemicals (synthetic and biological) is costly as it requires isolation or synthesis of chemicals, their purification, preservation, and packaging. Instead, many studies have exploited the possibility of using ESFB's natural enemies in their management. They belong to three major classes- parasites or entomopathogens, predators, and parasitoids. Some commonly used ones include entomopathogens like *Bipolaris tetramera* (fungus), baculovirus, and nuclear polyhedrosis virus (virus), etc., predator insects like *Chrysopa kulingensis*, *Cheilomenes sexmaculata*, *Coccinella septempunctata*, *Brumoides suturalis*, etc. and parasitoids like *Trathala flavoorbitalis*, *Diadegma apostate*, *Eriborus argenteopilosus*, *Xanthopimpla punctata*, *Trichogramma chilonis*, etc. (Srinivasan, 2008; Budhvat & Magar, 2014). However, natural enemies alone cannot always provide desirable pest-load reduction.

1.6.5. Integrated pest management (IPM)

Many of the above-mentioned biorational techniques alone cannot bring desirable ESFB control. As a result of which, IPM has gained more attention. IPM employs combinations of many methods with minimum adverse effects on society and the environment; (Mandal *et al.*, 2008; Srinivasan, 2008; Chatterjee & Mondal, 2012; Dar *et al.*, 2017; Yadav *et al.*, 2017). Asian Vegetable Research and Development Center (AVRDC) developed, validated, and promoted the IPM strategy for ESFB management in 2003 (Alam *et al.*, 2003). Their advice is to select resistant eggplant varieties/ cultivars, use ESFB-traps for continuous pest removal, frequent removal of infested shoots and fruits, restrict indiscriminate pesticide usage to allow the proliferation of natural enemies, and use biorational compounds.

1.7. Motivation

Detailed literature survey and communication with scientists of the Indian Council of Agricultural Research (ICAR), farmers, and vendors helped us to understand a few drawbacks of currently established techniques. ESFB resistance is claimed in a few varieties by seed vendors and farmers. However, the basis and degree of resistance are not documented. Many studies pointed out certain eggplant resistance factors. However, their selection and maintenance are labor- and cost-intensive processes. Many times, resistant varieties are less productive. The resistance factors determined by most of the studies are based on correlation analysis and lack experimental validation. Many studies are based on

the plant's phenotype. Their genomic, transcriptomic, proteomic, and metabolomic details are missing. These details can help in the successful and targeted incorporation and maintenance of resistance factors in breeding programs to generate high-yield and resistant varieties.

The application of biorational compounds as a part of IPM has been gaining attention recently. However, implementing this eco-friendly technique remains limited most of the time due to the cost associated, which is often many-fold more than synthetic pesticides, the lack of availability of biorational formulations in the markets, and the lack of scientist-to-farmer knowledge transfer. Considering the potential of biorational compounds as a replacement for hazardous synthetic chemicals, extensive interdisciplinary research is required to integrate omics, nanotechnology, synthetic biology, and ecology.

ESFB behavioral studies are limited. Most of the studies focus on controlling the less mobile larval stage of the pest. Basic information on their winged mobile adults is obscure. The questions on ESFBs' egg-laying behaviors, host location in a complex-aromatic environment, how ESFB moths refrain from oviposition on plant species closely related to eggplants, etc., are missing. Even though a very recent study showed that eggplant-volatiles attract ESFBs, they did not pinpoint the essential bioactive components of eggplant blend in attraction (Nusra *et al.*, 2021). More studies of this type will help to understand the full potential of hostplants' constitutive/ induced volatiles in ESFB management.

Importantly, ESFB larvae are non-folivorous. As discussed earlier in section 1.4.1, plenty of studies are available on plant-folivore interactions. The details of plants' response to folivory helped researchers to select or develop resistant/ tolerant varieties (Gimenez *et al.*, 2018). However, the knowledge of plant's response (recognition of oviposition, boring on leaves/ shoots/ fruits, OS/ excreta deposition, the transmission of signals, mounting direct/ indirect defense, interaction with natural enemies, and alerting neighbors) to ESFB attacks and ESFBs' counter adaptations to plant's response, is obscure. From a chemical, ecological perspective, the eggplant-ESFB interaction provides an excellent opportunity to understand plant-frugivore interactions with agricultural implications.

1.8. Groundwork, hypothesis, and objectives

Before taking up the problem, we contacted ICAR scientists and farmers to understand the situation better and visited research institutes and agricultural fields. We collected >20

eggplant varieties from agricultural institutes and commercially available ones. We planted them in the experimental field at IISER, Pune, following a complete randomized block design and noted observations on this interaction. Some striking phenomena were noticed when we initiated field studies and collected ESFB eggs and larvae to initiate insect culture. These were-

(1) We found ESFB eggs and larvae on solitary plants in the open or under a thick tree canopy cover.

(2) The number of eggs and larvae found varied across varieties when different varieties were planted together. No ESFB eggs or larvae were found on the Himalayan eggplant variety RC-RL-22 (RL22).

(3) We observed more flower buds at the apical buds of eggplant branches with ESFB-infested fruits than at healthy fruit branches.

These three observations served as the origin of hypotheses for my work. Based on the first two observations, we hypothesized that ESFB utilizes olfactory cues which influence ovipositing female's host choice; these cues vary between eggplant varieties; RL22 contains deterrents. Based on the third observation, we hypothesized that infested eggplant fruits respond to frugivory by flowering induction. Signal transduction from fruit to shoot, especially apical bud, is required. Based on these observations involving both the host-interacting stages (adults and larvae) of ESFB and the literature background, the objectives of my Ph.D. work were formulated. These were (1) To understand the role of eggplant volatiles in this interaction and (2) to understand how the host responds to the frugivorous larval stage. In this thesis, I have attempted to attain these two objectives using field studies, insect choice and no-choice assays, molecular biology, and metabolomics-based techniques.

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Chapter 2

Understanding the role of eggplant volatiles in ESFB adult's host location

2. Understanding the role of eggplant volatiles in ESFB adult's host location

2.1. Introduction

Interactions of herbivore insects with their host plants are often influenced by plant chemistry, which contributes to the evolution of differential host preference and host specialization in insects. Several butterflies and moths, which indiscriminately feed on the floral nectars of various species, selectively oviposit on the host species of their specialist herbivore larvae. Ovipositing females often distinguish between hosts and nonhosts with the help of these plant volatile organic compounds (VOCs) (Witzgall *et al*., 2005; Thöming *et al*., 2014). Generally, host VOCs act as attractants, while non-host ones act as repellents or neutrals. Insects' remarkably sensitive olfactory systems provide fine resolution of the complex vegetation's odorscape during the flight (Tasin *et al*., 2005; Najar-Rodriguez *et al*., 2010; Pickett *et al*., 2012; Leppik & Frérot, 2014). They can differentiate between background host odors to find their host (Schröder & Hilker, 2008; Karlsson *et al*., 2009; Thöming *et al*., 2014). In addition, herbivory-induced VOCs can deter insects from ovipositing plants already fed on by conspecific larvae, as these plants release repellent VOCs (De Moraes *et al*., 2001; Qiao *et al*., 2018). Furthermore, herbivory-induced VOCs of some plant species attract the natural enemies of the herbivore; this phenomenon is regarded as a natural biological control (De Moraes *et al*., 1998; De Boer *et al*., 2008; Qiao *et al*., 2018). Over the last few decades, several researchers have recommended the exploitation of these chemical ecology aspects of host selection for the management of insect pests (Pickett *et al*., 1997; Khan *et al*., 2000; Aldrich *et al*., 2003; Khan *et al*., 2008; James *et al*., 2012; Saha & Chandran, 2017; Cusumano *et al*., 2020).

Today, the protection of several crops depends on the heavy use of synthetic pesticides, which pose a serious threat to human health (NRC, 1993; Bissdorf, 2010; Alavanja *et al*., 2013; Mebdoua, 2018). Pesticides also deteriorate the environment by polluting soil, water, and air (Ucar & Hall, 2001; Devine & Furlong, 2007; Vymazal & Březinová, 2015; Hladik *et al*., 2018). To minimize the hazards of pesticides, chemical ecology-based eco-friendly pest management solutions are highly desired.

Eggplant (*Solanum melongena* L.; Solanaceae) is one of the crops which requires a serious and urgent reduction in pesticide application. Popularly called the king of vegetables, eggplant is the third most consumed solanaceous vegetable after potato and tomato (Chantella, 2011; Caruso *et al*., 2017; Cristol, 2018; Chapman, 2019). It is one of the

nutritionist-recommended vegetables with low calorie and fat content but high antioxidant, fiber, folic acid, calcium, phosphorus, potassium, and vitamins B and C content and various medicinal properties like analgesic, antipyretic, anti-inflammatory, anti-asthmatic, and spasmogenic (Daunay, 2008; Das & Barua, 2013; Caruso *et al*., 2017; Gürbüz *et al*., 2018; Stommel & Whitaker, 2019). From the farmers' point of view, eggplant is easy to grow, offers many varieties to suit vastly diverse geo-climatic zones that yield remarkably well in these different zones, and is profitable even when cultivated on small scales (Nakasuji & Matsuzaki, 1977; FAO, 2003; Frary *et al*., 2007; Singla *et al*., 2018). Due to these virtues, the area under eggplant cultivation is rapidly increasing (Chapman, 2019). Unfortunately, eggplant is one of the heaviest pesticide-applied vegetables. It receives up to 140 sprays per ~6-month season with a frequency as high as two per day (Rashid *et al*., 2003; Del Prado-Lu, 2015; Shelton *et al*., 2019). Although eggplant is attacked by >35 pests, the eggplant fruit and shoot borer (ESFB, *Leucinodes orbonalis* Guenee, Lepidoptera: Pyralidae) is mainly responsible for this high pesticide application. This pest is present in tropical regions of Asia, Africa, and North and South America (van der Gaag *et al*., 2005; EFSA Panel on Plant Health (PLH) *et al*., 2021; UK, 1976). ESFB attacks the vegetative as well as reproductive stages of eggplant, and its infestation can be so severe that it can cause a 45-100% loss of the marketable yield (FAO, 2003; Singla *et al*., 2018; Reshma *et al*., 2019). Most of the economic damage is inflicted by its frugivorous larval stage, which bores tunnels and deposits frass in fruits (Hautea *et al*., 2016). Since these larvae remain concealed in the fruit for most of their lifetime, several, especially non-systemic, pesticides show inadequate effects on them (FAO, 2003; Choudhary & Gaur, 2009). Due to such concealed habit, larvae are exposed to sublethal doses of pesticides, which facilitates the pesticide resistance development in them (Choudhary & Gaur, 2009). Various synthetic pesticides are often used in heavy doses and combinations for ESFB control (Choudhary & Gaur, 2009; Rahman, 2009; Reshma *et al*., 2019). Such pesticide abuse endangers the health of farmworkers, pollinators, and other non-target insects in the eggplant agroecosystem and can also cause a resurgence of secondary pests. Moreover, residues of these pesticides render eggplant harmful for human consumption (Kalawate & Dethe, 2011; Dasika *et al*., 2012; Del Prado-Lu, 2015; Shelton *et al*., 2019).

Assorted means of attaining ESFB control have been explored to lessen the dependence on pesticides; for example, screening and breeding for resistant or tolerant varieties (Lal, 1991; FAO, 2003; Prabhu *et al*., 2008; Khan & Singh, 2014; Kassi *et al*., 2019),

introgression of resistance traits from wild relatives (Dhankhar *et al*., 1982; Dhankar, 1988; Kumar & Sadashiva, 1996; Rotino, 1997), finding biophysical and biochemical bases of resistance traits for their introduction in susceptible varieties (Naqvi *et al*., 2009; Khorsheduzzaman *et al*., 2010; Wagh *et al*., 2012; Prasad *et al*., 2014; Nirmala & Vethamoni, 2016), intercropping with deterrent herbs (Khorsheduzzaman *et al*., 2010; Calumpang *et al*., 2013) and application of botanicals (Adiroubane & Raghuraman, 2008; Calumpang & Ohsawa, 2015), sex pheromones (Zhu *et al*., 1987; Cork *et al*., 2005), natural enemies (Naresh *et al*., 1986; Sasikala *et al*., 1999; FAO, 2003) and entomopathogens (Beevi & Jacob, 1982; Khorsheduzzaman *et al*., 1998; Mainali *et al*., 2013). While many of these techniques are utilized in integrated pest control, none has been found efficient for the management of ESFB (Alam *et al*., 2003; Mainali, 2014; Lalita & Kashyap, 2020). Consequently, the transgenic eggplant was created to control ESFB by incorporating a gene coding for the insecticidal Cry1Ac protein from *Bacillus thuringiensis* (*Bt*) (Choudhary & Gaur, 2009; Kumar *et al*., 2011; Hautea *et al*., 2016). Although these transgenics showed the desired resistance against ESFB, acceptance of them remained limited due to socioethical concerns (Krishna & Qaim, 2007; Seetharam, 2010; San-Epifanio, 2017; Glaab & Partzsch, 2018).

While collecting ESFB insects from eggplant fields for initiating a laboratory culture, we found eggs and larvae even on solitary plants that were far away from eggplant fields. Secondly, we observed that one of our germplasm collections from the Eastern Himalayas showed extremely low or no ESFB oviposition. Together, these observations suggested that ovipositing females' host finding and selection abilities were key to ESFB's occurrence. We hypothesized that the characteristics of the Himalayan variety eggplant's VOC blends influence ESFB's host selection. Lastly, we validated our findings by the reverse genetics-based characterization of the repellent biosynthesis gene in this variety.

2.2. Materials and methods

2.2.1. Eggplant field

Eastern Himalayan variety RC-RL-22 (RL22) [IC- 0634845; National Bureau of Plant Genetic Resources (ARIS Cell), India] and six popular Indian eggplant varieties Ankur Kavach (KV), Ankur Vijay (VJ), JK 6829 (JK), Riccia Hirvi Kateri (HK), KGN's pinstripe (KP) and Omaxe CVK MK 124 (CVK) were used in this work (Fig. 2.1a). All seven varieties were planted in the experimental field of the Indian Institute of Science Education

and Research (IISER), Pune (18.547669 ° N, 73.807636 ° E) in a randomized complete block design (Fig. 2.1b) with four blocks and each one containing 5 individuals of the abovementioned plants (n= 20 plants per variety, in four blocks) with 1 m spacing between individuals. Manures and fertilizers were provided as recommended for this region (Anonymous, 2010). No pesticides were applied in the field.

2.2.2. Insects

ESFB (Fig. 2.1c) eggs, larvae and pupae were collected from the eggplant fields in and around Pune to initiate the laboratory culture. Larvae were maintained in aerated polypropylene containers [(l) 30 cm× (b) 20 cm× (h) 10 cm] incubated inside a climate chamber (26± 1 °C temperature, 65± 5% relative humidity, 16 h light and 8 h dark photoperiod) and were reared on artificial diet (Salunke *et al.*, 2014). Pupae were maintained in the dark. For mating, moth pairs were kept in jars [(id) 10 cm× (h) 20 cm] containing healthy eggplant twigs as the oviposition substrates and were fed 10% (w/v) aqueous sucrose solution with the help of a cotton wick. Adults from the third generation of this culture were used in the experiments.

2.2.3. Natural occurrence of ESFB in the field

The incidence of ESFB on seven eggplant varieties in the randomized complete block design was measured. On each plant, the number of ESFB-infested fruits and shoots was counted. To understand whether the ovipositing ESFB females showed differential preferences to the seven eggplant varieties, the percentage of leaves (per plant) harboring eggs and the number of eggs per plant were recorded for all the varieties.

2.2.4. Determining the factors influencing the ovipositing females' host selection

Multiple-choice assays

All the insect behavioral assays were performed inside the climate chamber using potted (800 cm^3 pots) juvenile plants with five fully expanded leaves. In a multiple-choice oviposition assay, one plant of each eggplant variety was used. One artificial plant (AP) having five leaves made of a Whatman filter paper (grade 237 equivalent to grade 3 CHR; Sigma-Aldrich, India) was also used as an inert control. These plants were arranged inside a nylon mesh (160 µm) tent with a 30 cm interplant distance. One ESFB female, which had mated 18- 24 h before the assay, was released in this tent and allowed to oviposit for 10 h. A cotton wick dipped in a sucrose solution was provided to moths for feeding during

the assay. The assay was repeated 20 times with a different randomized hostplant arrangement every time to negate any hostplant positional effects. At the end of each assay, eggs on each plant were counted.

Multiple-choice assays using VOC blend-complemented APs

Since it is well known that ESFB females routinely oviposit on eggplant leaves, irrespective of the presence of flowers and fruits on plants (Prabhat & Johnsen, 2000; Ardez *et al.*, 2008; Mannan *et al.*, 2015), we hypothesized that the putative attractants or repellents were mainly from leaves. To find whether the putative attractants from the ESFB-preferred hosts and putative repellants from the ESFB-disliked hosts were present in their VOC blends, we conducted multiple-choice assays using APs and complemented them with the VOC blend extracts of different varieties. The cumulative area of the abaxial and adaxial surfaces of each filter paper leaf was 100± 10 $cm^{2,}$ and its weight was 1.0± 0.1 g, which was similar to the average surface area and the average weight of eggplant leaves. For complementation, VOC blends of the seven varieties were obtained as follows. 3.0± 0.5 g of freshly harvested fully expanded healthy leaves were immersed in 20 ml of GCMS grade dichloromethane (DCM; RANKEM, India) and extracted for six hours by rolling on a tube roller. Leaves were discarded, and the DCM extract was dehydrated using anhydrous sodium sulfate (Sigma-Aldrich, India). The leaf VOC extract of each eggplant variety was coated on the leaves of different APs in such a way that the extract from 1 g eggplant leaf was used for every 1 g of filter paper leaf. DCM was allowed to evaporate for 10 min, and then females were released in the assay set-up. DCM-complemented plants were used as controls. Assays were conducted, and results were analyzed using the same procedure as assays using living plants, mentioned above.

Dual-choice assays to determine the effects of individual VOCs

To determine whether the candidate VOCs (those found either only in RL22 or significantly higher in RL22 than other varieties) repelled ESFB females, a series of dual-choice assays were conducted. These assays were conducted in three ways: 1) using APs, 2) using plants of all seven varieties, and 3) using APs coated with eggplant leaf blend of all seven varieties. In the plant-based assay, two individuals of a variety were taken; one was complemented with the candidate compound (100 µl g^{-1}; diluted in its solvent) and the other with the candidate compound's solvent (100 µl g^{-1}). Details of the preparation of solutions for these compounds are given in table 2.1. These control and test plants were

kept in a nylon mesh tent inside the climate chamber into which, one mated female was released and allowed to oviposit; these assays were conducted for six hours. Plants of six eggplant varieties or APs, coated with their leaf blend, were complemented to match the candidate compound's physiological concentration (nmol g^{-1}) in RL22 (mean nmol g^{-1}). After the assay, the eggs were counted. For each candidate compound, twenty such assays were conducted with each variety. Pure standards of these compounds used in complementation assays were obtained from Sigma-Aldrich, India. Compounds *o*-tolylcarbinol and *p*-tolylcarbinol could not be procured or detected in headspace analysis and therefore, were not used for assays.

No-choice assays to determine the repellant's effective concentration

After determining the repellent compound, to determine its effective concentration, serially increasing concentrations were coated on leaves of the highly preferred CVK variety and AP. By considering the physiological concentration of the repellent compound in RL22 (nmol g^{-1}) as 1×, CVK and APs were complemented to attain 0.5, 1.0, 1.5, 2.0, and 2.5× concentrations. These plants were presented to the mated females for oviposition and the number of eggs laid on each choice was enumerated.

Table. 2.1 List of solvents used to dissolve candidate compounds.

Compound name	**The solvent used for 10× stock preparation**	**The diluent used for 1× working stock preparation**	**Effective ethanol concentration (%) in 1× working stock**
1-Hexanol	**Water**	**Water**	**NA**
(*E*)-3-Hexen-1-ol	Ethanol (0.01%, aqueous)	Water	0.001
(*Z*)-3-Hexen-1-ol	Ethanol (0.01%, aqueous)	Water	0.001
(*Z*)-3-Nonen-1-ol	Ethanol (0.01%, aqueous)	Water	0.001
Guaiacol	Water	Water	NA
Eugenol	Ethanol (absolute)	Water	0.1
Geraniol	Water	Water	NA

Dual-choice assays using geraniol synthase (*Sm*GS)-silenced RL22 plants

Dual-choice assays were performed using mated females as described above. Females were provided a choice between 1) *Sm*GS-silenced and WT, 2) *Sm*GS-silenced and empty pTRV2 vector transformed (EV), and 3) *Sm*GS-silenced and *Sm*GS-silenced geraniol-complemented plants.

2.2.5. Characterization and quantification of leaf VOCs using gas chromatography (GC)

For GC-based leaf VOC analysis, fully expanded leaves were collected (n= 6 plants per variety or treatment) between 9.00 pm and 1.00 am, which is the peak oviposition time of ESFB females (Mannan *et al.,* 2015). VOCs were extracted from the leaves using our previously described solvent extraction method (Pandit *et al.,* 2009). Two eggplant leaves of each plant were collected in a glass vial containing 20 ml DCM spiked with 44 µg of nonyl acetate as internal standard (IS). The glass vial was rolled for six hours followed by the removal of the leaves from the solvent. Then the extract was concentrated into 1 ml. Lipids with high molecular weight were removed from the extracts by precipitation at -80 °C followed by centrifugation (12000x g, 20 min, 4 °C). The solution was further concentrated to 200 µl and it was stored in GC autosampler vials at -20 °C until injection into GC. Qualitative and quantitative analysis of the extracted volatiles was conducted using a 7890B GC and 7000D triple quadrupole mass spectrometer (MS) system coupled with a flame ionization detector (FID) (Agilent Technologies, India). GCMS system was used for the identification of hostplant VOCs by comparing their mass spectra with the ones in mass spectral databases (Integrated Wiley Registry 11th Edition- NIST 2017 Mass Spectral Library) using the NIST mass spectral search program (ver. 2.0).

Sample extract (2 µl) was injected in a spitless injection mode via multimode autosampler and compounds were separated on a DB5 column (30 m l× 0.32 mm ID× 0.25 µm film thickness) (Agilent J&W Scientific, India). Inlet temperature was constant at 250 °C and carrier helium gas flow was 2 ml min^{-1}. The GC oven program was as follows: the temperature was held for 1.5 min at 40 °C, increased to 180 °C at the rate of 2.5 °C min^{-1}, increased to 280 °C at the rate of 20 °C min^{-1} and held at 280 °C for 5 min. MS parameters were: electron impact (EI) ionization with 70 eV energy and scanning m/z 30- 600 at scan speed 7 cycle sec^{-1}. For GCFID, which was used for the quantification of volatiles, GC parameters were the same as above. The detector temperature was maintained at 250 °C. Relative quantification of all the VOCs was done by normalizing the peak area of the analytes with that of the internal standard nonyl acetate (Sigma-Aldrich, India). Kovats indices of all compounds were calculated and matched with reported values. Procured standards of seven candidate/assay compounds (used in dual-choice assays) were also used for compound identification and quantification. Standard curves of each of these seven compounds were prepared. For that, 0.02- 2 µg of the pure standard of each compound

was added into 20 ml DCM and it was concentrated up to 200 µl. 2 µl was injected into GCMS. For each concentration, three technical replicates were performed.

The RL22 headspace was analyzed using the solid-phase microextraction (SPME) technique, which used SPME fiber assembly divinylbenzene/ carboxen/ polydimethylsiloxane with needle size 24 ga (Sigma-Aldrich, India), to detect headspace volatiles. For the headspace sampling, potted RL22 juvenile plants with five fully expanded leaves were placed for one hour in glass jars (100 cm l× 50 cm ID) with a hole (1 cm diameter) at the center of the lid for inserting the SPME needle. After sampling, the SPME fiber was injected into the GCMS system for volatile detection.

To find whether assay compounds retain in the headspace of APs after six hours of assay duration, we performed an SPME-based headspace analysis of the assay container. For this, APs were coated with all seven assay compounds together at their physiological concentrations. The SPME fiber was inserted after six hours of assay start. The SPME-based collection of compounds was continued for two hours and SPME fiber was injected into the GCMS system for volatile detection.

2.2.6. Isolation of *geraniol synthase* gene (*SmGS*) from RL22

To identify the monoterpene synthase gene responsible for geraniol production in *S. melongena*, we first obtained known GS sequences of Solanaceae members from the NCBI database. *Petunia* x *hybrida* (MK159028.1), the only Solanaceae GS with complete CDS, was used as a query to search Eggplant Genome Project Database and Sol Genomics Network using Blastn. A complete CDS of SMEL_001g121430.1.01, which showed 74% identity with the *Petunia* x *hybrida GS* was used as a candidate (*SmGS*) for further reverse genetics characterization. Other eggplant monoterpene synthases *Sm*MTPS1 (Sme2.5_12717.1_g00002.1) and *Sm*MTPS2 (SMEL_001g121460.1.01), which were highly similar (92% and 76%, respectively) to the candidate geraniol synthase *Sm*GS were used to test whether the *SmGS* silencing was specific and did not cause any off-target co-silencing. Dendrogram analysis was performed comparing the three *Sm*MTPS amino acid sequences with previously characterized Solanaceae geraniol synthase (MK159028.1) and other Solanaceae monoterpene synthases following the neighbor-joining (Dayhoff matrix) method.

2.2.7. Expression and purification and in vitro characterization of *Sm*GS

Cloning, expression & purification of *Sm*GS

*Sm*GS ORF (1599 bp) encoding a predicted protein sequence of 532 amino acids (62.8 kDa) and a theoretical p*I* of 5.92 was amplified from RL22 leaf-cDNA. AccuPrime™ Taq DNA Polymerase, High Fidelity (ThermoFisher Scientific, India) was used for amplification (thermocycling conditions 95 ºC for two minutes, 39 cycles of 95 ºC for 30 seconds, 62 ºC for 35 seconds, 68 ºC for two minutes) of *SmGS* ORF and the amplicon was cloned into the pGEM-T (Promega, India) cloning vector using primers listed in table 2.2. The insert was transferred from pGEM-T to pET-28a (+) (Sigma-Aldrich, India) expression vector following NotI digestion and the sequence was confirmed. *E. coli* strain BL21-AI™ One Shot™ (ThermoFisher Scientific, India) transformed with pET-28a (+)/ *SmGS* were grown overnight in 5 ml terrific broth (TB) medium (kanamycin 30 mg l^{-1}) at 37 °C for primary culture. For the secondary culture, 2% inoculum was added to 500 ml TB (kanamycin 30 mg/ l) in 1 l flask till OD_{600}= 0.4 at 37 °C. The culture was induced with 1 mM isopropylthio-β-galactopyranoside (IPTG; HiMedia, India) and 0.2% L-arabinose (Sigma-Aldrich, India). The culture was kept at 18 °C overnight post-induction.

The cells were harvested by centrifugation at 3000 rpm for 30 minutes at 4 °C. The cell pellet from the 500 ml culture was resuspended in a 40 ml lysis buffer (50 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol). The cells were sonicated on ice with 60% amplitude with pulse 1 s on and 3 s off for 20 minutes (on cycle). The cell-free extract was obtained by centrifugation at 18000 rpm for 30 minutes at 4 °C. The Ni-NTA resin was obtained from TaKaRa, Japan. The beads were pre-equilibrated with lysis buffer. The supernatant was loaded on Ni-NTA resin and kept overnight for binding at 4 °C. The beads were washed twice with 30 ml lysis buffer supplemented with 10 mM and 25 mM imidazole. The proteins were eluted with 5ml fractions of lysis buffer with an increasing concentration of imidazole (50 mM, 100 mM, 250 mM, 500 mM). The eluted protein was further purified and transferred to 1x PBS (pH 7.4) with the help of a pre-equilibrated Pierce™ Protein Concentrator, 30 K MWCO column. The column was washed with 1x PBS (pH 7.4) five times followed by centrifugation at 4000 rpm for 15 minutes at 4 °C. The protein concentration was measured by Bradford reagent (HiMedia, India). Empty and no vector controls were also inoculated separately and similar steps of purification were performed.

*Sm*GS enzyme assay

The substrate geranyl pyrophosphate ammonium salt (GPP) was obtained from Sigma-Aldrich. The substrate was lyophilized and reconstituted in 10 mM ammonium bicarbonate (pH 8.0) to a final concentration of 1 mM and stored at -80 °C until further use. Enzyme assays were done in 250 µl 50 mM Tris -buffer (pH 8), 10% glycerol, 20 mM MgSO4, 1mM $MnSO_4$ containing 50 µM GPP and 50 µg of the purified enzyme incubated in a 2 ml GC autosampler vial at 28 °C for 1 hour. The mixture was overlaid with 160 µl of hexane after incubation. The tube was vortexed vigorously and kept on ice for 5 minutes to allow phase separation. No enzyme controls at each condition were used to account for the hydrolysis of GPP in presence of divalent cations (Vial *et al.*, 1981). The hexane layer was collected and 2 µl was injected into the GCMS. The GC oven program was as follows: the temperature was held for 3 min at 75 °C, increased to 135 °C at the rate of 5 °C min^{-1}, and increased to 270 °C at the rate of 60 °C min^{-1}. All other parameters were kept constant as described in section 2.2.8. For quantitative analysis, the standard curve of geraniol (31.25 nM- 2 µM) was prepared. The optimum conditions of pH (6.5- 9.0) and temperature (20 °C- 48 °C) for *Sm*GS activity were measured. The *Sm*GS activity was assessed with an increase in substrate concentration (10 µM to 150 µM) and enzyme amount (10 µg to 150 µg). For determination of the Michaelis-Menten constant the assay was carried out with 50 µg of the purified enzyme at optimum temperature (32 °C), and pH (pH 8.0) with increasing concentration of GPP from 50 µM to 175 µM. Lineweaver-Burk plot was made to obtain the K_m value.

Phosphatase Assay

The phosphatase activity of non-specific phosphatases in the purified *Sm*GS enzyme was measured as described by Hernández and Whitton,1996) with the modifications described by Ijima *et al.*, 2004. The yellow color developed by the conversion of p-nitrophenyl phosphate to p-nitrophenol was measured at 405 nm by a spectrophotometer. The substrate p-nitrophenyl phosphate and product p-nitrophenol was obtained from HiMedia. Crude cell-free extract from *E. coli* was used as a positive control for the assay. The buffer composition was the same as the *Sm*GS enzyme assay. The activity (µM min^{-1}) was calculated by using the standard curve of p-nitrophenol (1.6 mM- 50 µM).

2.2.8. Virus-induced gene silencing (VIGS) of *Sm*GS

To ascertain that RL22's geraniol was responsible for the repellence of ovipositing ESFB females, we conducted a reverse genetics-based functional characterization of the *SmGS* using VIGS. For this purpose, tobacco rattle virus (TRV) based vectors were used. The genome of TRV, a bipartite virus contains two positive single-stranded RNAs- RNA1 and RNA2. RNA1 and RNA2-based vectors are termed pTRV1 and pTRV2, respectively. The gene of interest was cloned into pTRV2.

Gene silencing strategy should be designed in such a way that siRNA must not co-silence the non-target genes. For this, no ≥20 b stretch of the candidate mRNA section selected for siRNA generation should be 100 % similar to the homologous regions of the non-target mRNA (Kumar *et al.*, 2012). The *Sm*GS ORF section not showing such ≥20 b identity with the *SmMTPS1* and *SmMTPS2* transcripts could not be found. Therefore, along with a 91 b of the 3'-section of *SmGS* ORF, we used a (153 bp) section of *SmGS* 3'-UTR. This 244-bp *SmGS* fragment was amplified from the leaf cDNA (primers given in table 2.2). It was cloned in the pTRV2 vector (Liu *et al.*, 2002; Liu *et al.*, 2012) in an antisense orientation to generate the pTRV2-*SmGS* construct. pTRV1, pTRV2, and pTRV2-*SmGS* constructs transformed in *Agrobacterium tumefaciens* strain GV3101 were independently infiltrated into eggplant leaves, as described by Saedlar and Baldwin (2004). After four weeks of infiltration, plants were used for choice assays, and leaves were collected for analyzing the target *SmGS* transcript abundance and geraniol concentrations. In all the VIGS experiments, empty pTRV2 vector (EV) transformed plants and wild-type RL22 (WT) plants were used as negative controls (Kumar *et al.*, 2012).

2.2.9. RNA isolation and quantitative real-time PCR (qPCR)

VIGS-mediated silencing of the *SmGS* gene was confirmed using qPCR. Leaves harvested from pTRV2-*SmGS*, EV, and WT plants were flash-frozen in liquid nitrogen and were stored at -80 °C until further use. RNA was isolated from 500 mg pulverized tissue using RNAiso Plus reagent (Takara, Japan), as recommended by the manufacturer. cDNA was synthesized using the PrimeScript Reverse Transcriptase kit (Takara, Japan) as per the manufacturer's instructions. To determine *Sm*GS silencing efficiency, qPCRs were performed using the SYBR Premix Ex Taq II reagent kit (Takara, Japan) and CFX96 Touch Real-Time PCR Detection System (Biorad, USA). Thermocycling conditions were: 95ºC for one minute, 39 cycles of 95 ºC for 45 seconds, 60 ºC for 45 seconds, and 72 ºC

for 45 seconds. The presence and abundance of the virus were analyzed with the help of qPCR of viral coat protein transcripts. To determine whether the closely related gene was co-silenced, transcripts of *SmMTPS1* and *SmMTPS2* were analyzed. For calculating the relative abundance of these transcripts, a housekeeping gene coding for a peptidyl-prolyl isomerase was used as an internal reference (Kanakachari *et al.*, 2016). Details of all the primers used in these analyses are given in table 2.2. All results were obtained from six biological and two technical replicates.

Table. 2.2 Details of primers used for the amplification of VIGS cloning fragment, qPCRs, and open reading frame.

Gene name	**Primer orientation**	**Primer sequence**	**Used for amplification of**
SmGS	Forward	AAATCCCCACATAATAAGTCG	*SmGS* VIGS-fragment
	Reverse	TATTGGATGAAAAGTATATCTACATCG	
SmGS	Forward	TAGGGATGATGGTGACCTC	qPCR
	Reverse	GTAGGCTCGTAATTCCCTG	
pTRV coat protein coding gene	Forward	ACTCACGGGCTAACAGTGCT	qPCR
	Reverse	TCTTCCAAAGTCGAGCCAGT	
SmMTPS1	Forward	CGACTTCAAGGATGAGAAGGGTA	qPCR
	Reverse	GAGGTTTCAGAGTCCGTTGACAG	
SmMTPS2	Forward	TGTTCAAGCAGCACACCAACA	qPCR
	Reverse	CAGCCCAAAACAAATTCTCCACC	
Cyclophilin A	Forward	CAAAACCGCTGAGAACTTCCG	qPCR
	Reverse	CTTGACACATGAACCCTGGGA	

Gene name	Primer orientation	Primer sequence	Used for amplification of
SmGS	Forward	ATGGAGCGGTTTGACGAATTG	*SmGS* ORF
	Reverse	TCATCTTTCTCTCATGCATGC	

2.2.10. Statistical analyses

In the field experiments involving the randomized complete block design, data from each block were analyzed cumulatively as well as separately. The homogeneity of quantitative data (mean± SE) was tested using Levene´s test. Homogenous data were analyzed by one-way ANOVA and the statistical significance was determined by Tukey's *post hoc* test ($P \leq 0.05$). Normal non-homogenous data were analyzed using Welch ANOVA and the Games-Howell *post hoc* test ($P \leq 0.05$). Non-parametric data were analyzed using the Kruskal-Wallis test and Dunn's *post hoc* test with Bonferroni correction ($P \leq 0.05$). Data from dual-choice assays were analyzed using Student's t-test (2-tailed, $P \leq 0.05$). For this, quantities of compounds not detected in one or more varieties (mentioned as ND in table 2.4) were considered to be zero.

We used correlation analysis to understand whether a female's preference for eggplant variety for oviposition influences the larval occurrence in shoots and fruits of different varieties. Directions and strengths of correlations between the natural abundance profiles of larvae in shoots and fruits, eggs, and host preferences of ovipositing females in multiple-choice assays were calculated using Spearman's Rho (r_s) tests. The significance of obtained correlations was determined using a 2-tailed test ($P \leq 0.05$).

Non-metric multidimensional scaling (NMDS) following the Bray-Curtis dissimilarity matrix and analysis of similarities (ANOSIM) was performed (Rosenstiel, 2012) to discriminate eggplant types based on their VOC composition using Past 3.26 (Hammer, 1999).

2.3. Results

2.3.1. Himalayan eggplant variety RL22 shows very low ESFB oviposition and infestation in the field

To assess whether ESFB larvae show differential occurrence on different varieties, fruits, and shoots of all plants in our field were surveyed. JK (80.07± 4.33%), CVK (76.85± 5.32%), and HK (68.98± 5.03%) showed the highest fruit infestation, followed by KP (35.66± 4.75%), VJ (31.56± 5.15%) and KV (26.12± 5.39%) (Fig. 2.1d). RL22 showed no ESFB infestation in the 472 examined fruits (Fig. 2.1d). Shoot boring (Fig. 2.1e) was highest in CVK (24.71± 4.23%) and JK (26.27± 3.55%), followed by KP (11.41± 2.48%), VJ (9.94± 1.94%), KV (8.53± 2.36%) and HK (5.96± 1.55%). No shoot infestation was found in the inspected shoots of RL22 (Fig. 2.1e).

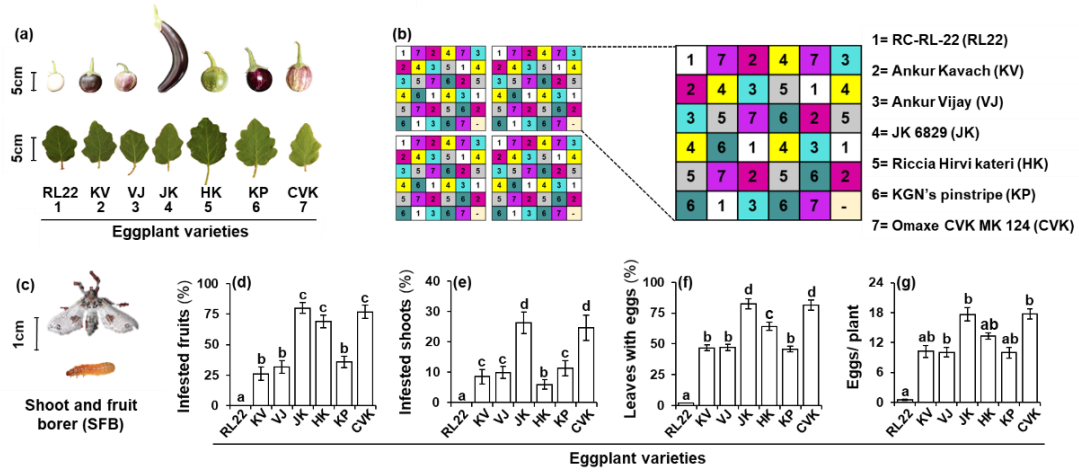


Fig. 2.1 ESFB does not prefer the Himalayan eggplant variety RL22. (a) Fruits and leaves of seven eggplants (*Solanum melongena*) varieties, eastern Himalayan eggplant variety RL22, and six others (KV, VJ, JK, HK, KP, and CVK) were used in this study. (b) Schematic of the experimental field with the randomized complete block design. (c) Moth and larva of ESFB (*Leucinodes orbonalis*). ESFB infestation (%) in (d) fruits (F= 133.7, df= 50.67, P< 0.0001) and (e) shoots (F= 25.65, df= 50.67, P< 0.0001) of seven varieties, indicating that RL22 is the least preferred. (f) Oviposition preference based on leaves (%) harboring eggs (F= 280.5, df= 54.89, P< 0.0001) and (g) number of eggs laid per plant (F= 134.6, df= 53.49, P< 0.0001) on different varieties. Significant differences (P≤ 0.05) were determined using the Welch F test with the Games-Howell *post hoc* test.

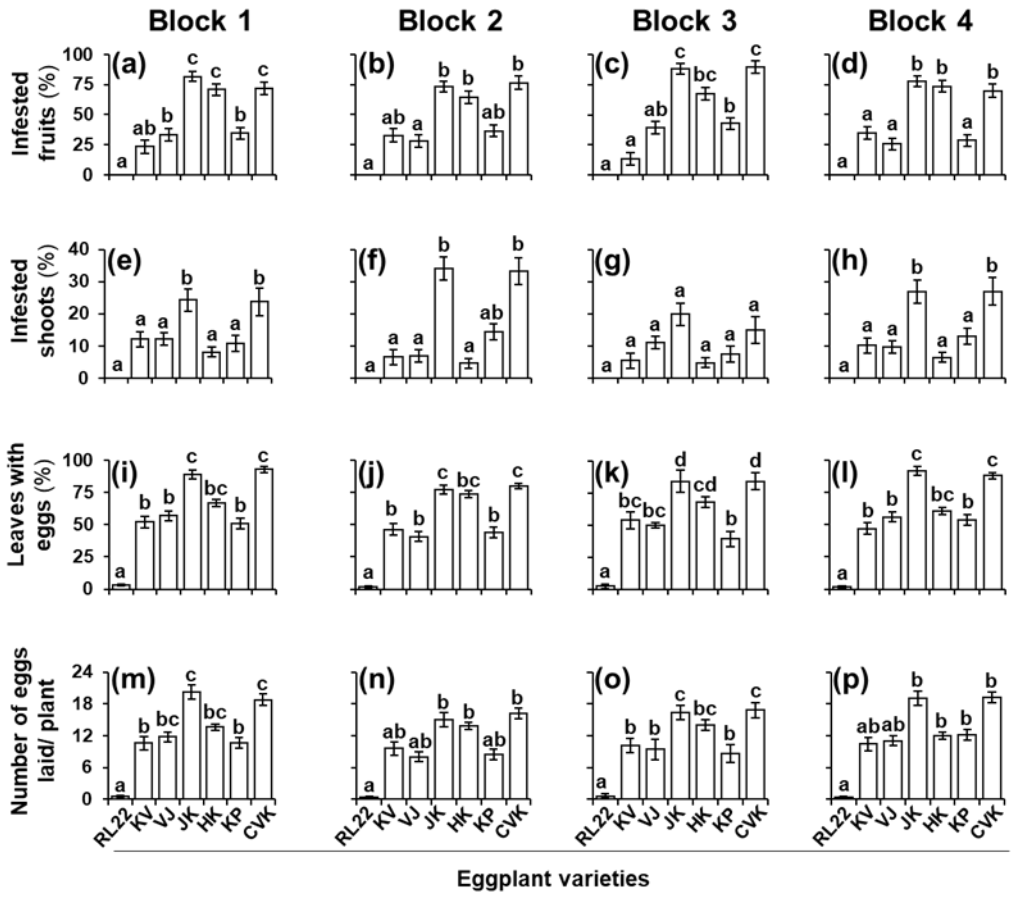


Fig. 2.2 ESFB host preference in different blocks of the randomized complete block design. ESFB infestation (%) in fruits of seven eggplant varieties in (a) Block 1 (F= 151.4, df= 10.67, P< 0.0001), (b) Block 2 (F= 17.74, df= 10.67, P< 0.0001), (c) Block 3 (F= 51.88, df= 10.67, P< 0.0001) and (d) Block 4 (F= 13.65, df= 10.67, p= 0.0002) and in shoots of seven varieties in (e) Block 1 (F= 6.254, df= 10.67, p= 0.005), (f) Block 2 (F= 5.234, df= 10.67, P= 0.01), (g) Block 3 (F= 4.456, df= 10.67, p= 0.017) and (h) Block 4 (F= 5.164, df= 10.67, P= 0.01). Oviposition preference based on leaves (%) of different varieties harboring eggs in (i) Block 1 (F= 76.04, df= 11.94, P< 0.0001), (j) Block 2 (F= 48.19, df= 11.74, P< 0.0001), (k) Block 3 (F= 66.77, df= 11.96, P< 0.0001) and (l) Block 4 (F= 166.7, df= 11.88, P< 0.0001) and eggs (mean± SE) laid per plant on different varieties in (m) Block 1 (F= 39.85, df= 11.47, P< 0.0001), (n) Block 2 (F= 17.36, df= 11.25, P< 0.0001), (o) Block 3 (F= 48.76, df= 11.75, P< 0.0001) and (p) Block 4 (F= 20.28, df= 11.16, P< 0.0001). Significant differences ($P \leq 0.05$) were determined using the Welch F test with the Games-Howell *post hoc* test.

We also tried to understand 1) whether the ovipositing ESFB females' resolution of the field is robust even when it contains mixed cultivation of multiple eggplant varieties, 2) whether they can differentiate between different eggplant varieties and 3) whether they can identify RL22, which was found to be the least preferred variety in the preliminary observations, even in a mixture of eggplant varieties. For this, we analyzed the oviposition in the randomized plantation. When we tried to analyze the females' host preferences based on the percentage of leaves of each variety they selected for oviposition, a similar trend was observed; CVK (81.47± 4.07%) and JK (82.62± 4.13%) were found to be the most preferred eggplants for oviposition, followed by HK (64.25± 2.78%), VJ (47.13± 2.35%), KV (46.75± 2.33%), and KP (45.8± 2.29%) (Fig. 2.1f). Congruent with the preliminary observation from its native habitat, the preference for RL22 was found to be the least (2.28± 0.11%) preferred host. Most numbers of eggs (per plant) were found on CVK (17.75± 1.04) and JK (17.65± 1.33) followed by HK (13.35± 0.65), KV (10.2± 1.22), VJ (10.05± 0.94), and KP (9.95± 1.02) (Fig. 2.1g). Very little (0.5± 0.18) oviposition was observed on RL22 (Fig. 2.1g). The same ESFB preference trends were observed when these data from different blocks were separately analyzed (Fig. 2.2a-p).

Since the occurrence of eggs showed a similar trend to that of larval occurrence, we attempted to find whether the larval occurrence was correlated to that of eggs. Shoot and fruit infestation of ESFB larvae on seven eggplant varieties, which showed a strong positive correlation with each other (rs= 0.79, P= 0.04) also showed strong positive correlations with the oviposition profile (rs= 0.86, P= 0.01, and rs= 0.64, P= 0.1, respectively). Such strong positive correlations indicated that the differential larval occurrence across eggplant varieties could be attributed to the differential oviposition.

2.3.2. Even in the controlled environment, ESFB females do not oviposit on RL22 or APs complemented with its VOC blend

To ascertain that the field-observed preference of ESFB was not influenced by the field's environmental factors, we conducted multiple host choice assays with the ovipositing females in a controlled environment. Negative control APs (Fig. 2.3a) were used in the assays. Similar to the field results, when mated females were presented with a choice of the seven eggplant varieties in a controlled environment (Fig. 2.3b), the highest oviposition was observed on CVK (21.35± 2.73) and JK (18.8± 2.12), followed by VJ (7.75± 1.96), KP (7.1± 2.33), KV (6.95± 1.22) and HK (6.84± 1.75) (Fig. 2.3c). Females laid a few eggs on AP (0.65± 0.27) but laid no eggs on RL22 (Fig. 2.3c).

We wanted to determine if RL22's repellency could be contributed to its VOCs or other characteristics such as morphological features. For this VOC blend extracts of different varieties were separately coated on APs. Females did not differentiate between untreated and DCM-complemented APs (Fig. 2.3d). APs were presented simultaneously to mated females (Fig. 2.3e). The highest number of eggs was found on plants complemented with the blends of CVK (19.25± 2.36) and JK (17.85± 2.44), followed by VJ (8.4± 1.46), KV (7.1± 1.59), KP (6.8± 1.86), HK (6.45± 1.51) (Fig. 2.3f). RL 22 (0.05± 0.05) and DCM-complemented artificial plant controls (0.6± 0.21) showed extremely low oviposition (Fig. 2.3f).

That the ovipositing females' host choices in the climate chamber were similar to that in the field was ascertained by the strong positive correlation between them (rs= 0.75, P< 0.06; Fig. 2.3g). Correlation analysis also ascertained that the oviposition on VOC extract complemented APs was highly similar to that on actual plants used in the multiple-choice assays (rs= 1.0, P< 0.0004; Fig. 2.3g) and also to that observed on the plants in the field (rs= 0.75, P< 0.06; Fig. 2.3g). The similarity in oviposition preference in all these assays suggested that ovipositing ESFB female's host preference is indeed influenced by leaf VOCs; more importantly, the preference-influencing VOC was a constituent of the extract.

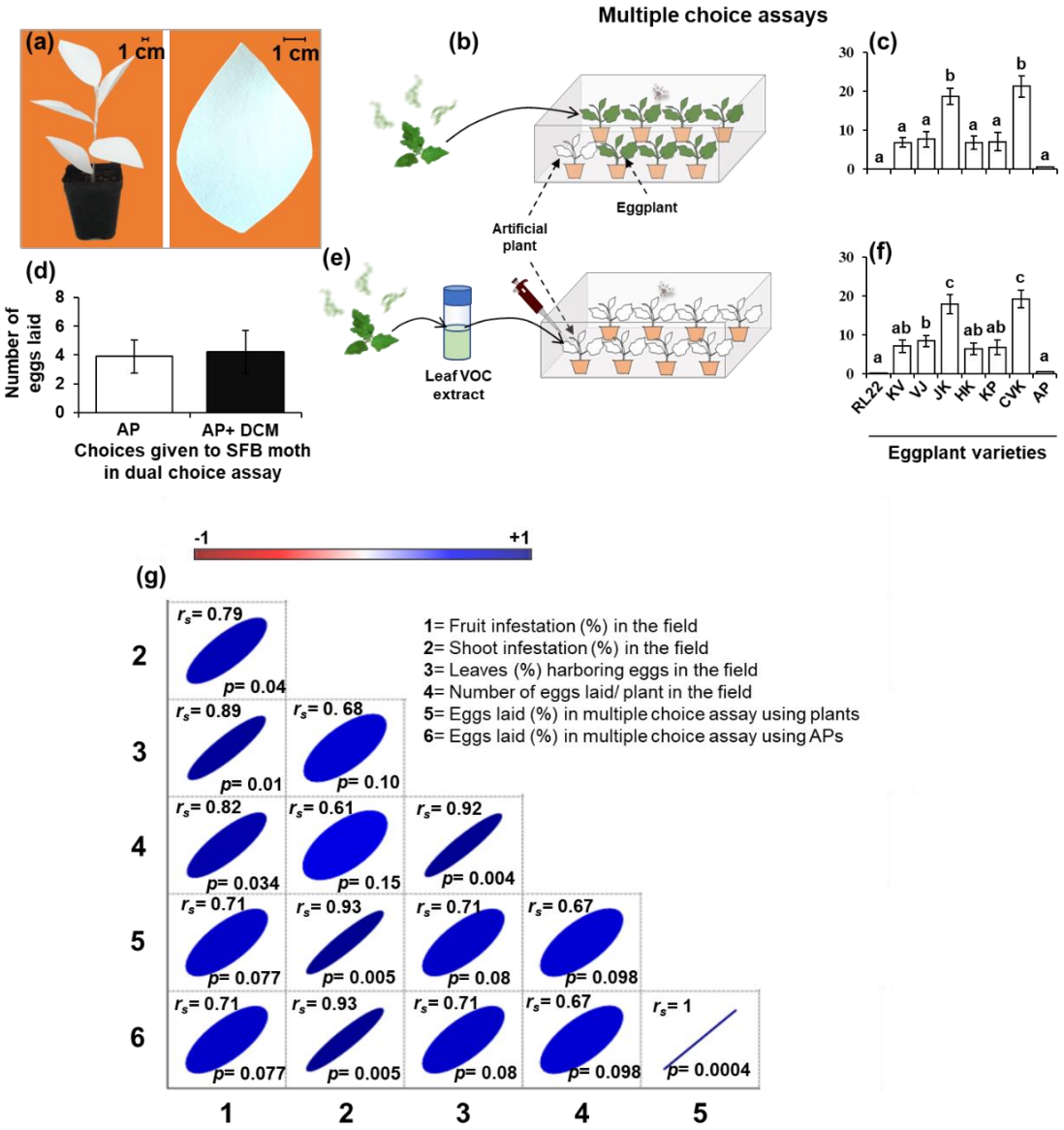


Fig. 2.3 Ovipositing ESFB's host preference observed in the field remains unchanged in the controlled environment. (a) Artificial plants (APs) made up of filter paper (grade 237 equivalent to grade 3 CHR; Sigma-Aldrich, India) were used in this study. (b) A schematic of multiple-choice assays was conducted to analyze ovipositing ESFB (*Leucinodes orbonalis*) females' host preference, using eggplants and a control artificial plant. (c) Eggs (mean± SE) laid on different plants used in the assay (F= 29.16, df= 57, P< 0.0001). (d) Eggs (mean± SE) laid on AP and AP+ DCM in a dual-choice assay, showing that the solvent (DCM) does not affect ESFB's preference. (e) A schematic of multiple-choice assays conducted using APs complemented with the VOC extracts of different eggplant (*Solanum melongena*) varieties. (f) Eggs (mean± SE) laid on different VOC extract-complemented APs used in the assay (X^2= 88.09, P< 0.0001). Significant differences ($P \leq 0.05$) in (c) were determined using the Welch F test with the Games-Howell *post hoc* test and in (f) Kruskal-Wallis followed by Dunn's *post hoc* test with Bonferroni correction. (g) Correlogram showing Spearman's (r_s) correlations between larval occurrence and oviposition profiles in the field and oviposition profiles in the multiple-choice assays conducted using plants and VOC extract-complemented APs.

2.3.3. RL22 leaf VOC blend is different from that of other eggplant varieties.

To understand the role of leaf volatile cues in ESFB female host selection, a detailed GCMS/ FID-based volatile analysis was conducted. A total of 21 compounds were detected (Fig. 2.4a). Compound identification was done based on their Kovats indices (Table. 2.3). The quantification of all compounds based on the internal standard is given in table 2.4.

Total eggplant volatile content varied from 68 nmol g^{-1} in KP to 146 nmol g^{-1} in RL22. Eggplant VOC blends were found to be mainly comprised of benzene derivatives and C6 and C9 alcohols and aldehydes. Qualitatively, VOC blends of all the eggplant varieties except RL22 were quite similar. They all contained 14 compounds, which showed quantitative differences between different varieties. Henceforth, we refer to this blend as a 'common eggplant blend' (CEB). In addition to CEB, the RL22 blend contained six compounds *viz*. 1-hexanol, guaiacol, *o*-tolylcarbinol, *p*-tolylcarbinol, (*Z*)-3-nonen-1-ol and geraniol, which were not detected in the other varieties; RL22 was also found to contain (*Z*)-3-hexen-1-ol, (*E*)-3-hexen-1-ol and eugenol in >4.0-, >1.5- and >1.5-fold higher concentrations than the other varieties, respectively (Fig. 2.4b-h, Table. 2.4). Hereafter, we refer to these nine VOCs as 'RL22 compounds'. For accurate quantification of RL22 compounds, the standard curve method was used. The comparison between the quantification done using the internal standard and standard curve of these compounds is given in table 2.5.

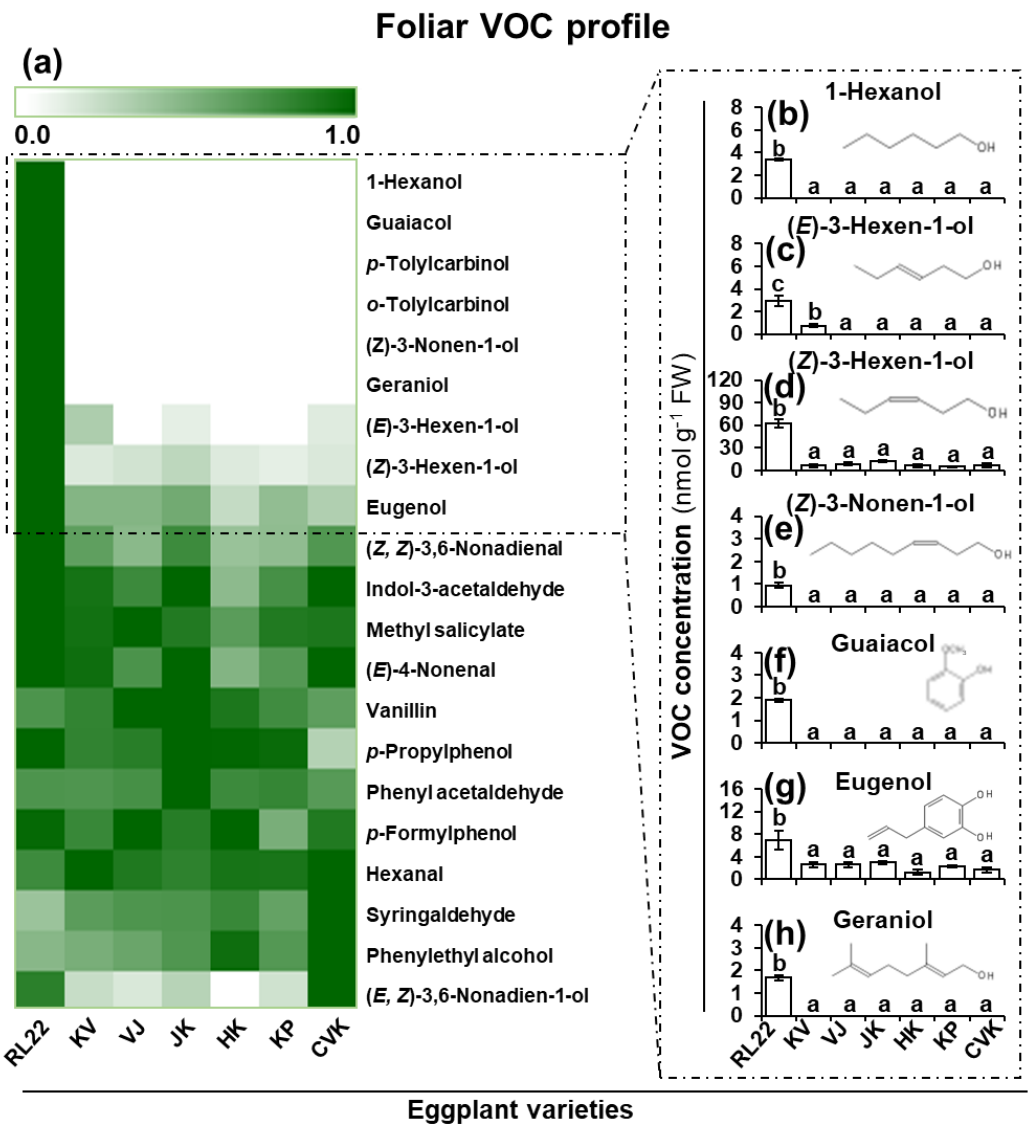
To understand how the seven eggplant varieties relate to each other based on their VOC blend compositions, we performed an NMDS. This multivariate analysis clearly showed that RL22 was different from the other six eggplant varieties (Fig. 2.4i). The first two PCs accounted for >98% of the total variation. (*Z*)-3-hexen-1-ol, which was >3-fold higher in RL22 than the other eggplant varieties, contributed the most in the grouping along the first PC (Fig. 2.4i). phenylacetaldehyde, which was found in high concentrations in all seven varieties, contributed the most to the grouping along the second PC (Fig. 2.4i).

The total volatile content of RL22 was found to be significantly higher than that of all other eggplant varieties (Fig. 2.5a). The CEB was dominated by benzene derivative compounds, which constituted >60% of the total volatile content (Fig. 2.5b). RL22 blend contained 39.5% of benzene derivative compounds (58.16 nmol g^{-1}). Major compounds were phenylacetaldehyde, methyl salicylate, eugenol, and vanillin, of which phenyl

acetaldehyde dominated the CEB (highest in JK= 43.47± 6.09 nmol g^{-1} and lowest in CVK= 21.71± 1.27 nmol g^{-1}); it's alcohol derivative phenyl-ethyl alcohol was relatively less abundant (Table. 2.4). Eugenol concentration was significantly higher in RL22 (6.97± 1.25 nmol g^{-1}) than in other eggplant varieties (Table. 2.4). Guaiacol was uniquely present in RL22 (1.89± 0.26) (Table. 2.4).

The eggplant C6 compound blend consisted of aldehyde hexanal and alcohols *viz*. (*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, and 1-hexanol (Fig. 2.5c). The total C6 compound content of eggplants showed a large variation ranging from 14 nmol g^{-1} in KP and 76 nmol g^{-1} in RL22 (Fig. 2.5c). C6 compounds represented 34% of the RL22's VOC blend. RL22 showed the highest abundance of (*Z*)-3-hexen-1-ol (61.82± 6.11 nmol g^{-1}) (Fig. 2.4d, Table. 2.4). C9 alcohols and aldehydes detected in the analysis were (*E*)-4-nonenal, (*Z*, *Z*)-3,6-nonadienal, (*Z*)-3-nonen-1-ol and (*E*, *Z*)-3,6-nonadien-1-ol. RL22 showed the highest C9 compound content (11.39 nmol g^{-1}) among all the varieties (Fig.2.5d, Table. 2.4). Geraniol, a monoterpene alcohol, the only terpenoid detected in the eggplant blend, was exclusively found in RL22 (1.68± 0.23 nmol g^{-1}) (Fig. 2.5h, Table. 2.4).

Further, from RL22 headspace analysis, a total of 10 volatiles were detected out of 21 compounds detected in RL22 leaf extracts. Three compounds (methyl salicylate, phenylacetaldehyde, and phenylethyl alcohol) from CEB and seven (1-hexanol, guaiacol, (*Z*)-3-nonen-1-ol, geraniol, (*Z*)-3-hexen-1-ol, (*E*)-3-hexen-1-ol and eugenol) RL22 compounds were detected in the headspace (Fig. 2.6, Table. 2.6).


Foliar VOC profile
(a)
0.0
1.0
1-Hexanol
Guaiacol
p-Tolylcarbinol
o-Tolylcarbinol
(Z)-3-Nonen-1-ol
Geraniol
(E)-3-Hexen-1-ol
(Z)-3-Hexen-1-ol
Eugenol
(Z, Z)-3,6-Nonadienal
Indol-3-acetaldehyde
Methyl salicylate
(E)-4-Nonenal
Vanillin
p-Propylphenol
Phenyl acetaldehyde
p-Formylphenol
Hexanal
Syringaldehyde
Phenylethyl alcohol
(E, Z)-3,6-Nonadien-1-ol
RL22
KV
VJ
JK
HK
KP
CVK
VOC concentration (nmol g-1 FW)
(b) 1-Hexanol
(c) (E)-3-Hexen-1-ol
(d) (Z)-3-Hexen-1-ol
(e) (Z)-3-Nonen-1-ol
(f) Guaiacol
(g) Eugenol
(h) Geraniol
Eggplant varieties

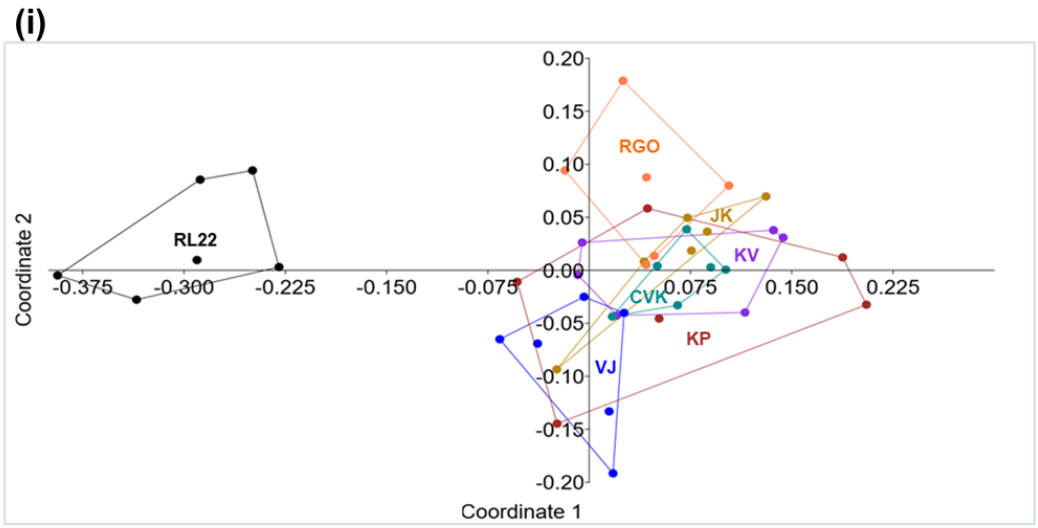

(i)
Coordinate 2
Coordinate 1
RL22
RGO
JK
KV
CVK
KP
VJ

Fig. 2.4 RL22's leaf VOC profile is different from the other eggplant varieties. (a) Heatmap showing a comparison between VOC profiles of seven eggplant (*Solanum melongena*) varieties. VOCs, which were found either only in or significantly higher in RL22 blend than other varieties: (b) 1-hexanol (F= 58.22, df= 15.48, P< 0.0001), (c) (*E*)-3-hexen-1-ol (F= 16.06, df= 15.38, P< 0.0001), (d) (*Z*)-3-hexen-1-ol (F= 13.64, df= 14.05, P< 0.0001), (e) (*Z*)-3-nonen-1-ol (F= 4.225, df= 15.48, P= 0.01), (f) guaiacol (F= 7.495, df= 15.48, p= 0.0007), (g) eugenol (F= 5.107, df= 15.31, P= 0.005), and (h) geraniol (F= 7.5285, df= 15.48, P= 0.0007). Significant differences ($P \leq 0.05$) were determined using the Welch F test with the Games-Howell *post hoc* test. (i) Non-metric multidimensional scaling (NMDS) showing the hostplant grouping based on their VOC compositions; RL22 separated from six eggplant varieties (one-way ANOSIM; p= 0.0001).

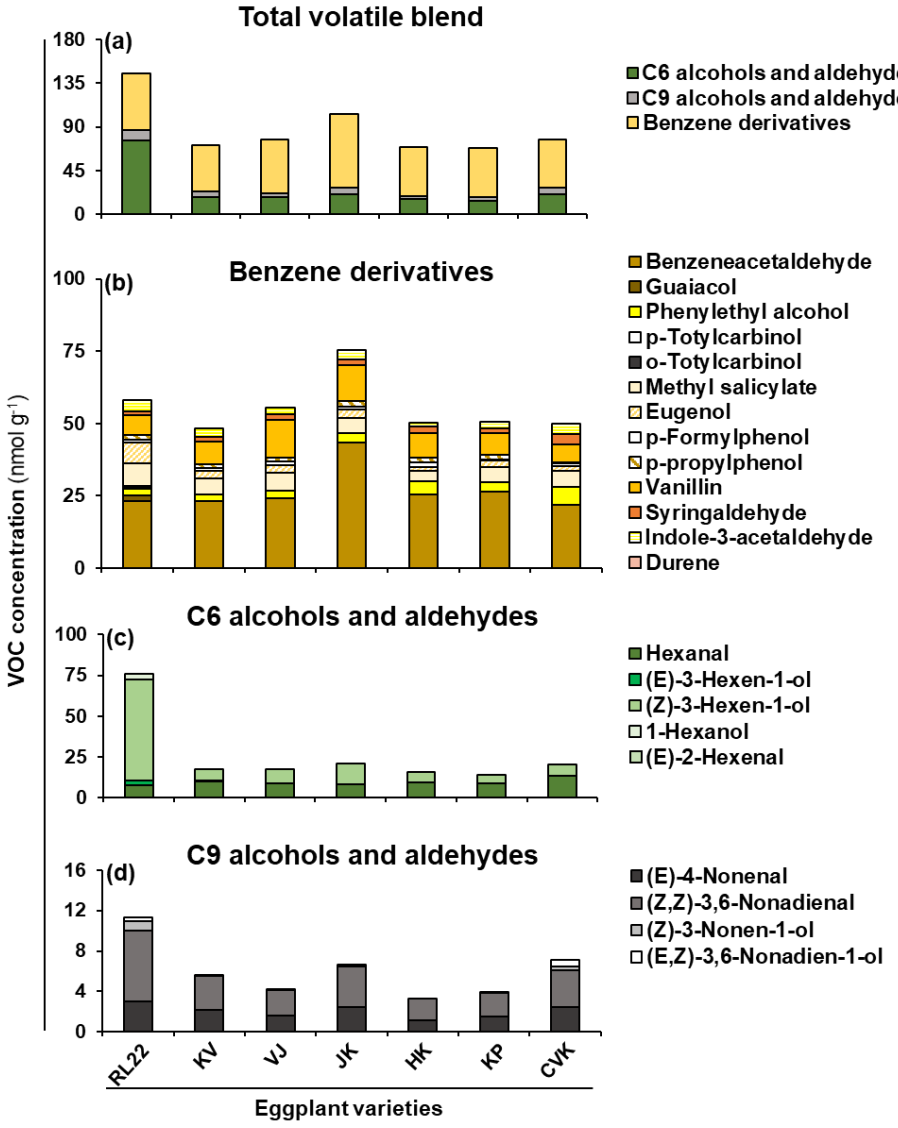


Fig. 2.5 Eggplant leaf VOC blend is mainly comprised of three groups of compounds. Proportions of (a) VOCs belonging to various chemical classes, (b) benzene derivatives (c) C6 alcohols and aldehydes, and (d) C9 alcohols and aldehydes in the blends of seven eggplant varieties.

Table. 2.3 Compounds with their reported and calculated Kovats indices.

Sr. No.	Compound name	Kovats index reported for semistandard nonpolar columns	Kovats index calculated
1	1-Hexanol*	868	874.504
2	Guaiacol*	1090	1093.683
3	*p*-Tolylcarbinol	1135	1142.328
4	*o*-Tolylcarbinol	not reported	1151.521
5	(*Z*)-3-Nonen-1-ol*	1156	1161.655
6	Geraniol*	1255	1263
7	(*E*)-3-Hexen-1-ol*	852	853.9914
8	(*Z*)-3-Hexen-1-ol*	857	855.2018
9	Eugenol*	1357	1364.516
10	(*Z*,*Z*)-3,6-Nonadienal	1104	1108.499
11	Indol-3-acetaldehyde	1713	1711.756
12	Methyl salicylate	1192	1197.134
13	(*E*)-4-Nonenal	1105	1105
14	Vanillin	1404	1406.013
15	*p*-Propylphenol	1260	1384.614
16	Phenyl acetaldehyde	1045	1050.083
17	*p*-Formylphenol	1364	1369
18	Hexanal	780	785
19	Syringaldehyde	1663	1670.619
20	Phenylethyl alcohol	1116	1120.233
21	(*E*,*Z*)-3,6-Nonadien-1-ol	1156	1165.406

* Retention times of these compounds were verified using pure standards.

Table 2.4 VOC composition of seven eggplant varieties. Volatile compounds and their concentrations (nmol g^{-1}; mean± SE) in the leaves of seven eggplant varieties. For homogenous datasets were analysed by one-way ANOVA followed by Tukey's *post hoc* test. Non-homogenous normal datasets were analysed by Welch F test followed by Dunn's *post hoc* test with Bonferroni correction.

Compound class	Compound name	Concentration (nmol g^{-1}) in hostplant leaves							One-way ANOVA; Tukey's *post hoc* test		Welch's F test; Dunn's *post hoc* test with Bonferroni correction		
		RL22	KV	VJ	JK	HK	KP	CVK	$F_{6, 35}$	*P*	*F*	*df*	*P*
C6 alcohols and aldehydes	Hexanal	7.68^{a}± 1.14	10.09^{ab}± 2.19	8.86^{ab}± 1.93	8.20^{a}± 1.03	9.20^{ab}± 0.87	9.11^{ab}± 1.17	13.20^{b}± 1.85	1.203	0.328	-	-	-
	(*E*)-3-Hexen-1-ol	2.94^{c}± 0.34	0.72^{b}± 0.11	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	-	-	16.06	15.38	<0.0001
	(*Z*)-3-Hexen-1-ol	61.82^{c}± 6.11	6.69^{ab}± 1.47	8.64^{ab}± 1.79	12.26^{b}± 2.66	6.38^{ab}± 0.99	4.84^{a}± 0.31	6.96^{ab}± 2.37	-	-	13.64	14.05	<0.0001
	1-Hexanol	3.38^{b}± 0.16	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	-	-	58.22	15.48	<0.0001
C9 alcohols and aldehydes	(*E*)-4-Nonenal	3.04^{ab}± 1.18	2.21^{ab}± 0.49	1.64^{ab}± 0.31	2.44^{b}± 0.29	1.05^{a}± 0.18	1.56^{ab}± 0.28	2.24^{ab}± 0.29	-	-	3.55	15.3	0.02
	(*Z,Z*)-3,6-Nonadienal	6.92± 3.16	3.33± 0.83	2.45± 0.67	4.03± 0.49	2.05± 0.34	2.31± 0.48	3.56± 0.77	-	-	2.138	15.27	0.10
	(*Z*)-3-Nonen-1-ol	0.94^{b}± 0.17	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	-	-	4.23	15.48	0.01
	(*E,Z*)-3,6-Nonadien-1-ol	0.40^{c}± 0.06	0.11^{b}± 0.01	0.07^{ab}± 0.05	0.14^{b}± 0.02	ND^{a}	0.09^{b}± 0.01	0.57^{bc}± 0.39	-	-	45.75	13.33	<0.0001
Benzene derivatives	Phenylacetaldehyde	23.31^{a}± 2.55	23.05^{a}± 3.25	24.20^{a}± 5.60	43.47^{b}± 6.09	24.06^{a}± 2.83	26.43^{a}± 2.38	21.71^{a}± 1.27	3.984	0.0038	-	-	-
	Phenylethyl alcohol	2.18^{a}± 0.15	2.46^{a}± 0.32	2.72^{a}± 0.96	3.20^{b}± 0.42	4.19^{b}± 0.80	3.13^{b}± 0.29	$7.11a^{b}$± 2.52	-	-	2.68	14.81	0.06
	p-Totylcarbinol	0.48^{b}± 0.07	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	-	-	6.13	15.48	0.002
	Unknown 1	0.60^{b}± 0.13	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	-	-	3.09	15.48	0.03
	p-Formylphenol	1.06^{ab}± 0.10	0.85^{ab}± 0.14	1.37^{ab}± 0.26	0.93^{ab}± 0.18	1.40^{b}± 0.31	0.57^{a}± 0.06	0.95^{ab}± 0.15	2.44	0.05	-	-	-
	Methyl salicylate	7.84± 1.94	5.58± 0.99	6.08± 0.87	5.20± 0.67	3.94± 0.93	5.22± 0.69	4.97± 1.32	1.139	0.3607	-	-	-
	Vanillin	6.94^{b}± 1.41	7.94^{ab}± 1.85	12.92^{b}± 2.05	12.45^{b}± 2.28	8.43^{ab}± 1.26	7.41^{a}± 1.13	6.08^{a}± 0.49	-	-	10.69	14.72	0.0001
	Guaiacol	1.89^{b}± 0.26	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	-	-	7.5	15.48	0.0007
	Eugenol	6.97^{c}± 1.25	2.58^{ab}± 0.49	2.58^{ab}± 0.55	2.96^{b}± 0.28	1.16^{a}± 0.30	2.28^{b}± 0.22	1.59^{a}± 0.42	-	-	5.12	15.31	0.005
	Syringaldehyde	1.05± 0.06	1.72± 0.13	1.86± 0.36	1.88± 0.13	2.12± 0.16	1.62± 0.23	3.28± 1.47	1.343	0.2647	-	-	-
	Unknown 2	1.76^{b}± 0.20	1.32^{ab}± 0.22	1.37^{b}± 0.18	2.13^{b}± 0.33	1.82^{b}± 0.57	1.57^{b}± 0.28	0.40^{a}± 0.25	3.075	0.01595	-	-	-
	Indole-3-acetaldehyde	4.08^{ab}± 1.09	2.86^{ab}± 0.42	2.39^{ab}± 0.76	3.33^{ab}± 0.52	1.45^{a}± 0.55	2.26^{a}± 0.19	3.29^{b}± 1.02	1.451	0.2237	-	-	-
Monoterpenoid	Geraniol	1.68^{b}± 0.23	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	-	-	7.53	15.48	0.0007

Table. 2.5 Relative (compared to IS based on GCFID) and absolute quantification of RL22 compounds.

Compound name	Compound concentrations used to prepare standard curve (nmol 20 ml^{-1} DCM extract)		Concentration (in nmol g^{-1} FW) based on standard curve	Concentration (in nmol g^{-1} FW) relative to IS
	Lowest	Highest		
1-Hexanol	0.01	19.58	2.42	3.38
Guaiacol	0.01	16.11	1.23	1.89
(*Z*)-3-Nonen-1-ol	0.01	14.06	1.10	0.94
Geraniol	0.01	12.97	1.99	1.68
(*E*)-3-Hexen-1-ol	0.01	19.97	2.54	2.94
(*Z*)-3-Hexen-1-ol	0.01	19.97	67.49	61.82
Eugenol	0.01	12.18	5.51	6.97

Table. 2.6 List of compounds detected in RL22-headspace.

Compound name	SPME detection
1-Hexanol*	Yes
Guaiacol*	Yes
p-Tolylcarbinol	No
o-Tolylcarbinol	No
(*Z*)-3-Nonen-1-ol*	Yes
Geraniol	Yes
(*E*)-3-Hexen-1-ol*	Yes
(*Z*)-3-Hexen-1-ol*	Yes
Eugenol*	Yes
(*Z*,*Z*)-3,6-Nonadienal	No
Indol-3-acetaldehyde	No
Methyl salicylate	Yes
(*E*)-4-Nonenal	No

Compound name	SPME detection
Vanillin	No
p-Propylphenol	No
Phenyl acetaldehyde	Yes
p-Formylphenol	No
Hexanal	No
Syringaldehyde	No
Phenylethyl alcohol	Yes
(*E*,*Z*)-3,6-Nonadienol	No

* RL22 compounds.

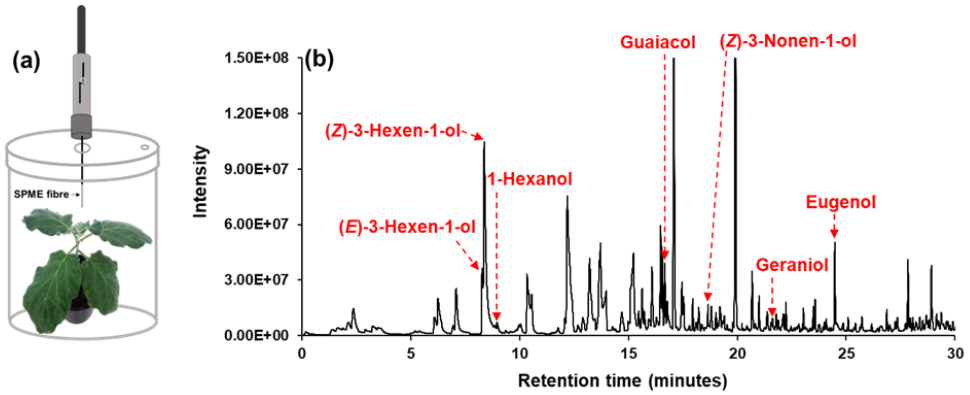


Fig. 2.6 Solid phase microextraction (SPME) based analysis of RL22-headspace. (a) Schematic of the SPME set-up. (b) Total ion chromatogram showing the presence of seven RL22 compounds.

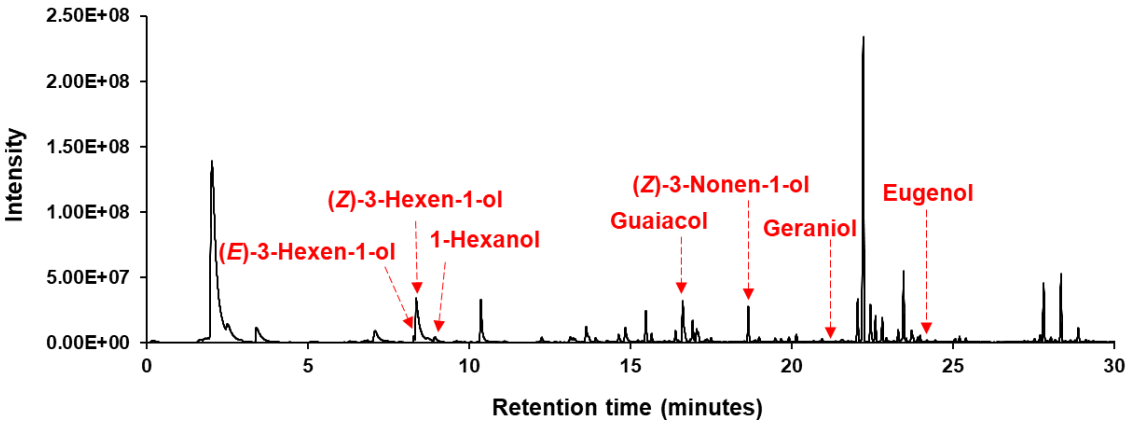


Fig. 2.7 Detection of compounds from the headspace of assay set-up. Total ion chromatogram showing the presence of all seven candidate compounds which were used in the assay when coated on APs in the physiological concentration of RL22.

2.3.4. RL22-specific geraniol repels ovipositing females

Since the GC analysis showed that the ESFB-repellent RL22 VOC blend contained six compounds that were not detected in the CEB and three compounds in significantly higher concentrations than that in CEB. Out of these nine compounds, seven compounds were detected in headspace analysis. We investigated whether these compounds were responsible for repellence. The assays were performed by individually complementing these seven compounds on APs. All of them could be detected in the headspace of the assay chamber even after six hours of assay duration (Fig. 2.7). The assay set-up is shown in Fig. 2.8a. 1-hexanol-complemented APs showed 1-fold increased oviposition than the control (Fig. 2.8b). Complementation of (*E*)-3-hexen-1-ol (Fig. 2.8c), (*Z*)-3-hexen-1-ol (Fig. 2.8d), (*Z*)-3-nonen-1-ol (Fig. 2.8e), guaiacol (Fig. 2.8f) and eugenol (Fig. 2.8g) did not influence oviposition. Geraniol-complemented APs showed no oviposition (Fig. 2.8h), indicating that geraniol was the repellent from RL22. Consequently, we tested whether geraniol retains its oviposition deterrent activity when it is complemented on the leaves of seven eggplant varieties (Fig. 2.8i); >90% reduction in oviposition was observed on geraniol-complemented plants (Fig. 2.8j- p). Remarkable reduction in the number of laid eggs took place in the most susceptible varieties JK (Fig. 2.8m) and CVK (Fig. 2.8p) (JK: 26.95± 2.78 in geraniol-deplete control and 1.35± 0.24 in geraniol-complemented plants; CVK: 28.4± 2.93 in geraniol-deplete control and 0.95± 0.22 in geraniol-complemented plants).

1-Hexanol showed no effect on oviposition when complimented on the leaves of RL22 (Fig. 2.9a), KV (Fig. 2.9b), VJ (Fig. 2.9c), and JK (Fig. 2.9d). Similar to its complementation on AP, 1-hexanol complementation on the leaves of HK (Fig. 2.9e), KP (Fig. 2.9f), and CVK (Fig. 2.9g) showed 1-fold increased oviposition than the controls. A similar result was documented when leaf-blend coated APs were complemented with 1-Hexanol (fig. 2.9h-n), Complementation of (E)-3-hexen-1-ol (Fig. 2.10a-n), (Z)-3-hexen-1-ol (Fig. 2.11a-n), (Z)-3-nonen-1-ol (Fig. 2.12a-n), guaiacol (Fig. 2.13a-n) and eugenol (Fig. 2.14a-n) on the leaves of seven eggplant varieties or leaf-blend coated APs did not influence oviposition. Geraniol complementation reduced oviposition on leaf-blend coated APs (Fig. 2.15a-g). In all the assays, females laid more eggs on geraniol-free controls than the geraniol-complemented leaves (Fig. 2.15h). RL22's geraniol concentration was found to be sufficient for deterring the oviposition (Fig. 2.15i, j).

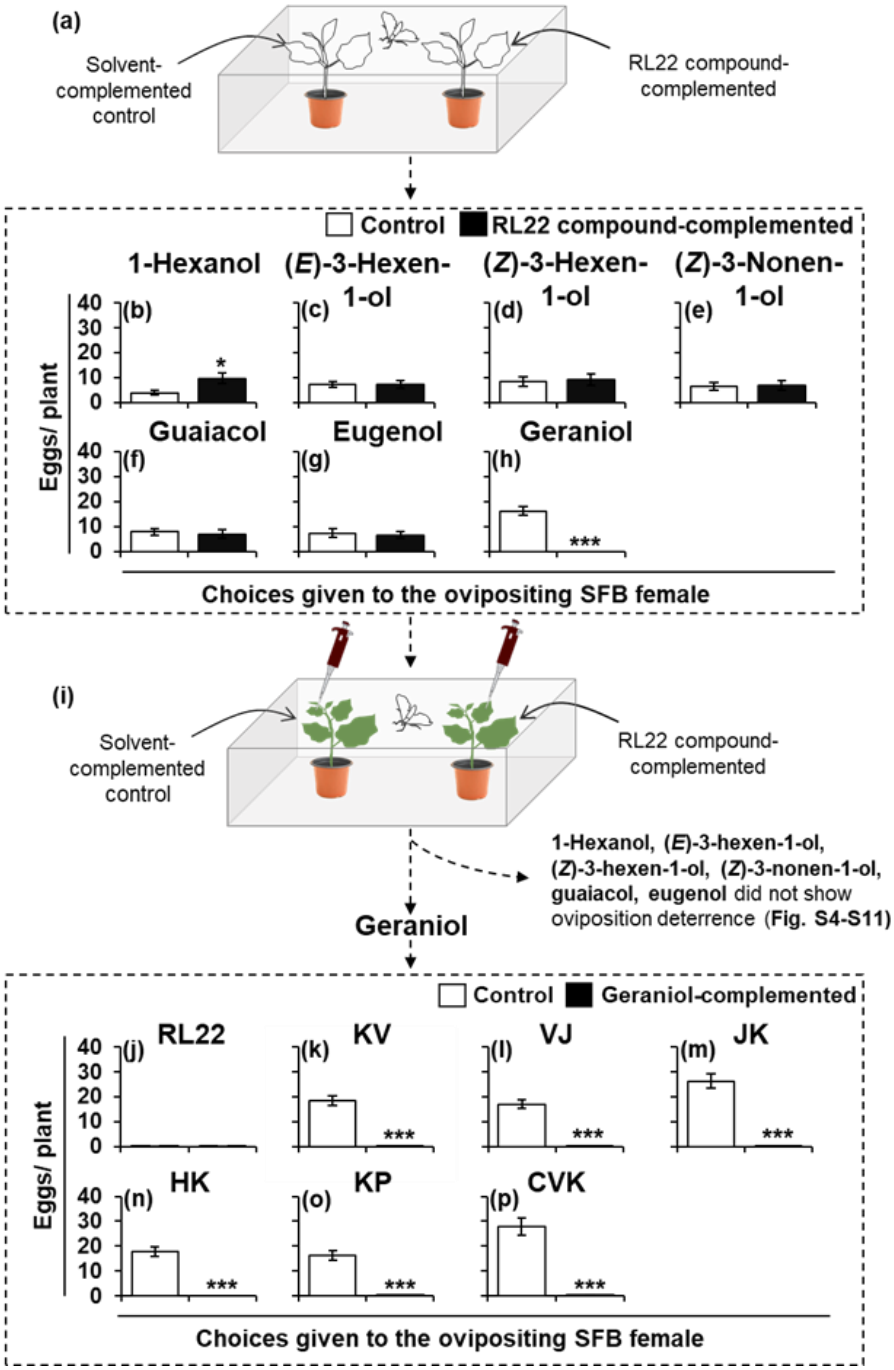


Fig. 2.8 Geraniol deters oviposition. (a) A schematic of dual-choice assays was conducted to analyze ovipositing ESFB females' host preference using control (solvent-complemented) and RL22 compound-complemented APs. Eggs (mean± SE;) laid on (b) 1-hexanol-, (c) (*E*)-3-hexen-1-ol-, (d) (*Z*)-3-hexen-1-ol-, (e) (*Z*)-3-nonen-1-ol-, (f) guaiacol-, (g) eugenol- and (h) geraniol-complemented APs and their respective controls, showing that only geraniol deterred oviposition. (i) A schematic of dual-choice assays conducted to analyze ovipositing females' host preference using control (solvent-complemented) and geraniol-complemented eggplant (*Solanum melongena*) saplings. Eggs (mean± SE;) laid on control (solvent-complemented) and geraniol-complemented (j) RL22, (k) KV, (l) VJ, (m) JK, (n) HK, (o) KP and (p) CVK, showing that geraniol complementation significantly decreases oviposition on the susceptible varieties. Asterisks indicate significant differences calculated using Student's two-tailed t-test (*≡ $P< 0.05$; **≡ $P< 0.001$; n= 20 assays).

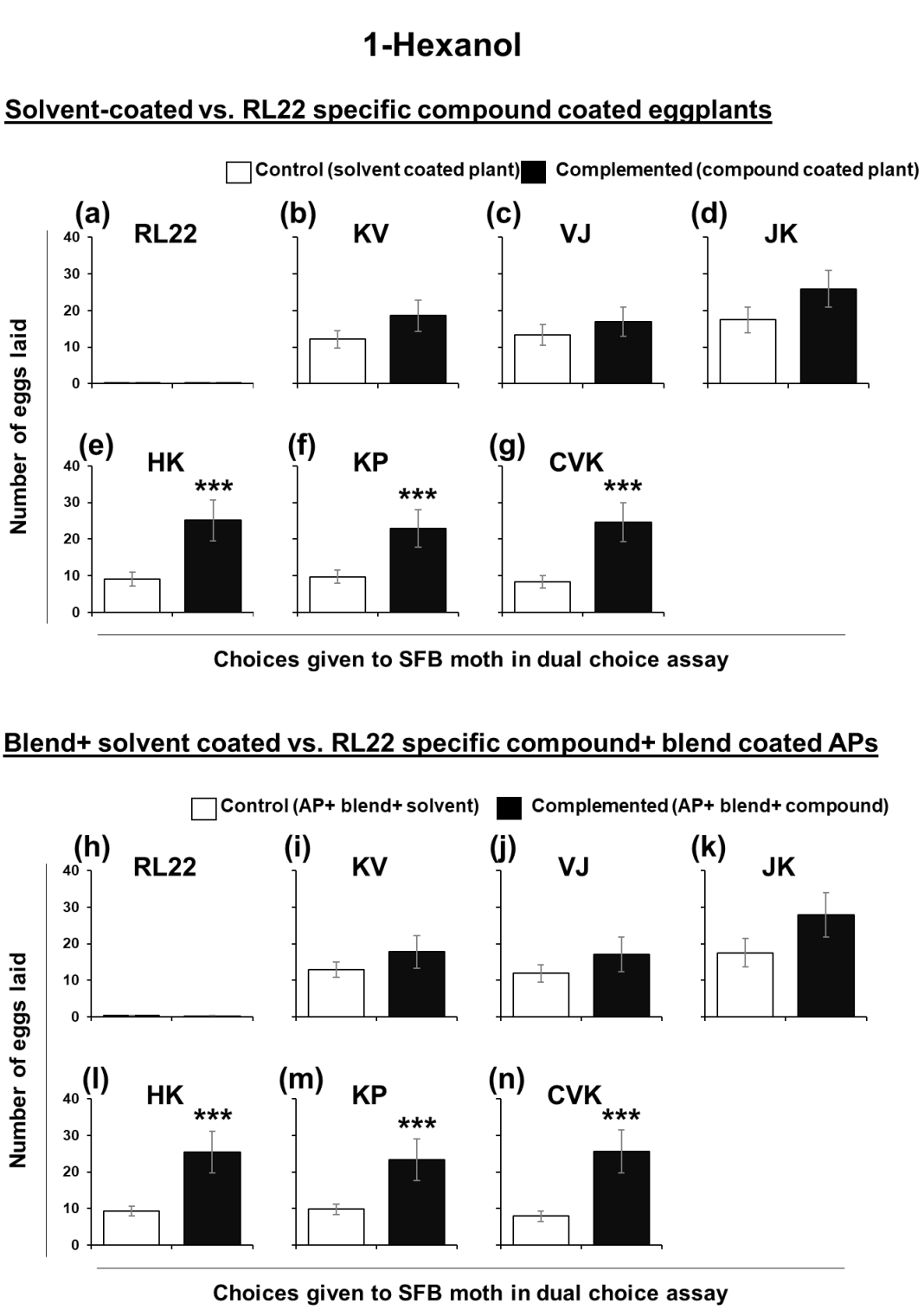


Fig. 2.9 1-Hexanol complementation attracts oviposition in three eggplant varieties. Eggs (mean± SE) laid on solvent- and 1-hexanol-complemented plants of (a) RL22, (b) KV, (c) VJ, (d) JK, (e) HK, (f) KP, and (g) CVK, and APs coated with leaf blend of (h) RL22, (i) KV, (j) VJ, (k) JK, (l) HK, (m) KP, and (n) CVK in dual-choice assays.

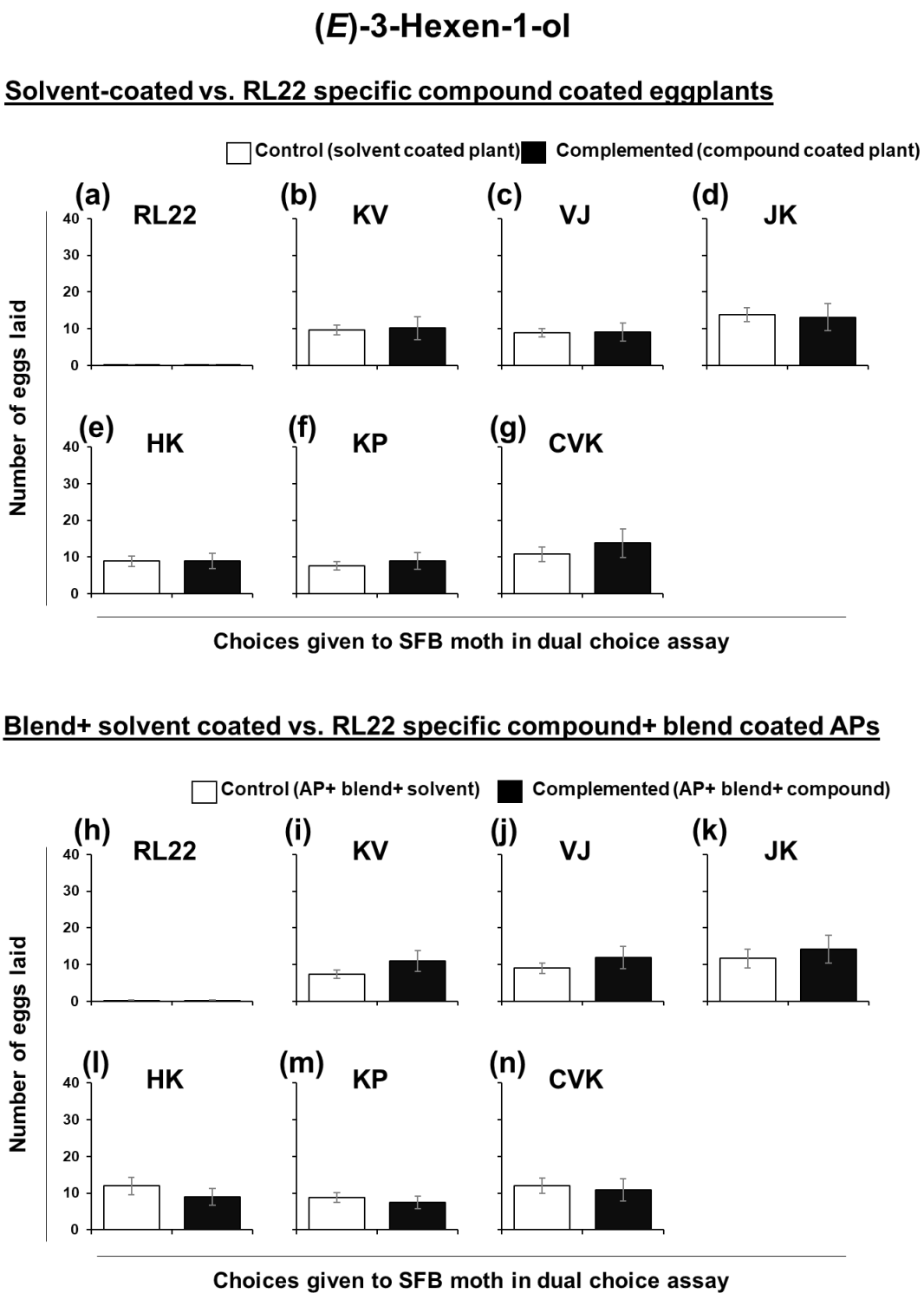


Fig. 2.10 (*E*)-3-Hexen-1-ol does not affect oviposition. Eggs (mean± SE) laid on solvent- and (*E*)-3-hexen-1-ol-complemented plants of (a) RL22, (b) KV, (c) VJ, (d) JK, (e) HK, (f) KP, and (g) CVK, and APs coated with leaf blend of (h) RL22, (i) KV, (j) VJ, (k) JK, (l) HK, (m) KP, and (n) CVK in dual-choice assays.

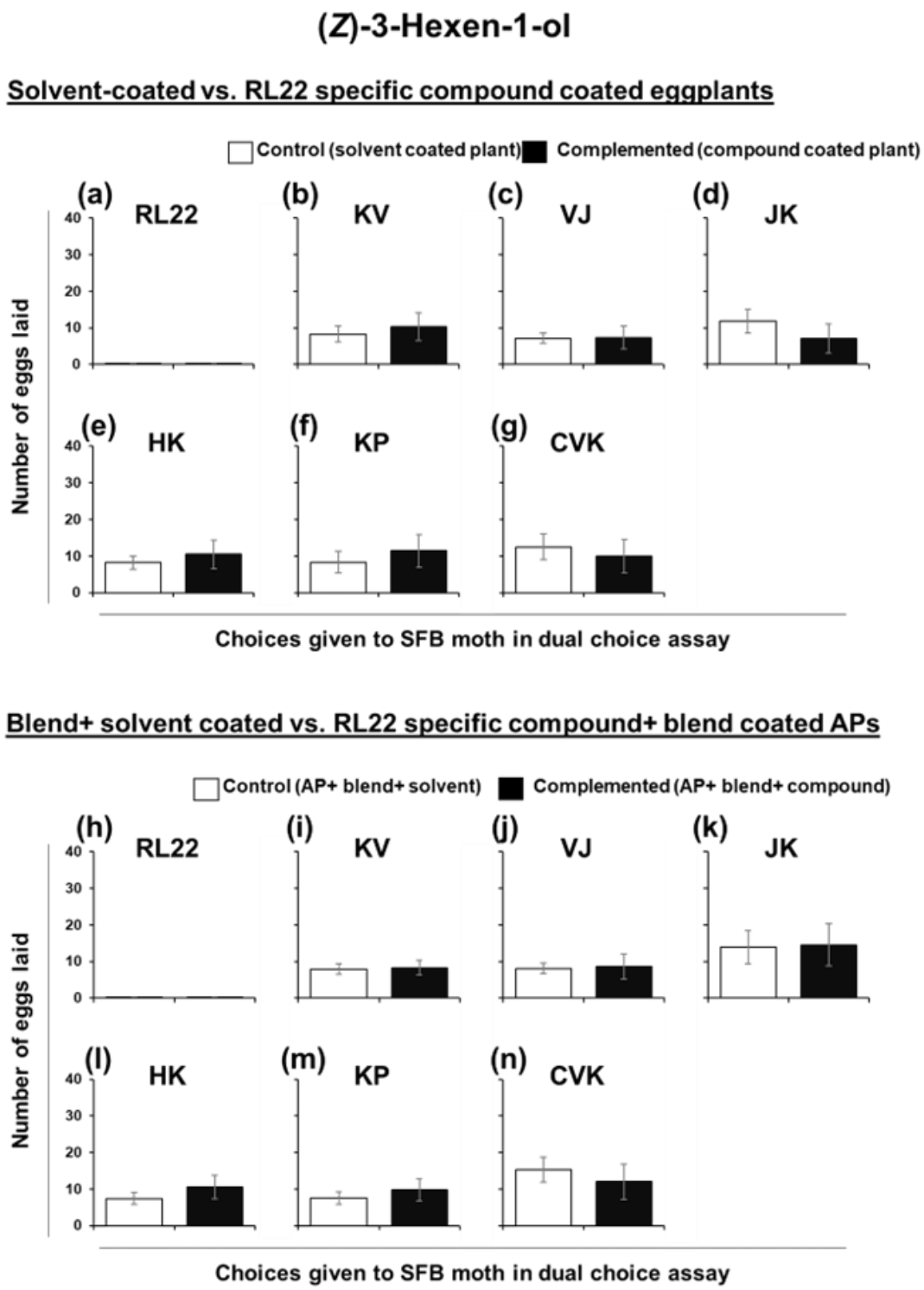


Fig. 2.11 (*Z*)-3-Hexen-1-ol does not affect oviposition. Eggs (mean± SE) laid on solvent- and (*Z*)-3-Hexen-1-ol-complemented plants of (a) RL22, (b) KV, (c) VJ, (d) JK, (e) HK, (f) KP, and (g) CVK, and APs coated with leaf blend of (h) RL22, (i) KV, (j) VJ, (k) JK, (l) HK, (m) KP, and (n) CVK in dual-choice assays.

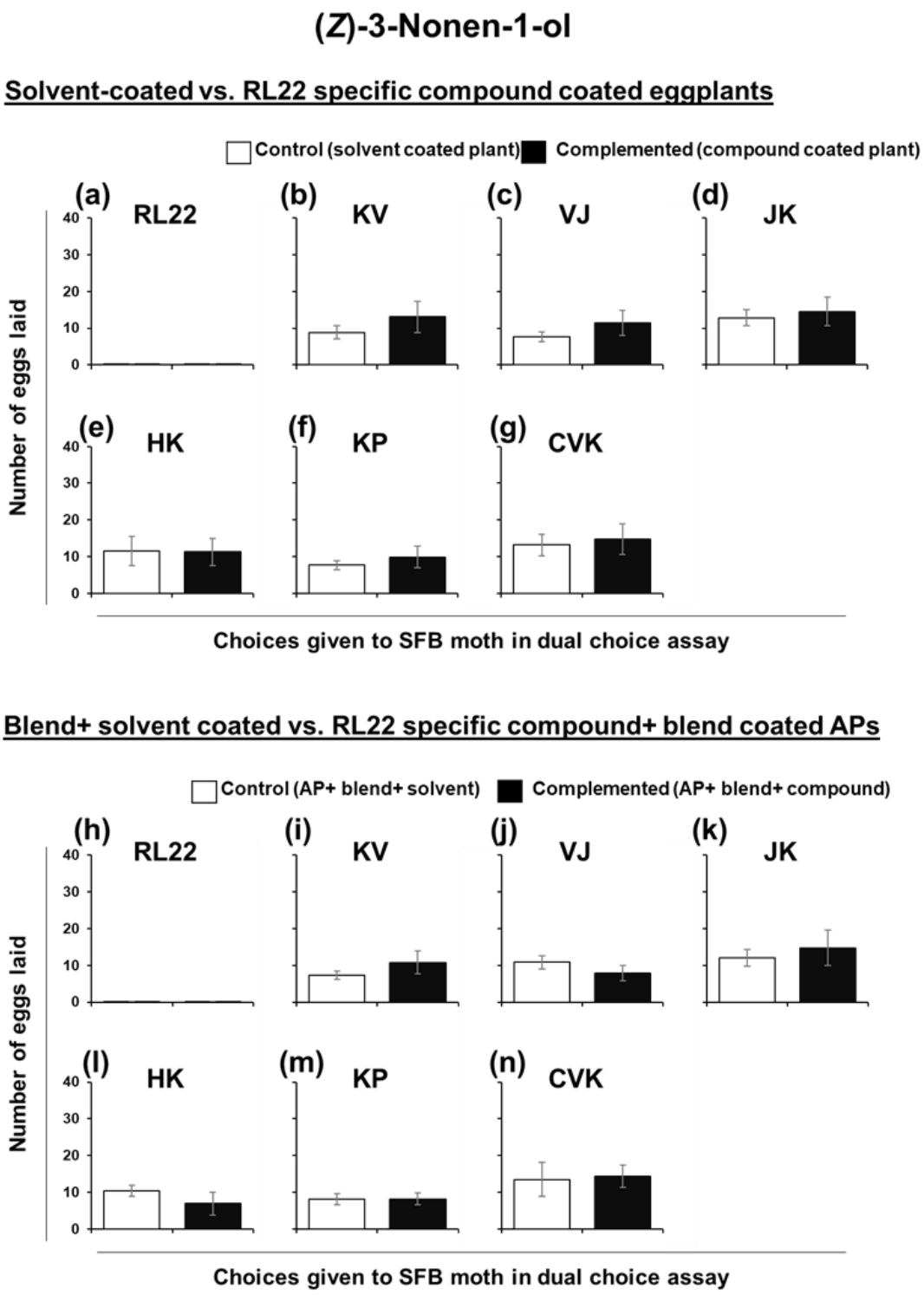


Fig. 2.12 (*Z*)-3-Nonen-1-ol does not affect oviposition. Eggs (mean± SE) laid on solvent- and (*Z*)-3-Nonen-1-ol-complemented plants of (a) RL22, (b) KV, (c) VJ, (d) JK, (e) HK, (f) KP, and (g) CVK, and APs coated with leaf blend of (h) RL22, (i) KV, (j) VJ, (k) JK, (l) HK, (m) KP, and (n) CVK in dual-choice assays.

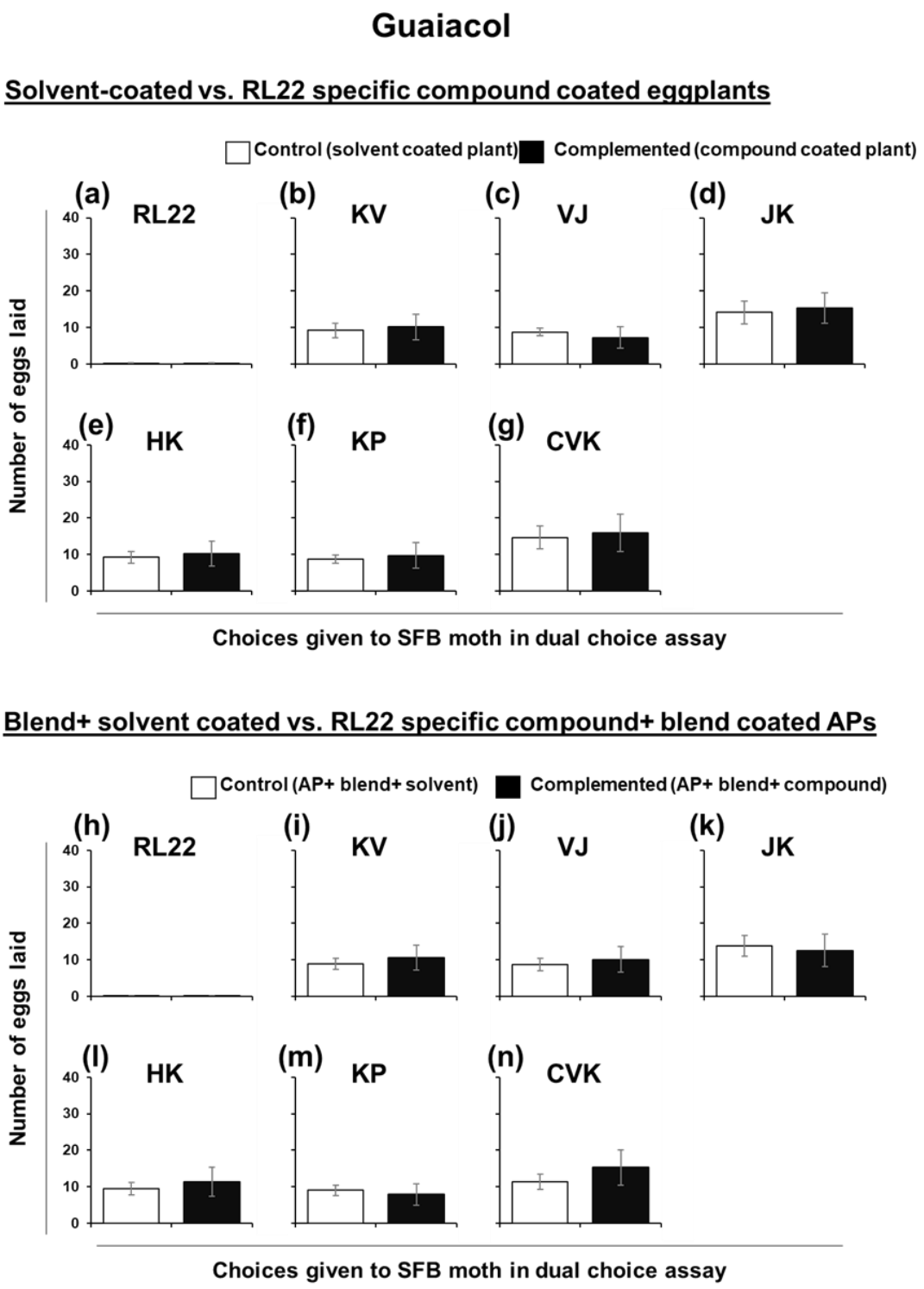


Fig. 2.13 Guaiacol does not affect oviposition. Eggs (mean± SE) laid on solvent- and guaiacol-complemented plants of (a) RL22, (b) KV, (c) VJ, (d) JK, (e) HK, (f) KP, and (g) CVK, and APs coated with leaf blend of (h) RL22, (i) KV, (j) VJ, (k) JK, (l) HK, (m) KP, and (n) CVK in dual-choice assays.

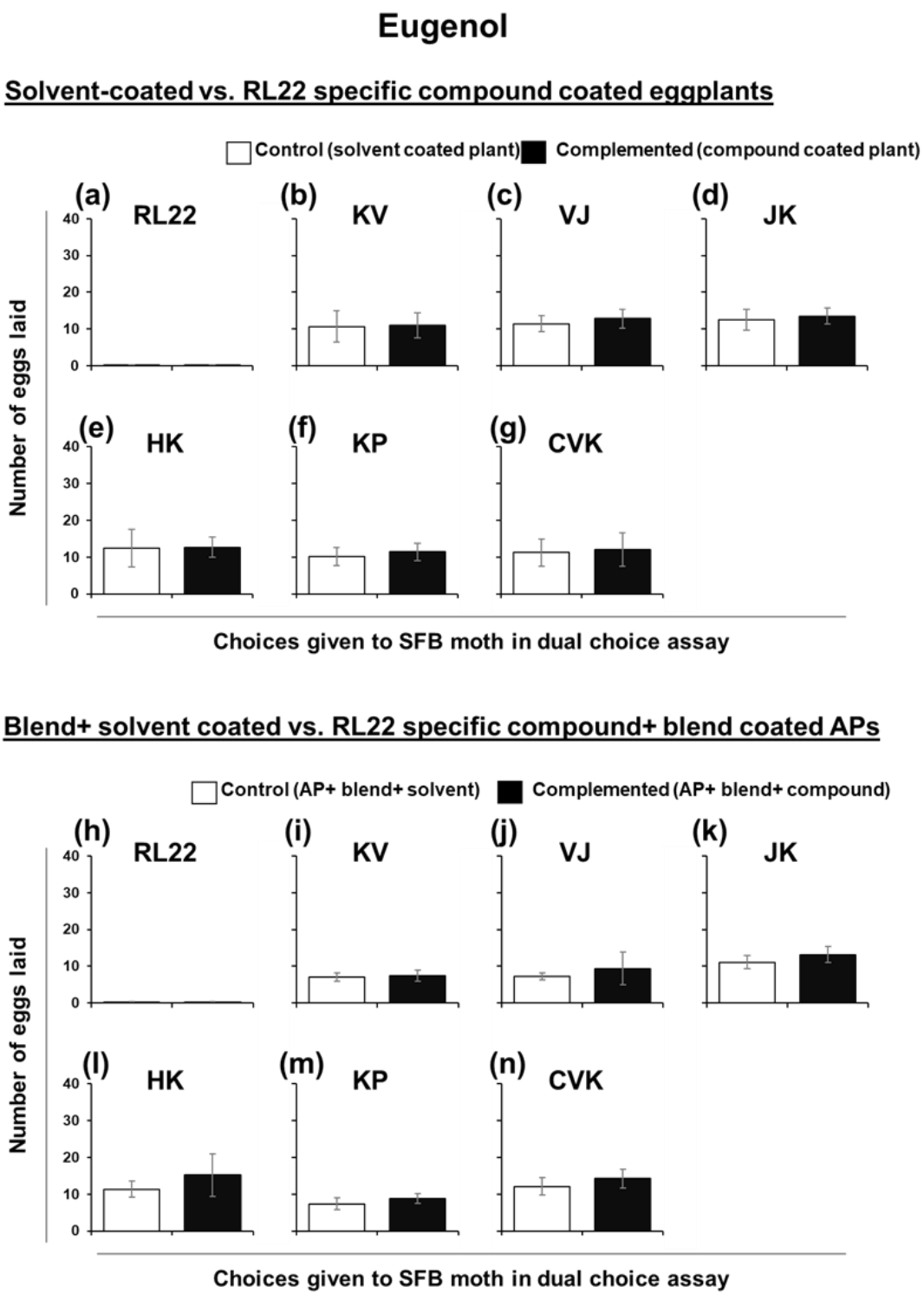


Fig. 2.14 Eugenol does not affect oviposition. Eggs (mean± SE) laid on solvent- and eugenol-complemented plants of (a) RL22, (b) KV, (c) VJ, (d) JK, (e) HK, (f) KP, and (g) CVK, and APs coated with leaf blend of (h) RL22, (i) KV, (j) VJ, (k) JK, (l) HK, (m) KP, and (n) CVK in dual-choice assays.

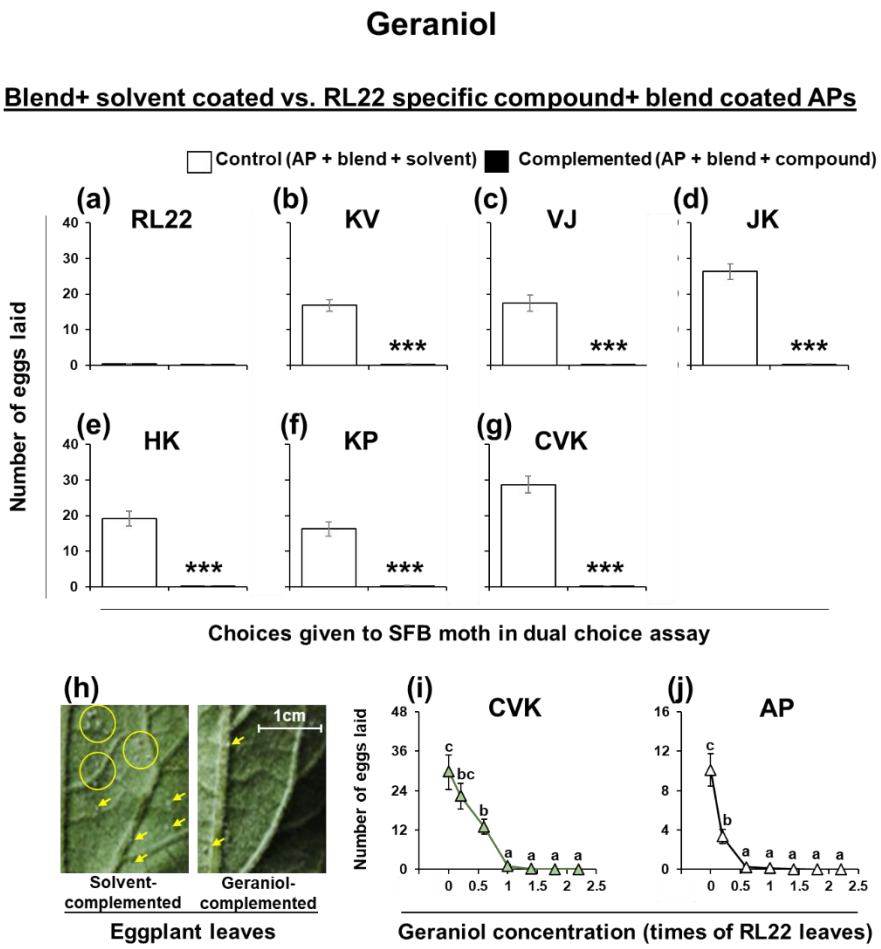


Fig. 2.15 Geraniol reduces oviposition. Eggs (mean± SE) laid on solvent- and geraniol-complemented APs coated with leaf blend of (a) RL22, (b) KV, (c) VJ, (d) JK, (e) HK, (f) KP, and (g) CVK in dual-choice assays. (h) Eggs laid on control and geraniol-treated eggplant leaves indicate that geraniol complementation reduces oviposition. Eggs (mean± SE) laid on (i) CVK (X_2= 85.68, P< 0.0001) and (c) AP (X_2= 65.56, P< 0.0001) leaves complemented with the increasing concentrations of geraniol (uniform complementation volume of 100µl g^{-1} leaf) showing that oviposition reduces with the increase in geraniol concentration (1 unit of concentration= 1.67 nmol g^{-1}, the average physiological concentration of geraniol in RL22); Significant differences (P≤ 0.05) were determined using Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni correction (n= 20 assays).

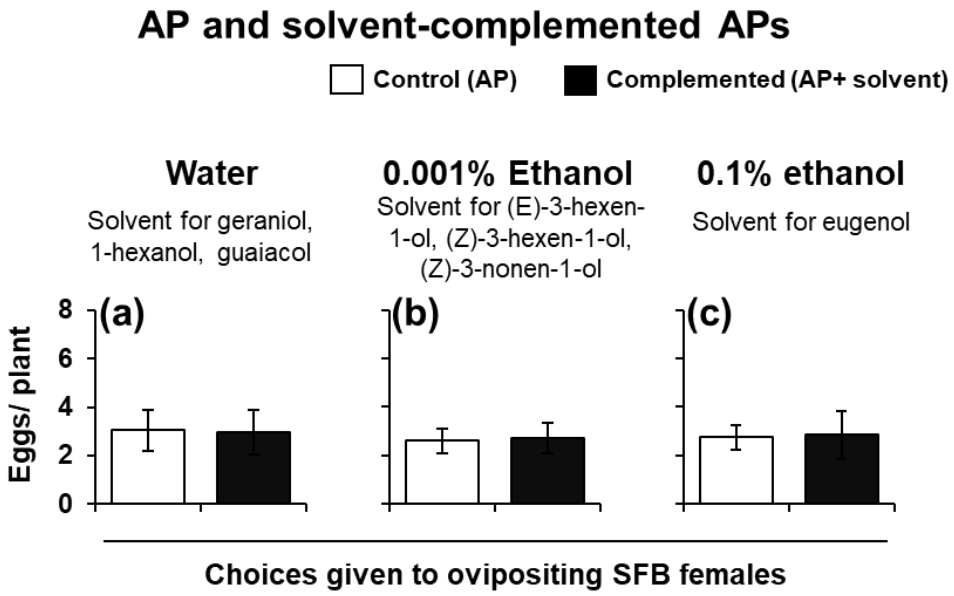


Fig. 2.16 Solvents do not affect oviposition preference. Eggs (mean± SE) laid on (a) AP and AP+ water, (b) AP and AP+ 0.001% ethanol, and (c) AP and AP+ 0.1% ethanol, in dual-choice assays.

Solvents of these compounds, water (geraniol, 1-hexanol, and guaiacol) (Fig. 2.16a), 0.001% ethanol [(*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol and (*Z*)-3-nonen-1-ol] (Fig. 2.16b) and 0.1% ethanol (eugenol) (Fig. 2.16c) did not influence females' oviposition.

2.3.5. Heterologously expressed *Sm*GS converts geranyl pyrophosphate (GPP) to geraniol

Based on sequence similarity and dendrogram analysis (Fig. 2.17) *SmGS* was identified and confirmed by heterologous expression. The construct map is shown in fig. 2.18a. The purified RL22 *Sm*GS catalyzed the conversion of GPP to geraniol. *Sm*GS did not act as nonspecific phosphatase, as it failed to catalyze the conversion of p-nitrophenyl phosphate to p-nitrophenol. The inability of purified protein to convert p-nitrophenyl phosphate concluded that purified protein is free of non-specific phosphatases. Empty and no vector controls failed to produce geraniol at similar conditions (Fig. 2.18b).

*Sm*GS activity was maximum at pH 8.0, with more than 50% activity in the pH range of 7.0- 7.5 (Fig. 2.18c). The optimum temperature for *Sm*GS activity was in the range of 32 °C- 36 °C. More than 50% of activity was observed in the 28 °C- 40 °C temperature range (Fig. 2.18d). Enzyme activity increased linearly with an increase in enzyme amount from 10 µg to 100 µg and saturated at 100 µg (Fig. 2.18e). The *Sm*GS activity also increased linearly with an increase in GPP concentration from 10 µM to 100 µM and saturated at

125 µM (Fig. 2.18f). The K_m value for *Sm*GS was found to be 31.39 µM at optimum conditions (Fig. 2.18g).

2.3.6. Silencing *geraniol synthase* renders RL22 susceptible to ESFB oviposition

To confirm that RL22's repellent nature is due to its geraniol content, we generated *SmGS*-silenced RL22 plants using VIGS (Fig. 2.19a). These plants showed >20-fold lower *SmGS* transcript abundance (0.05± 0.02) than that in EV (1.33± 0.27) and WT (1.03± 0.3) controls (Fig. 2.19b). The Geraniol content of these *SmGS*-silenced plants (14.63± 3.15 nmol g^{-1}) was about 2-fold lower than that of EV (46.56± 5.75 nmol g^{-1}) and WT (49.53± 8.73 nmol g^{-1}) controls (Fig. 2.19c). In choice assays, ESFB females preferred to oviposit on these *Sm*GS-silenced plants; ~98% of the oviposition occurred on these plants when they were used in dual-choice assays with the control WT (Fig. 2.19d) or EV (Fig. 2.19e). Lastly, the repellence could be restored by coating geraniol on *SmGS*-silenced plants as the number of eggs laid on these plants (0.1± 0.06) was <1% of that on *SmGS*-silenced geraniol-deplete plants (23.8± 2.78) (Fig. 2.19f).

Viral coat protein transcripts could be detected in the infiltrated *SmGS* and EV plants, in which they showed similar levels; these transcripts could not be detected in the un-infiltrated WT plants (Fig. 2.20a). Transcript abundance of *SmMTPS1* (Fig. 2.20b) and *SmMTPS2* (Fig. S2.20c) did not vary in WT, EV, and *SmGS*-silenced plants, indicating that the strategy to include *SmGS*'s unique 3'-UTR sequence in the silencing construct was successful. We inferred that the *SmGS* silencing was sufficiently specific. Geraniol peak area reduction in extracted ion chromatogram of fig. 2.20d-f confirms effective silencing.

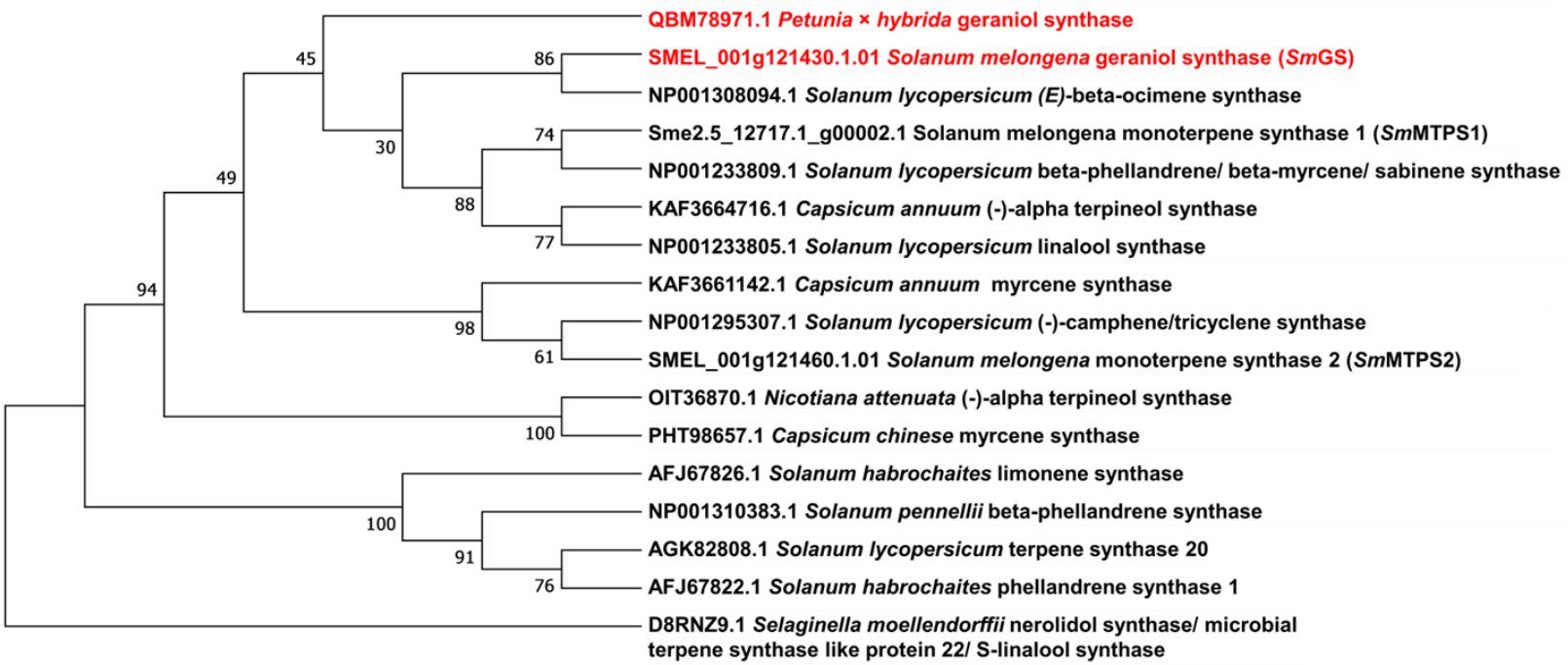


Fig 2.17 Phylogenetic analysis of three putative *Sm*MTPSs. The phylogenetic tree is drawn following the neighbour-joining (Dayhoff matrix) method using MEGA 7 software. JTT matrix-based method is used to calculate evolutionary distances and are in the units of the number of amino acid substitutions per site. All gaps were eliminated. The final dataset had 335 positions. Bootstrap percentage values are given for each branch (1000 replicates). Trees were build using monoterpene synthases of the Solanaceae family (the optimal tree with the sum of branch length equal to 6.65 is shown). Geraniol synthases are highlighted in red font.

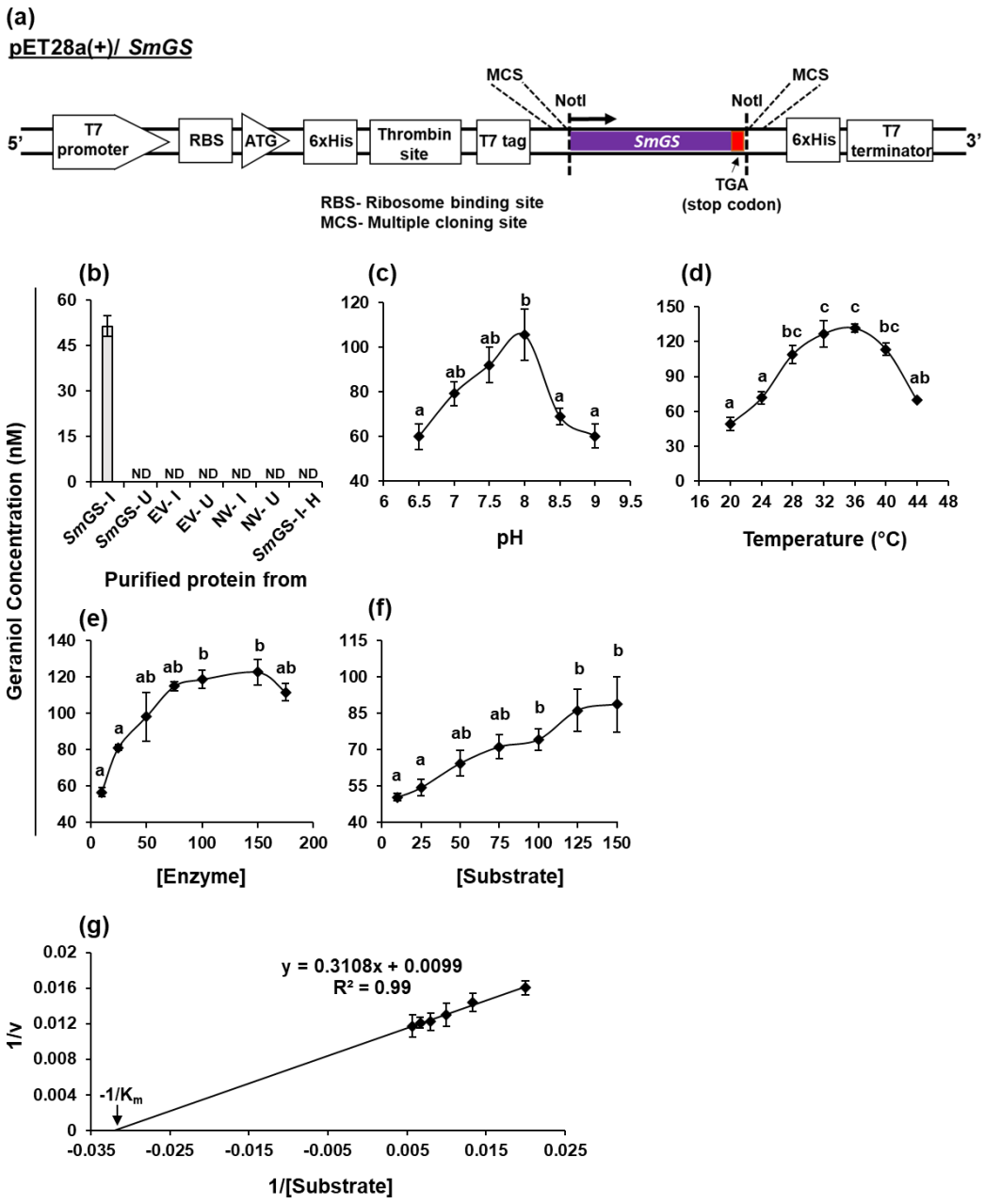


Fig. 2.18 *In-vitro* characterization of *Sm*GS. (a) Schematic of pET28a(+)/ *SmGS* construct used for heterologous expression of *Sm*GS in *E. coli*. (b) Bar-plot (mean± SE) represents protein activity (geraniol concentration) purified from induced (I) and uninduced (U) cultures of pET-28a (+)/ *SmGS*, empty vector (EV), and no vector (NV) transformed BL21-AI *E. coli* cultures and heat-denatured *Sm*GS (*Sm*GS-I-H) (n= 6). Optimum pH (c) (F= 6.22, df= 5, 28, P< 0.001), temperature (d) (F= 14.32, df= 6, 27, P< 0.0001) for *Sm*GS was determined (n= 6). Increasing the concentration of enzyme (e) (F= 28.74, df= 4.79, P< 0.01) and substrate (f) (F= 4.731, df= 7, 36, P< 0.001) increased the product formation at optimum pH and temperature and reached saturation (n= 6). Lineweaver-Burk plot (g) (F= 6.086, df= 7, 34, P< 0.001) was used to determine K_m (n= 6). Significant differences ($P \leq 0.05$) were determined using the Welch F test with the Games-Howell *post hoc* test.

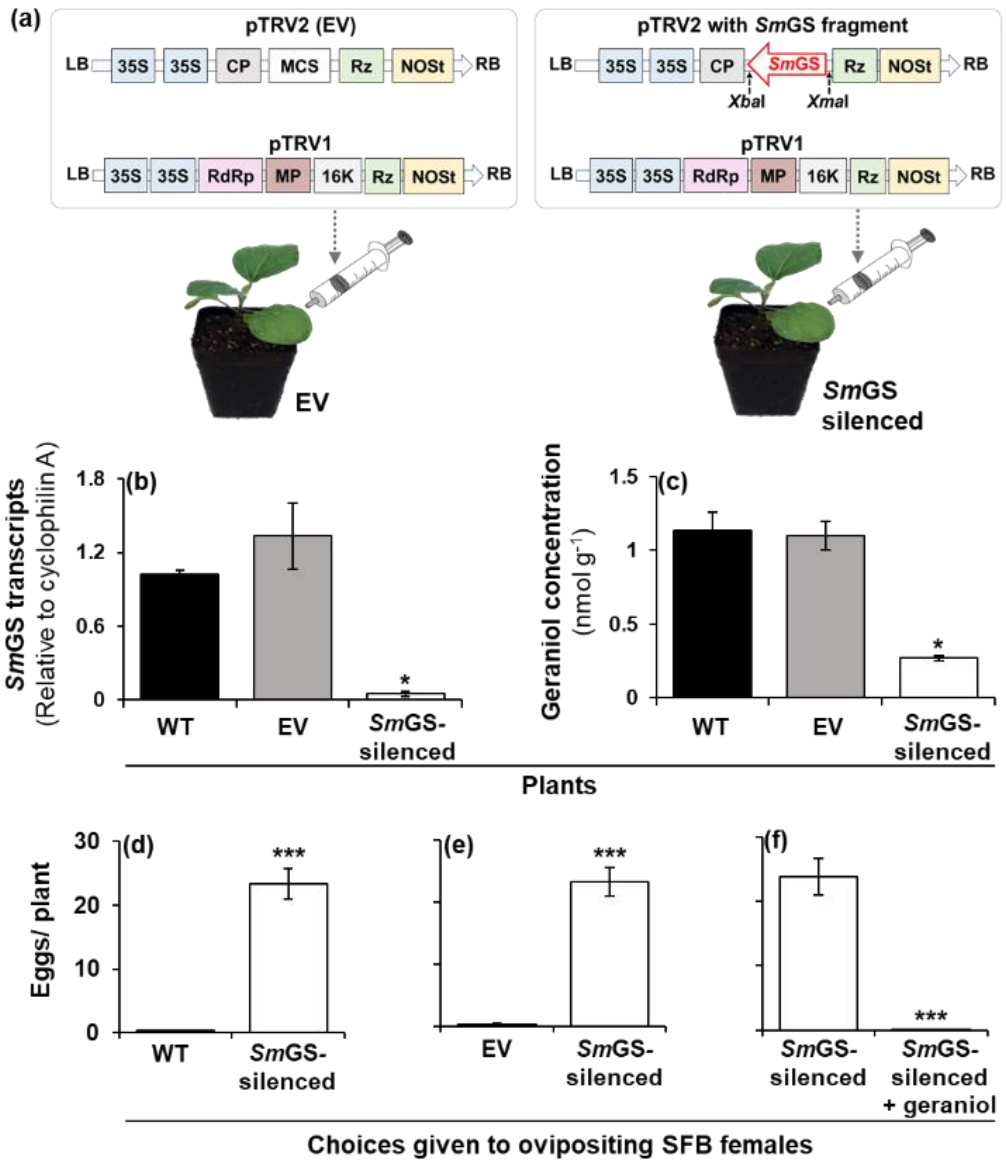


Fig. 2.19 Silencing of geraniol synthase (*Sm*GS) renders RL22 susceptible to ESFB (*Leucinodes orbonalis*) oviposition. (a) Schematic of constructs and infiltration procedure for virus-induced gene silencing (VIGS). (b) *Sm*GS transcript abundance (relative to cyclophilin A) in the leaves of wild type (WT), empty vector-infiltrated (EV), and *Sm*GS silencing construct-infiltrated (*Sm*GS-silenced) eggplants (*Solanum melongena*) (F= 369.3, df= 7.527, P< 0.0001). (c) Geraniol concentrations (mean± SE) in WT, EV, and *Sm*GS-silenced RL22 leaves (F= 53.12, df= 6.959, P< 0.0001). Eggs (mean± SE) laid in dual-choice assays on (d) WT and *Sm*GS-silenced, (e) EV and *Sm*GS-silenced, and (f) *Sm*GS-silenced and geraniol-complemented *Sm*GS-silenced RL22 plants, showing that *Sm*GS silencing renders RL22 susceptible to ESFB oviposition and geraniol complementation in *Sm*GS-silenced plants restores the resistance. Asterisks in (b) and (c) indicate significant differences ($P\leq$ 0.05) determined using the Welch F test with Games-Howell *post hoc* test (*≡ P< 0.05) and in (d), (e) and (f) indicate significant differences calculated using Student's two-tailed t-test (***≡ P< 0.001; n= 20 assays).

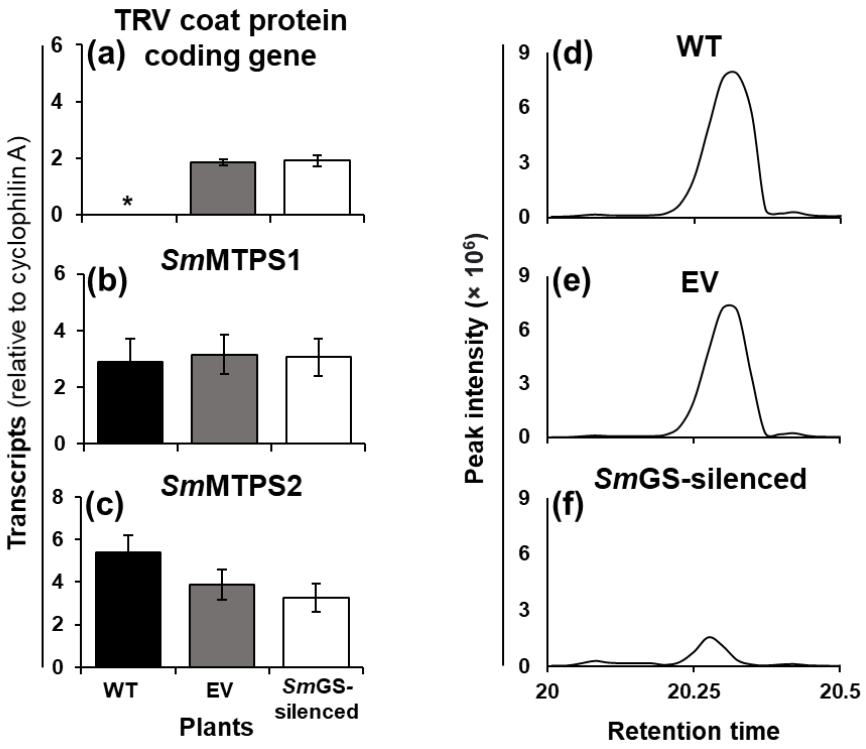


Fig. 2.20 *Sm*GS silencing does not co-silence highly similar *Sm*MTPS1 and *Sm*MTPS2 but reduces the geraniol content. Transcript abundance (relative to cyclophilin A) of (a) TRV coat protein biosynthetic gene (F=354.8, df= 6.667, P< 0.0001), (b) *Sm*MTPS1 (F= 0.03, df= 9.954, P= 0.9732) and (c) *Sm*MTPS2 (F= 1.967, df= 8.87, P= 0.191) in the leaves of WT, EV, and *Sm*GS-silenced plants. Asterisks indicate significant differences ($P \leq 0.05$) determined using the Welch F test with the Games-Howell *post hoc* test (*≡ P< 0.05). GCMS extracted ion (geraniol: m/z 69, 93,153) chromatograms of the VOC extracts of (d) WT, (e) EV, and (f) *Sm*GS-silenced plants showing that the geraniol concentration remarkably reduced upon *Sm*GS silencing.

2.4. Discussion

Several studies have shown that the extent of ESFB infestation varies in different eggplant varieties (Dadmal *et al.*, 2004; Elanchezhyan *et al.*, 2008; Javed *et al.*, 2011; Devi *et al.*, 2015; Kumar *et al.*, 2017). Some investigators searched for the resistance factors among the physicochemical properties of the varieties showing relatively less infestation. They reported morphological characters like spines (Shaukat *et al.*, 2018), trichomes (Panda & Das, 1974; Javed *et al.*, 2011) and silica deposition (Kale *et al.*, 1986) and contents of metabolites such as steroidal alkaloids (Doshi *et al.*, 1998; Preneetha, 2002; Jat & Pareek, 2003; Thangamani, 2003), phenolics (Kale *et al.*, 1986; Asathi *et al.*, 2002; Prabhu *et al.*, 2009), and crude fiber (Kale *et al.*, 1986) as putative ESFB-resistance factors. However, most of these findings were based on correlative analyses and did not empirically validate

the specific resistance factors for direct use in ESFB management (Shaukat *et al.*, 2018; Lalita & Kashyap, 2020).

Our results suggested that ovipositing female's host location was driven by the eggplant's foliar factors. Females showed a similar oviposition pattern on the VOC extract complemented APs as that on plants in both field and climate chambers. This ascertained that the female's host selection was associated with foliar VOCs and not with other features such as leaf area, shape, color, and surface morphology. Similar to the observations made in their native habitat and experimental field, females showed a distinct aversion to RL22 plants and RL22 VOC extract complemented APs in various choice assays. Although VOC-mediated insect resistance is known from several plants (De Moraes *et al.*, 2001; Degenhardt *et al.*, 2009; Staudt *et al.*, 2010), RL22 VOC blend's deterrence potential against a tenacious pest like ESFB was striking, mainly because eggplant genotype with such potential is not known.

In an attempt to find the repellence factor from RL22, we found that the RL22 VOC blend was distinctly polymorphic from other eggplant varieties. It was found to be rich in C6 and C9 alcohols and aldehydes, which frequently play an important role in mounting indirect plant defense to attract herbivores' natural enemies (Dicke, 2009; Zitzelsberger & Buchbauer, 2015). RL22 also showed a high content of some phenolics guaiacol and eugenol, which are known to confer resistance against lepidopteran herbivores in other plant species (Suckling *et al.*, 1996; Molnár *et al.*, 2017). However, these compounds showed no oviposition-deterring effect. Geraniol, the only terpenoid detected from eggplants, was found to be the ESFB repellant. Of the seven eggplant varieties used in this study, only one monoterpene, geraniol, was detected only in the RL22 leaves. This is congruent to the previous studies on several other eggplant varieties; Black beauty variety showed the presence of only one monoterpene, (3E)-4,8-dimethyl-1,3,7-nonatriene (Van Den Boom *et al.*, 2004). Similarly, Lena iri variety also showed the presence of only one monoterpene, linalool (Nusra et al., 2021). Eggplant leaves of Ikot Ekpene garden variety contained no monoterpenes (Chima Ogoko, 2020). Moreover, like this study's other varieties than RL22, Zomorrod F1 did not contain monoterpenes (Raed *et al.*, 2023); it did not emit any even after the whitefly infestation (Raed *et al.*, 2023). Likewise, Zheqie No. one variety did not contain monoterpenes and they were also not induced in this variety upon the lepidopteran folivory (Chen *et al.*, 2021). More important, none of these researchers analyzed eggplant volatiles during ESFB oviposition time, which is between

9:00 pm and 1:00 am, as we did. This was essential to identify the oviposition deterrence cues. Together, it can be inferred that (1) monoterpenes are rare volatiles of the eggplant, (2) their occurrence can be variety-specific, their emission can be under a temporal regulation.

Repellent properties of geraniol have also been reported against other lepidopteran pests like the light brown apple moth (*Epiphyas postvittana* Walker; Tortricidae) (Suckling *et al.*, 1996) and cabbage looper (*Trichoplusia ni* Hübner; Noctuidae) (Akhtar *et al.*, 2012). Moreover, recently it was discovered that in the fall armyworm, (*Spodoptera frugiperda* J.E. Smith; Noctuidae), geraniol reduces oviposition and also impairs embryo development (Guedes *et al.*, 2020). It is known that intercropping of aromatic plants like coriander (*Coriandrum sativum* L.) (Khorsheduzzaman *et al.*, 1997; Satpathy & Mishra, 2011; Singh *et al.*, 2016), fennel (*Foeniculum vulgare* Mill.) (Satpathy & Mishra, 2011; Singh *et al.*, 2016), lemongrass (*Cymbopogon citratus* Stapf.) (Calumpang *et al.*, 2013) and marigold (*Tagetes erecta* L.) (Calumpang & Ohsawa, 2015; Bhattacharyya, 2020) with eggplant reduces the ESFB incidence and therefore such intercropping has now become an important component of the integrated pest management. It is striking that geraniol is a prominent component of the VOC blends of all these crops [coriander (Bandoni *et al.*, 1998; Saim *et al.*, 2008; Pavlić *et al.*, 2015), fennel (Galambosi *et al.*, 1994; Oezcan & Chalchat, 2010), lemongrass (Anonymous, 2003; Ganjewala, 2009; Ganjewala & Luthra, 2009) and marigold (Singh *et al.*, 2003; Salinas-Sánchez *et al.*, 2012)]. Thus, our discovery of geraniol as an antixenosis factor is in agreement with these reports and suggests that albeit unknowingly and indirectly, this compound has already been in use for the protection of eggplant crops.

Breeding for the enrichment of resistance traits has been a commonly used method to develop pest resistance, involving germplasm screening for resistance traits, classical breeding, and characterization of the new phenotypes (Rotino, 1997; Elanchezhyan *et al.*, 2008; Javed *et al.*, 2011; Devi *et al.*, 2015). However, this method is time-demanding, labor-intensive, and most importantly, may have unpredictable results because of the unknown physicochemical basis of the desired traits (Kos *et al.*, 2009). Finding a single compound like geraniol as a basis of resistance is rare. For the management of lepidopteran pests, whose host location and selection are often performed by their adults (Renwick & Chew, 1994), disrupting adults' VOC-mediated host location has been suggested as a control measure (Städler, 1994; Bruce *et al.*, 2005; Kos *et al.*, 2009; Bruce & Pickett,

2011). Geraniol can be used for such disruption and ESFB control thereof. Geraniol is a U.S. Food and Drug Administration certified 'generally recognized as safe' (GRAS) food additive (Sinha *et al.*, 2014; Anonymous, 2020) and therefore, can be readily incorporated into the integrated pest management for a direct application to reduce the pesticide load.

Together, this work iterates the importance of insect behavioral and chemical ecology in pest management. It emphasizes that a focus on controlling ovipositing females rather than on controlling larvae by hazardous pesticide application can be a highly useful and eco-friendly strategy. Geraniol, the ESFB repellent discovered by studying the ESFB resistance in native Himalayan variety, can be directly incorporated into integrated pest management; geraniol overproducing eggplants or devices designed to constantly release geraniol can be thought of as durable options. Geraniol can also be used as a selection marker for developing ESFB-resistant eggplant varieties.

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Chapter 3

Understanding eggplant's response to ESFB frugivory

3. Understanding eggplant's response to ESFB frugivory

3.1. Introduction

Plants and insects frequently interact. Due to the plants' sessile nature, they are constantly challenged by several guilds of insect herbivores (Chen & Mao, 2020). To withstand insect herbivore-associated biotic stresses, plants have evolved interconnected regulatory pathways. These allow them to respond and adapt to their environment. Understanding how plants perceive stress signals and respond is important for us as this knowledge can be used for maintaining a sustained food supply to human society.

The most studied insect herbivory is lepidopteran folivory (leaf-eating herbivory). Folivory associated plant membrane degradation and the oral secretion (OS) components initiate jasmonic acid (JA)-mediated signaling (Baldwin *et al.*, 2002; Wasternack & Hause, 2002). Jasmonates (JA, intermediates, and derivatives) typically accumulate in leaves in the early hours and regulate local and systemic responses. It leads to massive transcriptional reprogramming followed by metabolome reconfiguration (Baldwin *et al.*, 2002; Doan *et al.*, 2004; Devoto *et al.*, 2005; Halitschke *et al.*, 2008; Pauwels *et al.*, 2008; Staswick, 2008; Zhang & Turner, 2008; Bruinsma *et al.*, 2009; Diezel *et al.*, 2009). Therefore, jasmonates are regarded as the master regulators of lepidopteran folivory-associated plant response (Creelman & Mullet, 1997; Yan *et al.*, 2013; Koo, 2018). Other phytohormones crosstalk with the JA-signaling and fine-tune this process (Liu & Timko, 2021). Salicylic acid (SA) mediates plants' response to phloem feeder infestations and mainly acts antagonistically with JA pathways. Conversely, lepidopteran folivory-associated JA induction suppresses SA (Diezel *et al.*, 2009). Ethylene (ET), a gaseous hormone, acts synergistically with JA (Von Dahl and Baldwin, 2007; Spoel *et al.*, 2003; Caarls *et al.*, 2015). While JA, SA, and ET are known for their pivotal roles in regulating insect herbivore-associated stress responses, abscisic acid (ABA) is predominantly involved in abiotic stress responses like drought, salinity, temperature, and wounding (Verma *et al.*, 2016). Only a few recent studies have demonstrated ABA's role in biotic stress. ABA can synergistically act with JA (Imai *et al.*, 1995). ET and ABA can independently modulate JA-mediated responses (Mauch-Mani & Mauch, 2005; Dinh *et al.*, 2013). Other phytohormones like auxins [indole acetic acid (IAA), indole butyric acid (IBA)], gibberellins (GAs), and cytokinins (CKs) with JA, SA, ET, and ABA maintain a fine balance and mediate effective plant responses (Bari & Jones, 2009).

Plants show remarkable phenotypic plasticity mediated by phytohormones to respond and adapt to continuous insect attacks. They produce defense-related enzymes and metabolites, including volatiles, secondary metabolites, silica, latex, quinones, proteinase inhibitors, lectins, and defensins upon attack. These chemicals act as insect repellants, antifeedants, and insecticides. These even attract natural enemies and alarm neighboring plants (Dicke & Baldwin, 2010; Holopainen, 2004; Holopainen & Blande, 2012; Mithöfer & Boland, 2012b; War *et al*., 2012; Zhou & Jander, 2022). These plant phenotype variations are also observed in flowers (Rusman et al., 2019a). There are only a few studies indicating that plants respond to the folivores, florivores, rhizovores, and phloem sap feeders by changing flowering time and floral metabolites composition. These changes are largely herbivore- and plant-species-specific (Schiestl *et al*., 2014; Rusman *et al*., 2019a, b). It is well-known that the phytohormones like ABA, GA, and CKs regulate flowering (Gawarecka & Ahn, 2021). However, how herbivory influences these phytohormones and flowering is largely unexplored.

Flowering is a complex multistep process that is regulated by various endogenous (phytohormone, age, etc.) and exogenous (photoperiod, temperature, etc.) cues (Song *et al*., 2013). These cues regulate the expression of flowering time genes (FTGs) which include flowering inducers (florigens) and repressors (anti-florigens). Some examples of FTGs are *FLOWERING LOCUS T* (*FT*; Solanaceae ortholog of *FT* is *SELF PRUNING 3D* or *SP3D*), *FLOWERING LOCUS D* (*FD;* Solanaceae ortholog of *FD* is *SELF-PRUNING G-BOX or SPGB*), *CONSTANS* (*CO*), *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *FLOWERING LOCUS C* (*FLC*), *GIGANTEA* (*GI*), and *TERMINAL FLOWER 1* (*TFL1*) (Searle *et al.*, 2006; Navarro *et al.*, 2011; Andrés & Coupland, 2012; Abelenda *et al.*, 2016; Shu *et al.*, 2016; Soyk *et al.*, 2017; Moraes *et al.*, 2019; Zhu *et al.*, 2020; Siemiatkowska *et al.*, 2022) (table 3.1). A balance between florigens and anti-florigens determines the meristem fate (Zhu *et al.*, 2020). Florigens induce floral meristem identity genes [like *APETALA1* (*AP1*) and *LEAFY* (*LFY*)] that are responsible for meristem fate alteration from vegetative to reproductive (Serrano-Mislata *et al.*, 2017; Seibert *et al.*, 2020; Jin *et al.*, 2021; Wang *et al.*, 2022; Moraes *et al.*, 2019; Gawarecka & Ahn, 2021) (table 3.1).

Table. 3.1 List of genes involved in flowering regulation and their functions.

Sr. No.	Gene name		Gene function	References
	Full form	**Short form**		
1	*FLOWERING LOCUS T/ SELF-PRUNING 3D/ SINGLE FLOWER TRUSS*	*FT/ SP3D/ SFT*	Flowering promoter, key to controlling the meristem transition	(Molinero-Rosales *et al.*, 2004)
2	*TERMINAL FLOWER 1/ SELF-PRUNING*	*TFL1/ SP*	Flowering repressor	(Pnueli *et al.*, 1998; Molinero-Rosales *et al.*, 2004)
3	*SELF-PRUNING 5G*	*SP5G*	Flowering repressor	(Soyk *et al.*, 2017; Cao *et al.*, 2018)
4	*MOTHER OF FT/ SELF-PRUNING 2G*	*SP2G/ MFT*	Flowering repressor	(Weng *et al.*, 2016)
5	*SELF-PRUNING 6A*	*SP6A*	Flowering promoter	(Navarro *et al.*, 2011; Abelenda *et al.*, 2014)
6	*SELF-PRUNING 9D*	*SP9D*	Associated with semi-determinate growth	(Carmel-Goren *et al.*, 2003)
7	*SELF-PRUNING G-BOX/ FLOWERING LOCUS D*	*SPGB/ FD*	*SP3D* binds to *SPGB* at the apex and promotes flowering	(Abelenda *et al.*, 2014)
8	*FLOWERING LOCUS C*	*FLC*	Flowering repressor	(Shimada *et al.*, 2009)
9	*CONSTANS 1*	*CO1*	Modulate flowering in a day-length dependent manner	(Navarro *et al.*, 2011)
10	*CONSTANS 2*	*CO2*		
11	*CONSTANS 3*	*CO3*		

Sr. No.	Gene name		Gene function	References
	Full form	**Short form**		
12	*SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	*SOC1*	Flowering inducer	(Lee & Lee, 2010)
13	*GIGANTEA*	*GI*	A flowering inducer	(Conti *et al.*, 2014; Odgerel *et al.*, 2022)
14	*APETALA1*	*AP1*	Key regulator of floral initiation and floral meristem establishment	(Shimada *et al.*, 2009; Jiang *et al.*, 2022)
15	*LEAFY-FLORICAULA/ FALSIFLORA*	*LFY/ FA*	Flowering promoter; regulates floral meristem identity and transition	(Molinero-Rosales *et al.*, 1999, 2004)

As discussed earlier, our knowledge of insect herbivory and plants' response to it is limited to folivory and, to some extent, rhizovory. Many other kinds of herbivores are present in an ecosystem, such as frugivores, granivores, florivores, etc. Studies on frugivory-associated plant responses are scarce. As fruits are reproductive organs, their protection must be of the plant's utmost priority, especially for unripe fruits with immature seeds. Generally, unripe fruits have hard pericarps, spines, and these are rich in secondary metabolites, acids, and essential oils, to avoid insect, pathogen, and parasite attacks (Mack, 2000; Schaefer *et al.*, 2003; Spadafora *et al.*, 2008; Sinniah *et al.*, 2013; Whitehead & Bowers, 2014a; Nevo *et al.*, 2017a). Despite that, specialized insects are adapted to feed on fruits (Stommel *et al*., 2015; Hanafy & El-sayed, 2013; Shukla *et al.*, 1997). Therefore, plants likely have evolved induced responses to deal with the adverse effects of frugivory. Generally, phytohormones are the central players in plant response regulation. Hence, their participation in frugivory-mediated response is highly anticipated.

Folivory or rhizovory-associated damages in vegetative parts lead to plant growth changes; plants induce defense by compromising growth, or they grow more to tolerate and compensate for stress (Johnson *et al*., 2016; Züst and Agrawal, 2017; Paige, 1992).

Similarly, plants may employ frugivory-associated defense or compensatory responses, which might include defense upregulation in fruits or production of more fruits. These studies are necessary and their results will enrich the knowledge of plant-insect interaction and help develop novel frugivore-management strategies.

Eggplant (*Solanum melongena,* Solanaceae) is the fifth most important vegetable crop of the tropics and subtropics (Taher *et al.*, 2017a; FAOSTAT, 2022), especially for the small-scale farmers and low-income consumers of Asia due to its cheap maintenance and easy growth conditions (Sidhu *et al.*, 2004; Kolady & Lesser, 2008; Huda *et al.*, 2009; Singla *et al.*, 2018). Chronic and widespread infestation by the eggplant shoot and fruit borer (ESFB, *Leucinodes orbonalis* Guenee, Lepidoptera: Pyralidae) causes massive (45- 90 %) yield losses (Deshmukh & Bhamare, 2006; Singla *et al.*, 2018). A detailed review of ESFB management practices and their drawbacks is given in chapter 1 of this thesis. With this background, it is high time to exploit the eggplant-ESFB system to increase our understanding of plant-frugivore interactions and utilize that insight into sustainable agriculture.

During agricultural field visits to understand ESFB biology, we observed increased flowering at ESFB-infested fruit-bearing branches compared to healthy fruit-bearing branches. We hypothesized that ESFB-frugivory led to flowering induction. We investigated the role of phytohormones in this frugivory-mediated plant response. We explored the details of phytohormone biosynthesis, accumulation, transport, and participation in flowering induction.

3.2. Materials and methods

3.2.1. Plants, growth conditions, and experimental set-ups

Eggplants of the variety PanchaGanga F1-hybrid Gaurav (PanchaGanga seeds Pvt. Ltd, Aurangabad) were used in this study. Seeds were germinated in an autoclaved mixture of red soil, cocopeat, vermiculite, perlite black, and red soil in 1:1:1:1 proportion respectively. One-month-old seedlings were planted in the experimental field of the Indian Institute of Science Education and Research (IISER), Pune (18.547669 °N, 73.807636 °E) with 1 m spacing between individuals. Fertilizers were provided as recommended for this region. Three months old field-plants were used for all experiments. For controlled field experiments, plants were caged [(length, l) 2 ft× (breadth, b) 2 ft× (height, h) 4.5 ft; mesh size- 1.5 mm] to avoid natural ESFB infestation. Cages provided adequate aeration and

undisturbed plant growth. Fruits (12 days after pollination; DAP) of field plants were used in experiments. All the field experiments were conducted between June to September, which is the most favorable season for ESFB infestation.

3.2.2. Insects

ESFB larvae and pupae, collected from the eggplant fields in and around Pune were used to initiate the laboratory culture. Larvae were maintained in aerated polypropylene containers [(l) 30 cm× (b) 20 cm× (h) 10 cm] incubated inside a climate chamber (25± 2 °C temperature, 65± 5 % relative humidity, 16 h light, and 8 h dark photoperiod) and were reared on eggplant fruits. Pupae were maintained in the dark. For mating, moth pairs were kept in jars [(inner diameter, id) 10 cm× (h) 20 cm] and provided with healthy eggplant twigs as the oviposition substrates and were fed 10% (w/v) aqueous sucrose solution with the help of a cotton wick. Early third-instar larvae, from the third generation of this culture, were used in the experiments.

3.2.3. Field observations

To understand the effect of frugivory-mediated plant phenotypic changes, field plants with natural infestations were studied. Numbers of flowers, flower buds, and fruits of the infested and healthy fruit-bearing eggplant branches (20 plants per fruit type) were compared.

3.2.4. Effect of controlled ESFB-infestation on eggplant's flowering and fruiting

To test whether flowering and fruiting induction is associated with frugivory, a field experiment was performed. Early third-instar ESFB larvae were introduced into healthy eggplant fruits of 12 DAP. For ESFB frugivory treatment, fruits were bored with a sterile stainless-steel borer. One larva was released in each mechanically bored fruit (ESFB+ MB). Fruits were bagged [net bags of (l) 20 cm× (b) 15 cm, mesh size- 0.5 mm] to avoid larval escape. The net bags were removed after one hour of ensuring that the larvae settled inside the fruits. Fruits with mechanical boring (MB) and untreated fruits were used as controls. Net bags were also used to cover control fruits for one hour. The number of flowers, flower buds, and fruits were counted on 0^{th}, 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th}, and 12^{th} days post-treatment (DPT) in apical and axillary buds. For each treatment, nine fruits on nine different plants were studied.

3.2.5 Plant tissue collection and phytohormone extraction

To find whether ESFB-associated flowering is due to phytohormonal regulation, the phytohormone profile of eggplant parts across three treatments (ESFB+ MB, MB, and untreated) was monitored. Plant tissues of the entire path from fruit to the apical bud (fruit mesocarp, pedicel, stem parts, source ($Leaves_{Source}$), and sink leaves ($Leaves_{Sink}$) were collected and flash-frozen in liquid nitrogen and stored at -80 °C. Tissues were collected on 0^{th}, 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} DPT. For each case, five biological replicates were collected. Plant tissues (250 mg) were pulverized in liquid nitrogen and extracted in 1 mL of 70 % methanol (aqueous, vol/ vol) with adonitol (200 ng ml^{-1}; used as an internal standard) for liquid chromatography-mass spectrometry (LCMS)-based phytohormone profile.

3.2.6. LCMS-based phytohormone profile

For the phytohormone analysis, our previously described procedure (Umesh *et al.*, 2022) was used. 20 µl of the extract was injected into an X500R UPLC-ESI-QTOF (AB SCIEX Pte. Ltd.) system. Phenomenex Gemini® C18 column (50 mm× 4.6 mm, 5 µm, 110 Å) was used for metabolite separation. A constant solvent [solvent 'A'- Milli-Q water with 0.1 % formic acid (Sigma-Aldrich, India) (vol/vol); solvent 'B'- methanol (J T Baker®, India) with 0.1 % formic acid (vol/vol)] flowrate (0.5 mL min^{-1}) with a gradient of 'B'- 5 % (0 min), 5 % (1.5 min), 95 % (10 min), 95 % (11 min), 5 % (12 min) and 5 % (15 min) was used. MS scans (100- 400 Da in negative ionization mode) were performed. MS parameters- 5000 V spray voltage, 400 °C curtain gas temperature, and 10 V collision energy with a spread of 45 V for fragmentation were used. Fragment masses (50- 400 Da) were scanned. Phytohormones were identified based on their retention time, and MS/ MS profile matches with the pure standard. All the major phytohormones [ABA, JA, SA, IAA, IBA, GA, cytokinins-Zeatin riboside (ZR), kinetin (K), and 6-benzyl amino purine (6BAP)] were studied. Quantitation was done using the standard curve (0.24 ng ml^{-1}- 500 ng ml^{-1}) method. Standards were prepared using pure compounds procured from Sigma-Aldrich, India.

3.2.7. ESFB oral secretion (OS) and excreta collection and their effect on fruit-phytohormone level

It is known that the plant defense is induced by the OS of folivores, especially the lepidopteran ones. ESFB larvae deposit both OS and excreta inside the fruit while feeding. We hypothesized that their OS and excreta induce response in the fruit. To test this

hypothesis, we infiltrated them into the mechanically bored fruits. For the OS treatment (treatment- OS), 5 µl OS of the early third instar larva mixed with 15 µl of sterile Milli-Q water was infiltrated. Similarly, a fresh 150 mg excreta (of the early-third instar larvae) was infiltrated into the mechanically bored fruits (treatment- E). The pH of OS was between 8.8 and 9.0, and that of excreta was between 5.0 and 5.2. Therefore, 20 µl sterile Milli-Q water of pH 8.8-9.0 and 20 µl sterile Milli-Q water of pH 5.0-5.2 were used as OS-solvent control (treatment- SOS) and excreta-solvent control (treatment- SE), respectively. For the OS+ excreta treatment, 20 µl of OS mixture (5 µl of OS+ 15 µl of Milli-Q water) and 150 mg excreta were mixed and infiltrated (treatment- OE). As a control, a mixture of 20 µl of OS-solvent and 20 µl of excreta-solvent was infiltrated (treatment- SOE). Untreated fruits were also used as negative controls (treatment- Un). Five replicates were used for each treatment (n= 5). Fruit tissues were collected on 3rd DPT for analyzing their phytohormone levels.

3.2.8. Exogenous ABA infiltration into fruits and its effect on eggplant's flowering

To ascertain the role of ABA in flowering, ABA was infiltrated into fruits. To prepare ABA main stock solution, 10 mg (37.83 µmoles) of ABA was dissolved in 1 N NaOH, and the pH was adjusted to 6.8. The concentration of ABA working stock solution was such that after infiltration, the total ABA content of fruit became equivalent to the induced amount [3 days post frugivory (DPF)≡ 5.75 µmol (mean± SE) of ABA induction per fruit of average weight 35 g]. 300 µl of ABA solution was infiltrated into mechanically bored fruits (ABA+ MB). As controls, we used only solvent (pH- 6.8) infiltrated fruits (solvent+ MB) and untreated fruits (untreated). Flowers, flower buds, and fruits were counted every alternate day till the 10th DPT. Ten biological replicates were analyzed per treatment.

3.2.9. ABA application on leaves adjacent to the apical buds and its effect on eggplant's flowering

To find whether ABA induction in $Leaves_{Sink}$ is associated with flowering, exogenous ABA application on leaves was made. The concentration of ABA solution (pH- 6.8) was such that after application, the total ABA content of the leaf became equivalent to the induced amount [0.09 µmol (mean± SE) per leaf of average weight 1.2 g]. 300 µl of ABA was applied on leaves with a pipette and gently spread over both leaf surfaces using a paintbrush. As controls, we used solvent (pH- 6.8) infiltrated fruits (MB+ solvent) and

untreated fruits (untreated). Flowers and flower buds were counted for the next two days. Ten biological replicates were analyzed per treatment.

3.2.10. Fruit-to-leaf transport of ABA

To determine whether frugivory-induced ABA moved from the fruits to the leaves adjacent to the apical bud, deuterium-labeled ABA, (+)-d6-ABA (d6-ABA), was infiltrated into fruits, and its level was monitored in $Leaves_{Sink}$.

Before the d6-ABA infiltration experiment, the time required for ABA transport from the fruit to the leaf was determined. Temporal kinetics of leaf ABA levels upon ABA infiltration into fruits was recorded. For that, ABA (5.75 µmol $fruit^{-1}$ as calculated in section 3.2.8., pH- 6.8) was infiltrated into fruit, and ABA concentration was measured in the $Leaves_{Sink}$ after 0.5^{th}, 1^{st}, 1.5^{th}, 2^{nd}, and 3^{rd} DPT using LCMS following the same method described previously in section 3.2.6. Solvent+ MB treated and untreated fruits were used as controls. Three to four biological replicates were used.

After the travel time of ABA from fruit to leaf was determined, d6-ABA was infiltrated into fruits, and its concentration was measured in leaves. d6-ABA was purchased from Cayman Chemical Company, USA. 450 µg (1.66 µmol) d6-ABA was dissolved in 300 µl 1 N NaOH, the pH of the solution was adjusted to 6.8, and it was infiltrated into each fruit. Solvent+ MB treated and untreated fruits were used as controls. Four biological replicates per treatment were collected.

ABA and d6-ABA were differentiated from each other based on their fragment masses, as described in table 3.1. The concentration of d6-ABA was measured using the standard curve method described in section 3.2.6. Solvent+ MB treated and untreated fruits were used as controls.

Table 3.2 List of fragment masses used to identify ABA and d6-ABA.

No.	ABA	d6-ABA
1	153.0924	159.1297
2	203.1081	207.1341
3	219.1394	225.1468
4	136.0533	141.0861
5	122.0378	128.0776
6	125.061	125.0562

No.	ABA	d6-ABA
7	248.1064	251.124

3.2.11. Identification of genes, RNA isolation, cDNA synthesis, and qRT-PCR-based transcript profile

To understand whether ABA is biosynthesized in the fruits and $Leaves_{Sink}$ or transported from other parts of the plant, we studied the transcript dynamics of the rate-limiting gene of ABA biosynthesis, *9-cis-epoxycarotenoid dioxygenase* (*NCED*) (Xiong & Zhu, 2003) across treatments (ESFB+ MB, MB, and untreated). We obtained known *NCED* sequences of Solanaceae members from the NCBI database. Those were used as queries to search Eggplant Genome Project Database (Barchi *et al.*, 2019) and Sol Genomics Network ('Sol Genomics Network (SGN)'; Fernandez-Pozo *et al.*, 2015) using the nucleotide-BLAST tool to identify eggplant homologs. Four *SmNCED* homologs were identified as given in table 3.2. qRT-PCR-based relative transcript level (compared with housekeeping gene cyclophilin A) analysis was conducted using primers listed in table 3.3. For every case, four to five biological replicates were studied.

To search for which flowering-related genes, as described in table 3.1, were involved in frugivory-induced flowering, their expression dynamics in $Leaves_{Sink}$, fruits and apical buds were studied. The source of the flowering induction signal was frugivory. To understand whether flowering-related genes were also expressed in the infested fruits, temporal transcript profiles of these genes at fruits were analyzed on the 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} DPT, which is when ABA level was induced. The treatments were ESFB+ MB, MB, and untreated, each with four to five biological replicates. These genes were identified from eggplant following the same protocol as described previously. Details of gene identification are given in table 3.5. qRT-PCR-based relative transcript level (compared with housekeeping gene cyclophilin A) analysis was conducted using primers listed in table 3.6.

For each sample, 150 mg of pulverized tissue was used for RNA isolation. RNA was isolated using RNAiso Plus reagent (Takara, Japan) following the manufacturer's instructions. 500 ng of total RNA was used for cDNA synthesis. cDNA was prepared using the PrimeScript Reverse Transcriptase kit (Takara, Japan) following the manufacturer's instructions. To the 10 µl of cDNA prepared, 90 µl of nuclease-free water was added. 5 µl of qRT-PCR cocktail contained 1 µl of cDNA, 0.25 µl of both forward and reverse primers

with a concentration of 5 µM, and 2.5 µl of SYBR Premix from SYBR Premix Ex Taq II reagent kit (Takara, Japan). CFX96 Touch Real-Time PCR Detection System (Biorad, USA) was set with thermocycling conditions: 95 ºC for one minute, 39 cycles of 95 ºC for 45 seconds, 60 ºC for 15 seconds, 72 ºC for 20 seconds.

3.2.12. Statistical analyses

The homogeneity of quantitative data (mean± SE) was tested using Levene´s test. Homogenous data were analyzed by one-way ANOVA, and the statistical significance was determined by Tukey's *post hoc* test ($P \leq 0.05$). Non-parametric data were analyzed using Kruskal-Wallis and Dunn's *post hoc* test with Bonferroni correction ($P \leq 0.05$). Data from dual-choice assays were analyzed using Student's t-test (2-tailed, $P \leq 0.05$). Values not detected were considered to be zero.

Table. 3.3 Identification of eggplant *9-cis-epoxycarotenoid dioxygenases* (Sm*NCEDs*), which catalyzes the rate-limiting step of ABA biosynthesis.

Sr. No.	Gene name	Details of gene sequence used as query		Eggplant homolog identified from Eggplant Genome Project Database				Eggplant homolog naming
		Organism	NCBI accession ID	Accession ID	Score	E value	Gene location (chromosome number)	
1	*NCED1*	*Lycopersicon esculentum*	NP_001234455.1	SMEL_007g292400.1.01	2097	0.00E+00	7	*SmNCED1_1*
				SMEL_003g177000.1.01	486	1.00E-136	3	*SmNCED1_2*
2	*NCED2*	*Solanum lycopersicum*	NP_001362822.1	SMEL_008g308280.1.01	1939	0.00E+00	8	*SmNCED2*
3	*NCED3*	*Nicotiana tabacum*	AFP57678.1	SMEL_008g308280.1.01	1574	1.00E-163	8	*SmNCED2*
4	*NCED6*	*Solanum lycopersicum*	XP_010321597.1	SMEL_005g227940.1.01	1263	0.00E+00	5	*SmNCED6*

Table. 3.4 List of primers used for quantitative real-time (qRT)-PCRs of S*mNCEDs*.

Gene name	**Primer type**	**Primer sequence**	**Amplicon size**
SmNCED1_1	Forward	ATGGCCACTACTTCTCATGC	167
	Reverse	TGGAGAATAGGAGGAGCTTGAA	
SmNCED1_2	Forward	TTTAGATGGCTGTTGGGATGC	162
	Reverse	CCCAGAAATCTGGACTCGTG	
SmNCED2	Forward	TTTCTCATTCCTTCCCACCA	124
	Reverse	TTTGGGAAAGTGAGTAGAGCAG	
SmNCED6	Forward	TTTGCCAGTGTCACCATCG	204
	Reverse	GGTGCAAAATTCCCTTCTAGC	

Table. 3.5 Identification of eggplant genes involved in flowering.

Sr. No.	Gene name	Details of gene sequence used as query		Eggplant homolog identified from Eggplant Genome Project Database/ Sol Genomics Network				Eggplant homolog naming
		Organism	NCBI accession ID	Accession ID	Score	E value	Gene location (chromosome number)	
1	*SP3D*	*Lycopersicon esculentum*	AY186735.1	SMEL_010g347840.1.01	733	0.00E+00	10	*SmFT*
2	*SP*	*Lycopersicon esculentum*	U84140.1	SMEL_006g266390.1.01	785	0.00E+00	6	*SmSP*
3	*SP5G*	*Solanum lycopersicum*	NM_001321052.1	SMEL_005g228350.1.01	654	0.00E+00	5	*Sm5G*
4	*SP2G*	*Solanum lycopersicum*	NM_001329446.1	SMEL_000g063740.1.01/ SMEL4.1_02g017350.1.01	573	1.00E-163	2	*SmSP2G*
5	*SP6A*	*Solanum lycopersicum*	NM_001329447.1	SMEL_005g231460.1.01	270	9.00E-72	5	*SmSP6A*
6	*SP9D*	*Solanum lycopersicum*	NM_001329448.1	SMEL_009g320660.1.01	654	0.00E+00	9	*SmSP9D*
7	*SPGB*	*Solanum lycopersicum*	NM_001247416.1	SMEL_000g031840.1.01/ SMEL4.1_02g021180.1.01	157	2.00E-37	2	*SmSPGB*
8	*FLC*	*Solanum lycopersicum*	NM_001365682.1	SMEL_005g235860.1.01	319	1.00E-86	5	*SmFLC_1*
				SMEL_005g235850.1.01	276	2.00E-73	5	*SmFLC_2*
9	*CO1*	*Solanum lycopersicum*	NM_001246910.2	SMEL_002g166200.1.01	1005	0.00E+00	2	*SmCO1*
10	*CO2*	*Lycopersicon esculentum*	AY490252.1	SMEL_002g166180.1.01	630	1.00E-180	2	*SmCO2*
11	*CO3*	*Solanum lycopersicum*	NM_001329782.1	SMEL_002g166190.1.01	692	0.00E+00	2	*SmCO3*
12	*SOC1*	*Nicotiana tabacum*	JQ686938.1	SMEL_001g130580.1.01	349	2.00E-25	1	*SmSOC1*
13	*GI*	*Solanum tuberosum*	XM_006358978.2 (*StGI.04*)	SMEL_004g214240.1.01	2541	0.00E+00	4	*SmGI_1*
		Solanum tuberosum	XM_006361554.2 (*St.GI.12*)	SMEL_005g235360.1.01	3915	0.00E+00	5	*SmGI_2*
14	*AP1*	*Solanum tuberosum*	GU220568.1	SMEL_010g351360.1.01	561	1.00E-159	10	*SmAP1*
15	*LFY*	*Solanum tuberosum*	EU371047.1	SMEL_003g195070.1.01	803	0.00E+00	3	*SmLFY*

Gene accession numbers are collected from the Eggplant Genome Project Database. For the genes whose chromosome numbers were not mentioned in this database, their accession numbers are collected from the Sol Genomic Network. To keep accession numbers from the Sol Genomic Network separate, red font is used.

Table. 3.6 List of primers used for qRT-PCRs of eggplant's flowering-related genes.

Gene name	Primer type	Primer sequence	Amplicon size
SmSP3D	Forward	TGGTAGTTGGTCGTGTGGTAGG	195
	Reverse	CATCAGGGTCCACCATAACC	
SmSP	Forward	ATTCTTTCCTTCCTCAGTCACC	160
	Reverse	GCCTGGAATGTCTGTGACG	
SmSP5G	Forward	ACAATAGGGTGGTCTACAATGG	150
	Reverse	TCAAGTTTGGGTTGCTAGGG	
SmSP2G	Forward	AATGGCTGATCCAGATGCTC	174
	Reverse	AACAGCGCGAACACGTATC	
SmSP6A	Forward	TCCATTGATTGTTGGTCGTG	205
	Reverse	GTTGCTGGGATATCTGTGACC	
SmSP9D	Forward	CAATGGCCATGAACTTTTCC	182
	Reverse	CATCAGTTGTGCCAGGAATG	
SmSPGB	Forward	GACACCAGGGGACATTCAAC	204
	Reverse	GCAATCTTGCAAAGTCATGC	
SmFLC_1	Forward	GCCCAAAGAGACTGAACACC	176
	Reverse	GGCTCTTGTATCGGTGAAGC	
SmFLC_2	Forward	ATGGGGCGAAGAAAGGTAG	196
	Reverse	TCCCTGTCACACTGTTAGTGC	
SmCO1	Forward	AGTGCCCCATAAGTCTACAGC	185
	Reverse	GGAATGGCAATTATCACACACC	
SmCO2	Forward	GACAGTTGCCATTCCACTG	168
	Reverse	CTTGCAAAGAAAGGGTGCAG	
SmCO3	Forward	GGATTCCACCTATTTGTGTTCG	185
	Reverse	GGAGGTTGGCAGAATGGATG	
SmSOC1	Forward	GAGTTCAACCTGAAAACCAAGC	132
	Reverse	TGCAGGACTGAAGACCTTCTC	
SmGI_1	Forward	CTGAACCATTGGAAGCACACC	207
	Reverse	TGCCCTGTATAACCTCGTTTG	
SmGI_2	Forward	GTTCCTGCACTTCTGCTTCC	208

Gene name	Primer type	Primer sequence	Amplicon size
	Reverse	CCACTAGTTGTACCGCAGCATC	
SmAP1	Forward	GGAACAACAGCTTGATTCTGC	142
	Reverse	CCTTCTCCTTGAGCTTCTTGG	
SmLFY	Forward	CAAGTGGGACCCAAGAGG	124
	Reverse	CAAGTGGGACCCAAGAGG	

3.3. Results

3.3.1. ESFB frugivory induces flowering and fruiting

To assess whether ESFB infestation causes flowering induction, flowers, flower buds, and new fruits of eggplant branches containing ESFB-infested and healthy fruits were counted. Two times induction in flowers+ flower buds and five times induction in new fruits were observed on eggplant branches with infested fruits (flowers+ flower buds- 10.15± 0.90 and new fruits- 1.45± 0.28) as compared to healthy fruits (flowers+ flower buds - 5.35± 0.68 and new fruits- 0.75± 0.14) (Fig. 3.1a, b).

Eggplants of the same age were used in all the experiments. Flowers, flower buds, and new fruits of eggplant branches containing fruits of age 10 DAP (Fig. 3.2a) were counted. The counting was done for treatments ESFB+ MB, MB, and untreated (Fig. 3.2b) in apical and axillary buds (Fig. 3.2c). Eggplant branches having ESFB+ MB treatment had a significantly high number of flowers+ flower buds in apical buds (>0.5-fold) on 6^{th} DPT (8.7± 0.65) when compared with MB (6.11± 0.62), and untreated (6.11± 0.74) (Fig. 3.2d). With time, the flowers+ flower buds count for ESFB+ MB increased to two-times on the 10^{th} DPT compared to other treatments (10^{th} DPT; ESFB+ MB- 13± 1.12, MB- 5.89± 1.02, untreated- 6.33± 0.6; Fig. 3.2d). Axillary buds did not show flowering induction (Fig. 3.2e). Like flowering, the apical buds showed induction in fruiting on 10^{th} DPT (ESFB+ MB- 1± 0.3, MB- 0.33± 0.14, untreated- 0.3± 0.21; Fig. 3.2f). Axillary buds did not show any fruiting induction (Fig. 3.2g).

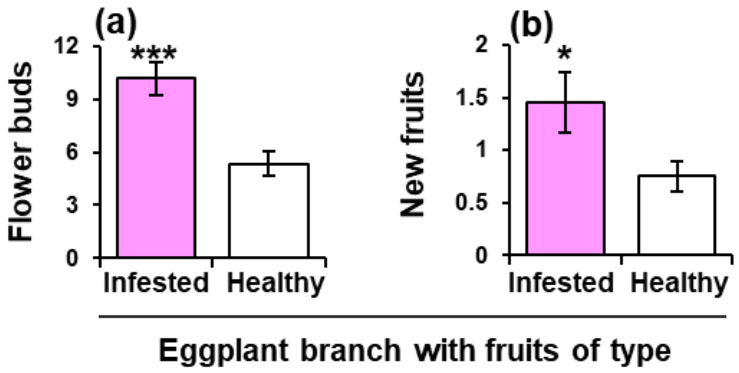

Fig. 3.1 Eggplant shoot and fruit borer (ESFB)-infested fruit-bearing eggplant branches have more flowers, flower buds, and fruits compared to healthy fruit-bearing ones. Counts of (a) flowers+ flower buds (mean± SE) and (b) new fruits (mean± SE) in apical buds of eggplant branches (n= 20), containing infested v/s healthy fruits, of PanchaGanga plants at agricultural fields in Pune, Maharashtra, India (18.5204° N, 73.8567° E), are plotted. Asterisks over the error bars indicate significant differences determined using Student's 2-tailed t-test (***≡ p< 0.001; *≡ p< 0.05).

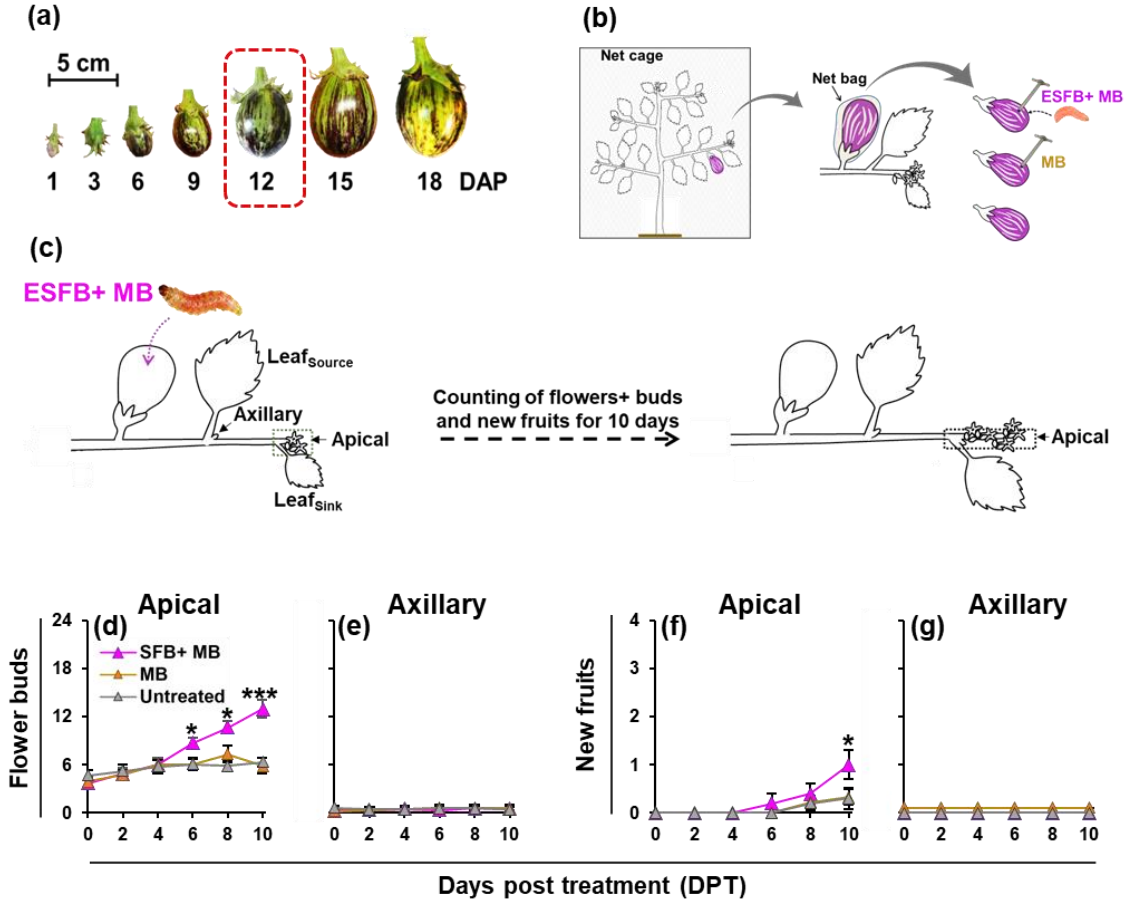

Fig. 3.2 Controlled ESFB frugivory induces flowering and fruiting at the apical buds. (a) Fruits, (12 days after pollination; DAP), were selected for all the experiments. (b) Schematic showing the experimental set-up. Plants were caged to avoid natural ESFB infestation, and predation on manually introduced ESFB larvae. Fruits were bored (mechanical boring- MB) for ESFB introduction (ESFB+ MB). Untreated fruits, and only mechanically bored (MB) fruits were used as controls. All fruits were bagged to avoid larval escape. (c) Schematic of eggplant branch with fruit. One early third instar ESFB Larva was introduced into fruits on 0^{th} day. For next ten days, flowers+ flower buds, and new fruits in the apical and axillary buds were counted for all three treatments (n= 8 or 9). Flower+ flower bud count (mean± SE) in (d) apical (6^{th} DPT- $F_{2, 24}$= 5.02, p=0.015; 8^{th} DPT- $F_{2, 24}$= 8.88, p= 0.001; 10^{th} DPT- $F_{2, 24}$= 14.7, p= 6.8E-05), and (e) axillary buds. New fruit count (mean± SE) in (f) apical (10^{th} DPT- $F_{2, 21}$= 5.39, p= 0.01), and (g) axillary buds. Asterisks above the error bars indicate significant differences determined using one-way ANOVA ($p \leq 0.05$) with Tukey's HSD *post hoc* test or Welch ANOVA with Games-Howell *post hoc* test.

3.3.2. Frugivory induces ABA in fruits, stem parts, and sink leaves

To determine the involvement of phytohormones in ESFB-associated fruit-shoot signaling that promotes flowering and track the movement of the signaling molecule, temporal phytohormone kinetics were generated. Out of nine phytohormones studied, three (ABA, JA, and SA) could be detected (based on retention time and MSMS fragmentation pattern matches with standards). The plant parts used for the LCMS-based phytohormone profile were fruit mesocarps, fruit pedicels, stem sections between the fruit to apical bud route ($Stem_1$, $Stem_2$, and apical bud), and leaves (both $Leaves_{Source}$, and $Leaves_{Sink}$) (Fig. 3.3a).

ABA was the only phytohormone found to be induced in ESFB-infested fruit mesocarps and pedicels compared to controls. In mesocarp, the significant ABA induction was observed on 2^{nd} DPT (~3-fold; ESFB+ MB- 154.33± 35.38 nmol g^{-1}, MB- 31.62± 3.25 nmol g^{-1}, and untreated- 35.38± 9.5 nmol g^{-1}), followed by the highest induction on 3^{rd} DPT (~7-fold; ESFB+ MB- 254.85± 36.31 nmol g^{-1}, MB- 30.73± 2.03 nmol g^{-1}, and untreated- 25.73± 3.29 nmol g^{-1}), and continued to 4^{th} DPT (~5-fold; ESFB+ MB- 122.55± 37.22 nmol g^{-1}, MB- 19.27± 2.93 nmol g^{-1}, and untreated- 13.61± 1.18 nmol g^{-1}) (Fig. 3.3b). In pedicel, the significant ABA induction was observed on 4^{th} DPT (>2.5-fold; ESFB+ MB- 657.95± 132.77 nmol g^{-1}, MB- 177.42± 4.86 nmol g^{-1}, and untreated-164.94± 9.05 nmol g^{-1}), and continued to 5^{th} DPT (>0.5-fold; ESFB+ MB- 286.78± 33.75 nmol g^{-1}, MB- 155.06± 11.07 nmol g^{-1}, and untreated- 163.8± 7.86 nmol g^{-1}) (Fig. 3.3c).

At $Stem_1$, ABA induction began on 2nd DPT for ESFB+ MB treatment (200.86± 15.64 nmol g^{-1}) in comparison to others (MB- 107.7± 20.52 nmol g^{-1}, and untreated- 75.69± 15.33 nmol g^{-1}), and continued till 4th DPT (>2-fold; ESFB+ MB- 185.23± 8.40 nmol g^{-1}, MB- 48.08± 11.82 nmol g^{-1}, and untreated-59.34± 14.73 nmol g^{-1}) (Fig. 3.3d). $Stem_2$ and apical bud did not show ABA induction (Fig. 3.3e, f).

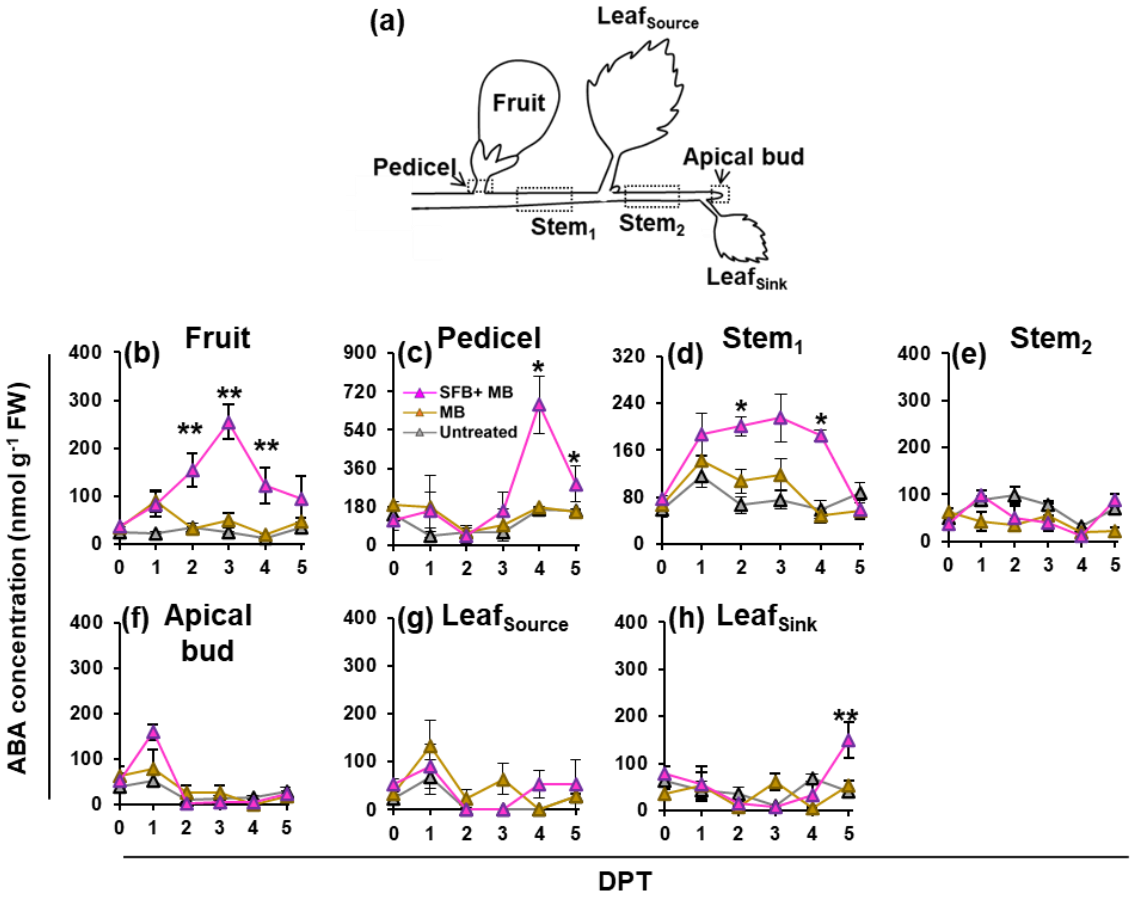

Fig. 3.3 ESFB infestation induces abscisic acid (ABA) in fruits, stems, and sink leaves. (a) Schematic of eggplant branch with parts labeled. Seven phytohormone levels were analyzed. Out of them, ABA showed significant induction in multiple plant parts. ABA concentration (mean± SE; in nmol g^{-1} FW) in (b) fruit mesocarp (2nd DPT- χ^2= 8.72, p= 0.01; 3rd DPT- F=8.86, df= 5.004, p= 0.023, 4th DPT- χ^2= 11.94, p= 0.002), (c) pedicel (4th DPT- χ^2= 10.5, p= 0.005, 5th DPT- χ^2= 7.5, p= 0.02), (d-e) stem parts ($stem_1$: 2nd DPT- $F_{2, 12}$= 9.392, p= 0.004; 4th DPT- χ^2= 7.538, p= 0.02), (f) apical bud, and (g-h) leaves ($Leaves_{Sink}$: 5th DPT- $F_{2, 9}$= 18.69, p= 0.0006) across treatments (ESFB+ MB, MB and untreated) for timepoints 0th to 5th DPT (n= 4 to 5). Asterisks above the error bars indicate significant differences ($p \leq 0.05$) determined using one-way ANOVA with Tukey's HSD *post hoc* test or Kruskal-Wallis with Dunn's *post hoc* test (sequential Bonferroni significance).

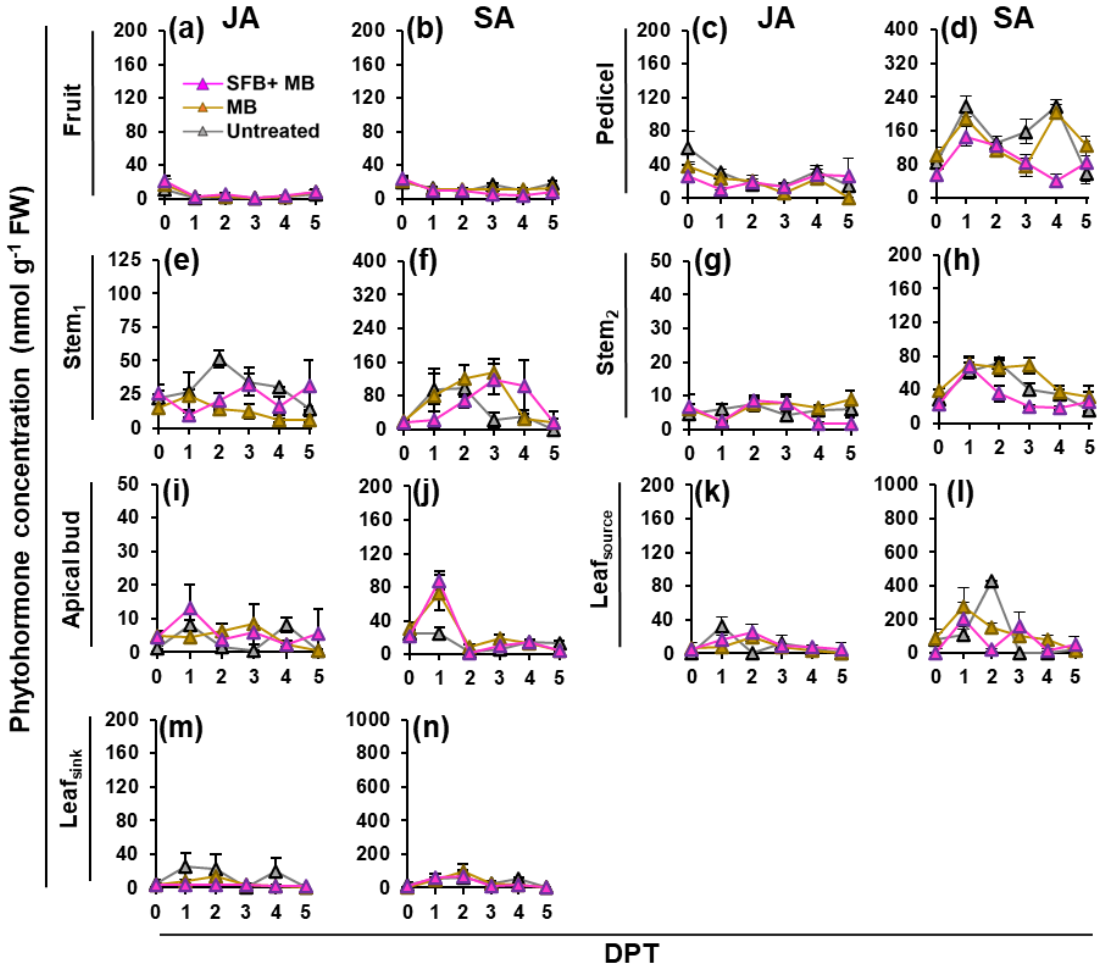

Fig. 3.4 Temporal phytohormone kinetics of eggplant parts. Seven phytohormones were analyzed. Out of them, jasmonic acid (JA), salicylic acid (SA), and ABA (figure 4) were detected. JA, and SA levels (mean± SE; in nmol g^{-1} FW) at (a, and b) fruit mesocarp, (c, and d) fruit pedicel, (e-h) stem parts, (i, and j) apical bud, and (k-n) leaves across treatments (ESFB+ MB, MB and untreated) for timepoints 0^{th} to 5^{th} DPT are plotted (n= 4 to 5).

$Leaves_{Source}$ did not show any ABA induction (Fig. 3.3g). However, the $Leaves_{sink}$ showed significant ABA induction compared to controls (>2.5-fold; ESFB+ MB- 149.95± 15.17 nmol g^{-1}, MB- 52.52± 11.82 nmol g^{-1}, and untreated- 39.52± 7.91 nmol g^{-1}; fig. 3.3h)]. JA and SA showed no significant differences in their concentrations at different plant parts across three treatments (Fig. 3.4a-n). From phytohormonal profiling, it could be inferred that frugivory influenced the phytohormone levels of fruits, stems, and sink leaves.

3.3.3. ESFB OS induced ABA synthesis and accumulation in fruit

ESFB deposits both OS and excreta into fruits. To find whether these are individually or together responsible for fruits' ABA induction, an experiment using field plants was conducted. OS (49.87± 1.67 nmol g^{-1}) infiltration alone and together with excreta (46.30±

1.43 nmol g^{-1}) showed a significant induction in ABA level when compared with excreta and all other controls (E- 36.49± 1.52 nmol g^{-1}, SOS- 34.84± 2.45 nmol g^{-1}, SE- 34.27± 1.86 nmol g^{-1}, SOE- 33.97± 1.35 nmol g^{-1} and Un- 36.28± 1.12 nmol g^{-1}) (Fig. 3.5a-b). Other phytohormone levels were also analyzed. JA and SA were detected in the mesocarp but did not show variations in their levels across different treatments.

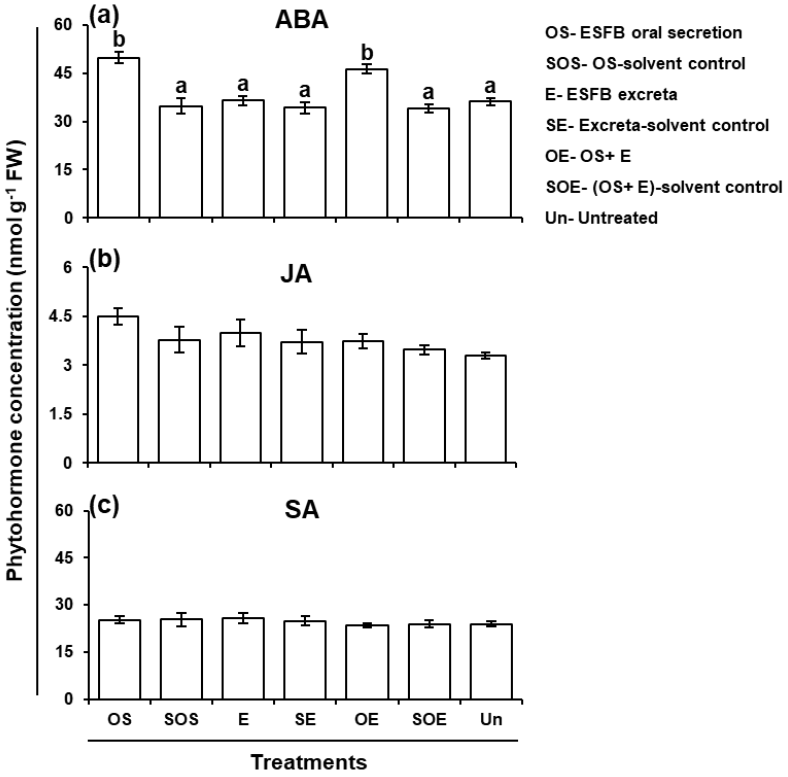

Fig. 3.5 ESFB oral secretion (OS) induces ABA in fruits. Phytohormone profile of mesocarp on 3rd DPT. Out of seven phytohormones profiled, ABA, JA, and SA were detected. Their concentrations (mean± SE; in nmol g^{-1} FW) are given in the following order (a) ABA (χ^2= 17.23, p= 0.008), (b) JA, and (c) SA for treatments OS, OS-solvent (SOS), E, E-solvent (SE), OS+E (OE), (OS+E)-solvent (SOE), and untreated (Un). Different letters above bars denote different significance levels ($p \leq 0.05$) determined using Kruskal-Wallis with Dunn's *post hoc* test (sequential Bonferroni significance).

3.3.4. Exogenous ABA infiltration in fruits induces flowering in apical buds

To assess the effect of fruits' ABA induction on flowering and fruiting, ABA was infiltrated into mechanically bored fruits (simulated frugivory; fig. 3.6a). Like frugivory, the ABA application induced flowering (>0.5-fold) in apical buds. The significant induction was observed on the 4th DPT (ABA+ MB- 6.1± 0.89, solvent+ MB- 3.7± 0.56, and untreated- 3± 0.42; fig. 3.6b). Axillary buds did not show flowering induction (Fig. 3.6c).

The ABA application caused the fruiting (two-times) increase on the 10th DPT in apical buds compared to controls (Fig. 3.6d). Axillary buds showed no fruiting induction (Fig. 3.6e). These results suggested that ABA infiltration into fruits is responsible for flowering and fruiting induction in apical buds.

3.3.5. Exogenous ABA application on leaves close to apical buds induces flowering in the apical bud

Frugivory induced flowering in the apical bud on the sixth DPT. Frugivory also induced ABA in fruits on the second DPT and leaves on the fifth DPT. Also, exogenous ABA infiltration into fruits induced flowering on the fourth DPT. With this background, to find whether ABA accumulation in leaves was associated with flowering, exogenous ABA was applied on leaves, and flowers+ flower buds in apical buds were counted, as shown in fig. 3.7a. Exogenous ABA application resulted in a ~0.7-fold increase in apical bud flowering compared to controls (ABA+ MB- 7.4± 0.77, solvent+ MB- 5.00± 0.49, and untreated- 4.90± 0.38) on 2 DPT (fig. 3.7b). Thus, we inferred that frugivory-associated ABA induction in leaves near apical buds was responsible for flowering induction in apical buds.

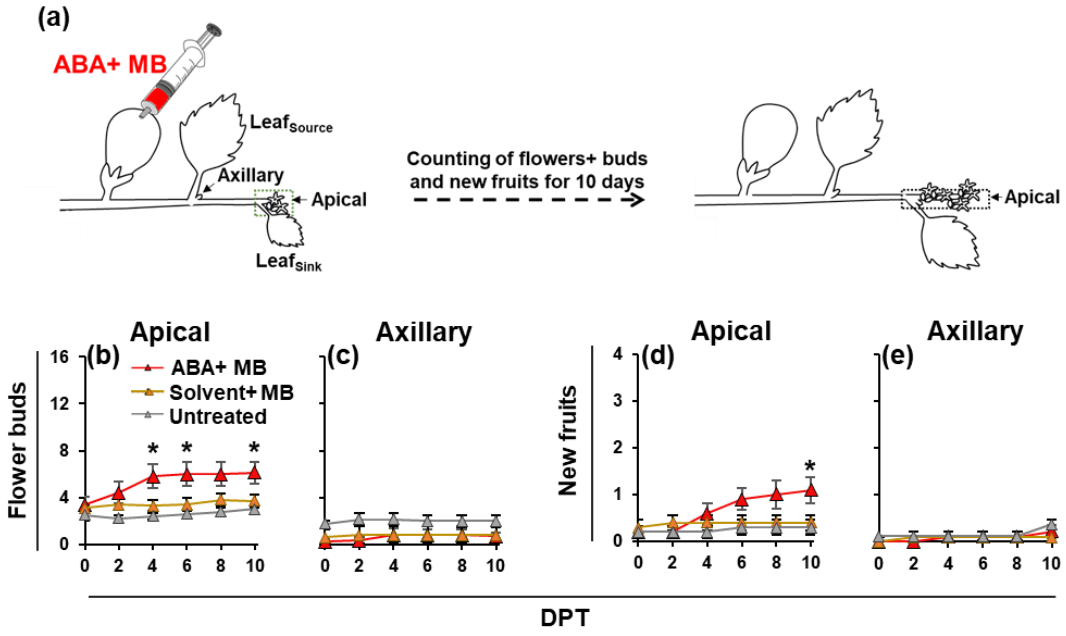

Fig. 3.6 Exogenous ABA infiltration into fruits induces flowering and fruiting. (a) Schematic showing ABA infiltration into fruits. Flowers+ flower buds and new fruits were counted for ten days for all three treatments, ESFB+ MB, MB, and untreated (n= 10). Flower+ flower bud count (mean± SE) of (b) apical (4th DPT- $\chi 2$= 12.5, p= 0.007; 6th DPT- $F_{2, 27}$= 6.62, p= 0.004; 10th DPT- $F_{2, 27}$= 6. 21, p= 0.006), and (c) axillary buds. New fruit count (mean± SE) of (d) apical (10th DPT- $\chi 2$= 5.36, p= 0.03), and (e) axillary buds. Asterisks above the error bars indicate significant differences ($p \leq 0.05$) determined using one-way ANOVA with Tukey's HSD *post hoc* test or Kruskal-Wallis with Dunn's *post hoc* test (sequential Bonferroni significance).

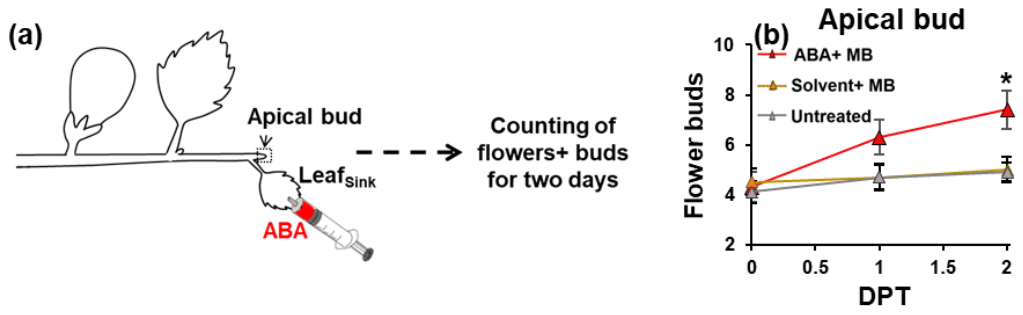

Fig. 3.7 Exogenous ABA application on sink leaves promotes flowering in the apical buds. (a) Schematic showing ABA application on leaves adjacent to the apical bud. Flowering in apical buds of solvent-applied and untreated leaves were used as controls (n= 10). (b) Flower+ flower bud count (mean± SE; 2nd DPT- χ^2= 8.255, p= 0.013) is given. Asterisks above the error bars indicate significant differences ($p \leq 0.05$) determined using Kruskal-Wallis with Dunn's *post hoc* test (sequential Bonferroni significance).

3.3.6. Upon frugivory, ABA is biosynthesized in fruit and then transported to the sink leaves

Since the ABA accumulation in fruits and $Leaves_{Sink}$ was associated with flowering, we asked whether ABA was transported from the fruits to the $Leaves_{Sink}$. For that we analyzed the transcript expression dynamics of the rate-limiting gene of ABA biosynthesis, *SmNCED*. The relative expression variation of all four *SmNCED* homologs was monitored across three treatments (ESFB+ MB, MB, and untreated). It was observed that in frugivory treatment, *SmNCED1_1* level started to increase on the 2nd DPT; it was significantly induced on the 3rd DPT (ESFB+ MB- 1.15± 0.25, MB- 0.42± 0.12, and untreated- 0.15± 0.03), and the induction continued till the 4th DPT (ESFB+ MB- 0.73± 0.19, MB-0.12± 0.03, and untreated- 0.10± 0.04) (Fig. 3.8A). The relative expression level of other *SmNCED* homologs showed no difference between treatments (Fig. 3.8b-d). This suggested that the ABA accumulation in fruit was associated with its frugivory-induced *SmNCED1_1* biosynthesis.

Frugivory also induced ABA levels in $Leaves_{sink}$ on the fifth day. To find whether ABA is synthesized there or moved from other parts, the *SmNCED* transcript levels were monitored in $Leaves_{sink}$. Surprisingly, *SmNCED1_1* was found to be suppressed on the fifth DPT upon frugivory treatment compared to controls (ESFB+ MB- 0.1± 0.02, MB- 0.28± 0.06 and untreated- 0.26± 0.07; Fig. 3.8e). Other *SmNCED* homologs showed no difference between treatments (Fig. 3.8f-h). We hypothesized that ABA is not biosynthesized in leaves; instead, it is transported from other parts. As it is already found that ABA concentration was induced in fruits upon frugivory and at different parts of the

fruits-to-leaf route successively, it was hypothesized that ABA was transported from the fruit to the leaves.

We infiltrated d6-ABA into fruits to test this hypothesis and traced it in $Leaves_{sink}$ (Fig. 3.9a). Firstly, we determined the travel time of ABA by exogenously infiltrating ABA into fruits and quantifying its level at $Leaves_{sink}$. The highest ABA level was monitored on the 1st DPT, and it decreased in later time points (Fig. 3.9b). Hence, when d6-ABA was infiltrated into fruits, its level in $Leaves_{sink}$ was monitored after one day. It could be detected in the leaves after one day (Fig. 3.9c). Thus, we inferred that ABA is transported to the $Leaves_{sink}$ from the fruits.

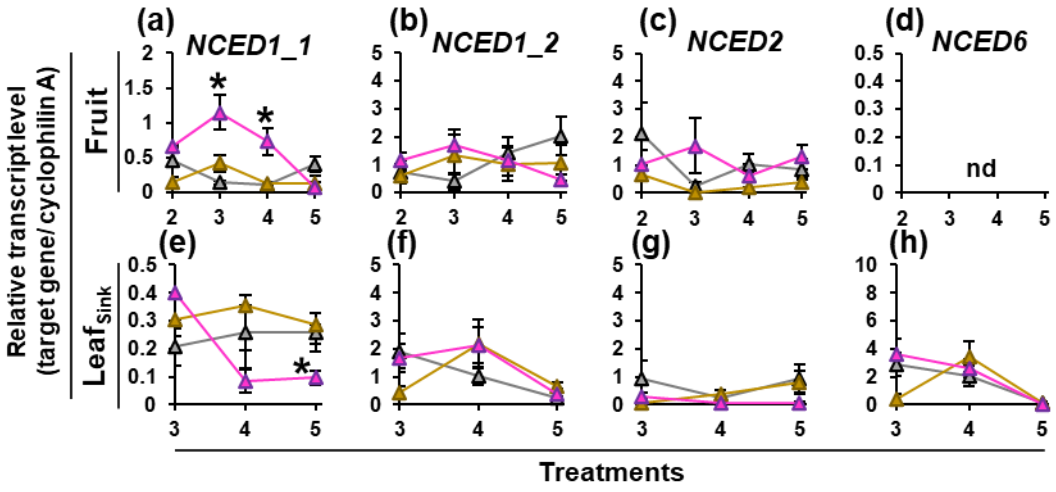

Fig. 3.8 ESFB frugivory induces Sm*NCED1_1* transcripts in fruits and reduces them in sink leaves. Transcript abundance (mean± SE; relative to cyclophilin A; n= 3 to 5) of the rate-limiting gene of ABA biosynthesis, *SmNCED*, across treatments ESFB+ MB, MB, and untreated on 2nd to 5th DPT [(a) *NCED1_1* (3rd DPT: χ^2= 6.038, p= 0.048; 4th DPT: χ^2= 6.08, p= 0.048), (b) *NCED1_2*, (c) *NCED2*, (d) *NCED6*] in fruits, and on 3rd to 5th DPT [(e) *NCED1_1* (5th DPT: χ^2= 6.02, p= 0.049), (f) *NCED1_2*, (g) *NCED2* and (h) *NCED6*] in sink leaves are plotted. Asterisks above the error bars indicate significant differences ($p\leq$ 0.05) determined using Kruskal-Wallis with Dunn's *post hoc* test (sequential Bonferroni significance).

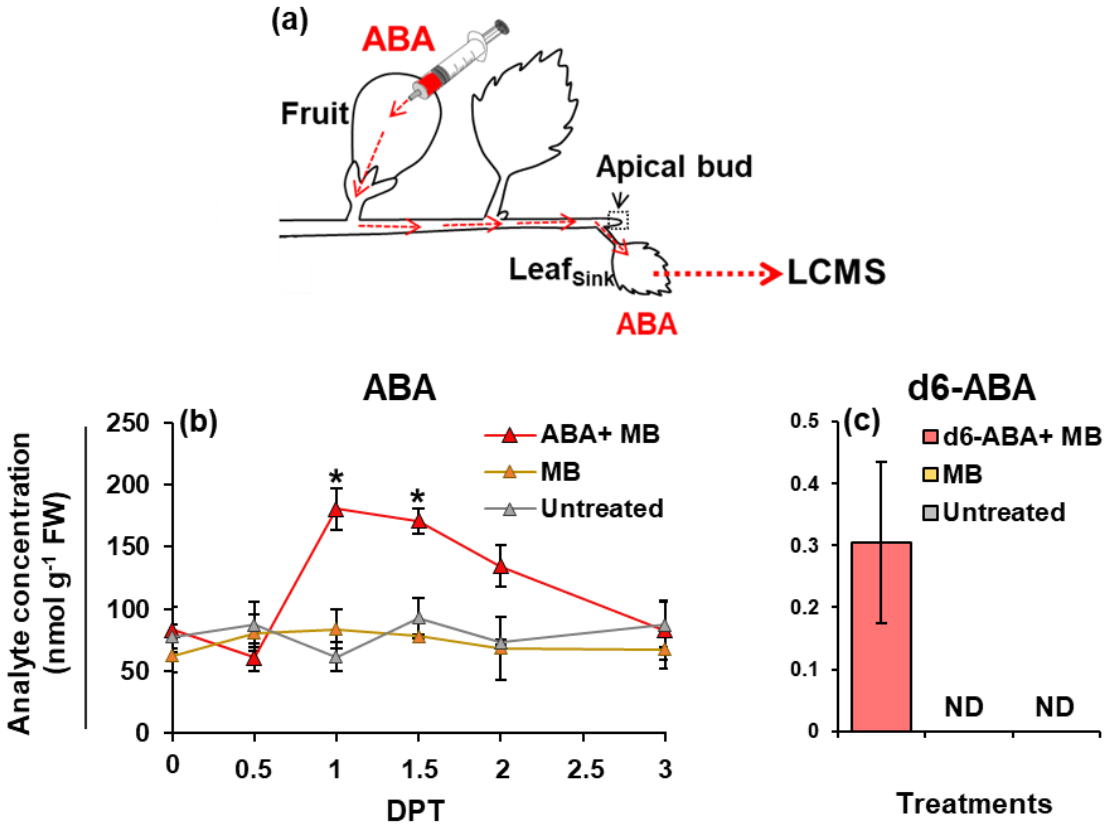

Fig. 3.9 ABA-transport from fruits to sink leaves. (a) Schematic of eggplant branch showing the possible route of ABA movement. ABA/ labelled ABA (d6-ABA) was infiltrated into fruits (ABA+ MB/ d6-ABA+ MB) and its concentration (mean± SE; in nmol g^{-1} FW) was measured in $leaves_{Sink}$. Solvent+ MB, and untreated controls were used. (a) ABA movement temporal kinetics: ABA concentration in $leaves_{Sink}$ on 0, 0.5, 1, 1.5, 2, 2.5, and 3rd DPT (1st DPT- $F_{2, 9}$= 13.92, p= 0.002; 2nd DPT- $F_{2, 9}$= 20.19, p= 0.0005). (b) d6-ABA concentration in $Leaves_{sink}$ on 1st DPT. ND denotes no detection. Asterisks above the error bars indicate significant differences ($p \leq 0.05$) determined using one-way ANOVA with Tukey's HSD *post hoc* test or Kruskal-Wallis with Dunn's *post hoc* test (sequential Bonferroni significance).

3.3.7. ESFB infestation changes flowering-related gene expression levels

Transcription dynamics of flowering-related genes were monitored to understand the mechanisms of frugivory-associated flowering. In $Leaves_{sink}$ (Fig. 3.10a-p), upon frugivory treatment (ESFB+ MB), the floral inducer *SmSP3D* (ESFB+ MB- 0.35 ± 0.18, MB- 1.98 ± 0.47, and untreated- 1.53 ± 0.34; Fig. 3.10a), and repressor *SmSP5G* (ESFB+ MB- 1.17 ± 0.37, MB- 22.75 ± 3.16, and untreated- 11.05 ± 4.69; Fig. 3.10c) were significantly reduced, when compared with controls- MB, and untreated. The floral repressor *SmSP* showed a two-times reduction. However, the difference between frugivory treatment and controls was insignificant (ESFB+ MB- 0.01 ± 0.001, MB- 0.03 ± 0.01, and untreated- 0.04 ± 0.01; Fig. 3.10b). Floral inducer *SmSOC1* showed a two-times nonsignificant induction (ESFB+ MB- 1.53 ± 0.52, MB- 3.59 ± 0.26, and untreated- 2.58 ± 0.56; Fig. 3.10l). In the apical buds (Fig. 3.10q-af), the floral inducer *SmSP3D* was induced upon frugivory (ESFB+ MB- 2.05 ± 0.55, MB- 0.37 ± 0.34, and untreated- 0.61 ± 0.3; Fig. 3.10ae). The floral homeotic gene, *SmAP1* showed a two-times induction upon frugivory (ESFB+ MB- 1.47 ± 0.37, MB- 0.88 ± 0.11, and untreated- 0.92 ± 0.14; Fig. 3.10ae). However, it was not significant. Floral repressor *SmSP5G* showed a two-times reduction upon frugivory (ESFB+ MB- 0.33 ± 0.06, MB- 0.47 ± 0.17, and untreated- 0.65 ± 0.09; Fig. 3.10s). However, it was not a significant difference. Floral inducer *SmCO2* showed significant induction in fruits upon frugivory treatment. *SmAP1* also showed a two-times induction upon frugivory (NS) (Fig. 3.11). Other genes did not show any differences in their transcript levels.

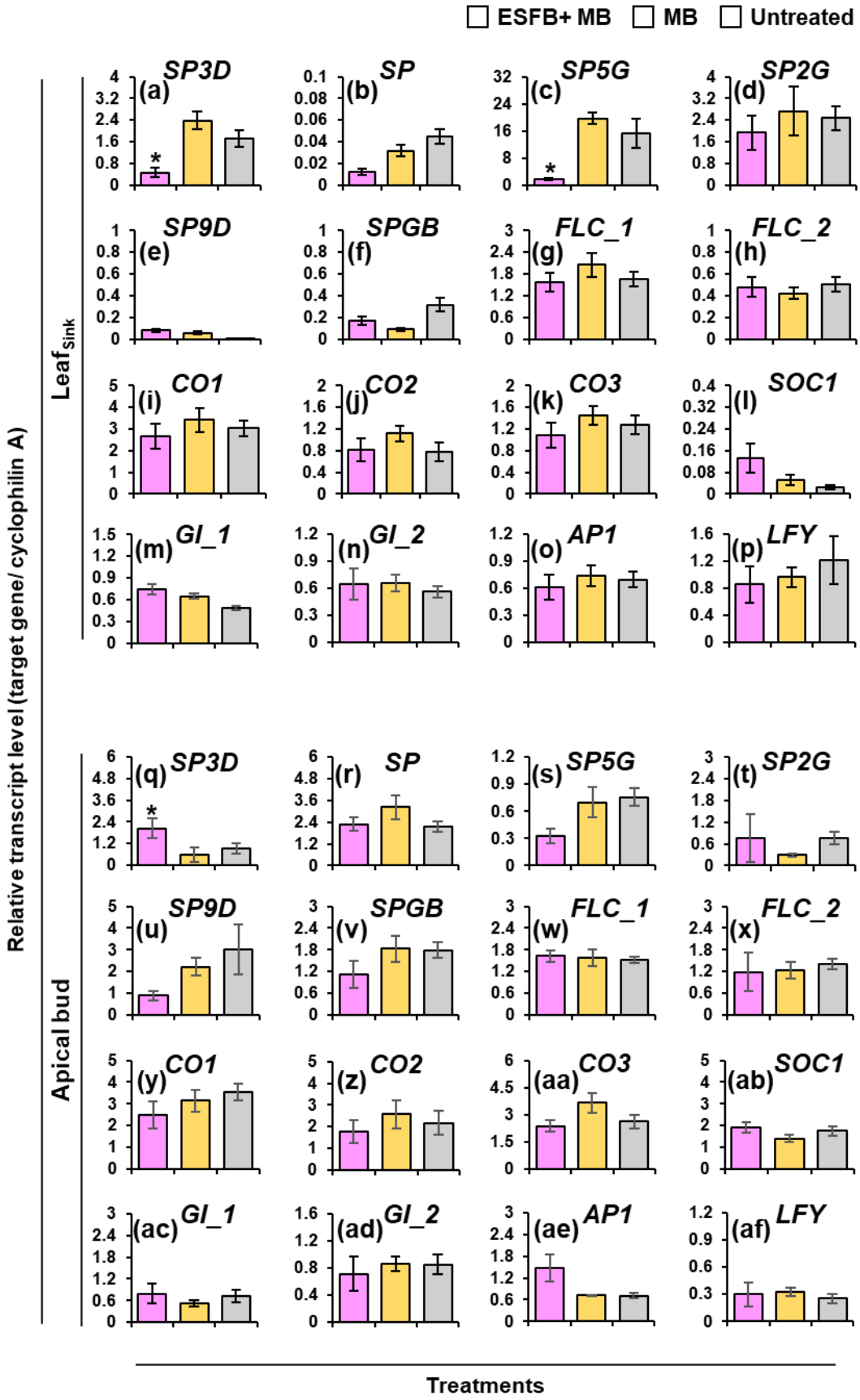
ESFB+ MB
MB
Untreated
Relative transcript level (target gene/ cyclophilin A)
Leaf Sink
Apical bud
(a) SP3D
(b) SP
(c) SP5G
(d) SP2G
(e) SP9D
(f) SPGB
(g) FLC_1
(h) FLC_2
(i) CO1
(j) CO2
(k) CO3
(l) SOC1
(m) GI_1
(n) GI_2
(o) AP1
(p) LFY
(q) SP3D
(r) SP
(s) SP5G
(t) SP2G
(u) SP9D
(v) SPGB
(w) FLC_1
(x) FLC_2
(y) CO1
(z) CO2
(aa) CO3
(ab) SOC1
(ac) GI_1
(ad) GI_2
(ae) AP1
(af) LFY
Treatments

Fig. 3.10 ESFB frugivory-associated transcript dynamics of floral genes in sink leaves, and apical buds. Transcript abundance (mean± SE; relative to cyclophilin A; n= 3 to 5) of eggplant genes of treatments ESFB+ MB, MB, and untreated (as described in fig. 2) for the time-point five DPT are plotted as follows- (a) *SP3D* ($F_{2, 6}$= 6.69, p= 0.03), (b) *SP*, (c) *SP5G* ($F_{2, 12}$= 9.27, p= 0.004), (d) *SP2G*, (e) *SP9D*, (f) *SPGB*, (g) *FLC_1*, (h) *FLC_2*, (i) *CO1*, (j) *CO2*, (k) *CO3*, (l) *SOC1*, (m) *GI_1*, (n) *GI_2*, (o) *AP1*, and (p) *LFY*, in the $Leaves_{sink}$; (q) *SP3D* ($F_{2, 12}$= 8.134, p= 0.006), (r) *SP*, (s) *SP5G*, (t) *SP2G*, (u) *SP9D*, (v) *SPGB*, (w) *FLC_1*, (x) *FLC_2*, (y) *CO1*, (z) *CO2*, (aa) *CO3*, (ab) *SOC1*, (ac) *GI_1*, (ad) *GI_2*, (ae) *AP1*, and (af) *LFY* in the apical buds. Asterisks above the error bars indicate significant differences determined using Student's 2-tailed t-test (***≡ p< 0.001; *≡ p< 0.05) or one-way ANOVA (p≤ 0.05) with Tukey's HSD *post hoc* test or Welch ANOVA with Games-Howell *post hoc* test.

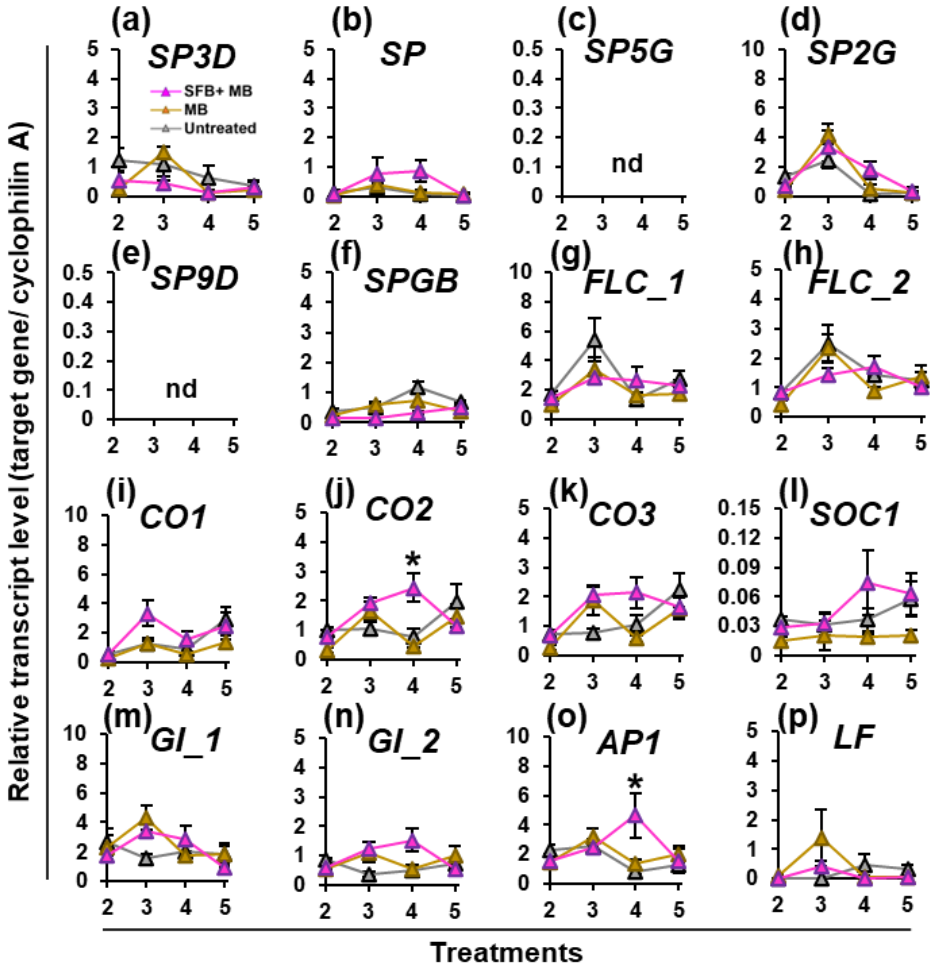

Fig. 3.11 ESFB frugivory-associated transcript dynamics of floral genes in fruit mesocarps. Transcript abundance (mean± SE; relative to cyclophilin A; n= 3 to 5) of eggplant genes of treatments ESFB+ MB, MB, and untreated (as described in fig. 2) for the time-point 2nd to 5th DPT are plotted as follows- (a) *SP3D* (b) *SP*, (c) *SP5G*, (d) *SP2G*, (e) *SP9D*, (f) *SPGB*, (g) *FLC_1*, (h) *FLC_2*, (i) *CO1*, (j) *CO2* (4th DPT: X_2= 6.26, p= 0.04), (k) *CO3*, (l) *SOC1*, (m) *GI_1*, (n) *GI_2*, (o) *AP1* (4th DPT: X_2= 11.38, p= 0.003), and (p) *LFY*, in the fruit mesocarps. Asterisks above the error bars indicate significant differences (p≤ 0.05) determined using one-way ANOVA with Tukey's HSD *post hoc* test or Kruskal-Wallis with Dunn's *post hoc* test (sequential Bonferroni significance).

3.4. Discussion

The plant reproductive organ is fruit. Fruits, an excellent energy source, are prone to attack by myriad antagonists, like microbes, insect frugivores, other invertebrates, and vertebrates (Nevo *et al.*, 2017b). Often, it is assumed that defense metabolite production in ripe fruits is ecologically costly as it can deter seed-dispersal mutualists (Whitehead & Bowers, 2014b). At the same time, plants must respond to protect unripe fruits from pre-dispersal antagonists (Nevo *et al.*, 2017b; Maynard *et al.*, 2020). While all the current knowledge regarding plant responses to insect herbivory is limited to lepidopteran folivory, this study tried to understand the fruit's induced responses to lepidopteran frugivory. We focused on eggplant fruits' responses to ESFB frugivory.

Our field observations suggested more flowering and fruiting in apical buds of ESFB-infested fruit-bearing eggplant branches. To test whether the increase in flowering is due to frugivory, we conducted a controlled ESFB infestation experiment. A direct association was documented between frugivore infestation and flowering induction in apical buds. Previously, several studies reported that insect herbivory induces plant species-specific changes in floral traits (Schiestl *et al*., 2014; Rusman *et al*., 2019a, b). Very few studies documented early flowering phenotype with herbivory. These reports showed that when herbivory happens at early plant growth stages, the plants tend to flower earlier (Hanley & Fegan, 2007; Hoffmeister *et al.*, 2016; Pashalidou *et al.*, 2020). The herbivory-mediated signaling pathways that bring floral plasticity are understudied. Notably, none of the studies reported herbivory-dependent flowering induction (Mothershed & Marquis, 2000; Hanley & Fegan, 2007; Rusman *et al.*, 2019a), as observed in this study.

Mammalian herbivory-associated overcompensation of vegetative and reproductive tissues is known, which increases plant fitness (Paige, 1992; Massad, 2013). Similarly, the flowering and later fruiting increase in eggplants upon ESFB-frugivory is significant. From an ecological perspective, flowering induction can be explained as compensation for fruit loss which can be directly associated with plants' reproductive fitness. Notably, the frugivory-associated induced flowering phenotype was more pronounced when plants were three to four months older than young plants (data not shown). It can be explained by the fact that in earlier growth stages, plants tend to invest more in growth, whereas, in later stages, reproduction is the priority (Massad, 2013).

Our results showed that the frugivory-induced signal transported from fruit to shoot. ESFB infestation in eggplant fruits causes defense responses by increasing the polyphenol

oxidase and phenol content, a local chemical response (Bhattacharya *et al.*, 2009). No studies have shown evidence of frugivory-mediated long-distance signaling generated from eggplant fruits or other species and transmitted to the apical buds to bring phenotypic changes. A recent study by Reissig *et al.* in 2021 showed that tomato fruits infested by frugivore *Helicoverpa armigera* transmit electrical signals, different from healthy fruits, to the pedicel, and there was a significant increase in reactive oxygen species content in the leaves (Reissig *et al.*, 2021). However, they could not link electrical signals with the leaf responses.

As phytohormones are crucial in regulating plant responses, we analyzed their levels in eggplant fruits, stem sections, apical buds, and leaves before and on 1st, 2nd, 3rd, 4th, and 5th DPF. ABA induction kinetics was monitored from fruits to other parts, starting in the fruits on the 2nd DPT, stem on the 4th DPT, and young leaves on the 5th DPT, which indicated an ABA movement from fruits to young leaves. When ESFB OS was infiltrated into fruits alone or with excreta to find their roles in the frugivory-associated fruits' ABA induction, OS induced ABA. This induced ABA level was lower than frugivory. This is obvious because the frugivore continuously feeds and deposits OS inside the fruit, whereas the OS infiltration treatment was administered only once.

Young leaves are heterotrophic, as they import photoassimilates like a sink along with autotrophy (Turgeon, 1989). No other phytohormone levels varied in the other plant parts. We inferred that frugivory induces ABA in fruits, and it moves to the sink leaves through stems; hence, it is a sink-sink transport. Together, we inferred that frugivory, or frugivore's OS, induces ABA in fruits and moves to the leaves. To our best knowledge, this is the first report of fruit's response to frugivory and frugivory-induced chemical signaling.

ABA induction in leaves is also known to induce flowering during drought escape (Riboni *et al.*, 2016). Exogenous ABA applications had positive and negative effects on flowering in different plants (Cui *et al.*, 2013; Conti *et al.*, 2014; Li *et al.*, 2018; Conti, 2019). We asked whether ESFB-mediated ABA induction in fruits and sink leaves is associated with flowering induction. Exogenous ABA applications both in fruits and leaves led to induction in flowering at the apical buds, which suggested an essential involvement of ABA in ESFB's frugivory-induced flowering.

Extensive research has been conducted to understand the flowering biology of angiosperms using the model plant system *Arabidopsis thaliana* (Arabidopsis) (Conti, 2019b; Martignago *et al*., 2020a, 2020b). ABA's role in flowering is yet unclear. ABA has

been shown to both induce and suppress Arabidopsis flowering depending on other endogenous and exogenous factors like photoperiod, age, temperature, etc. (Conti *et al*., 2014; Conti, 2019a; Martignago, 2020b; Schroeder & Kuhn, 2006). ABA binds to PYRABACTIN RESISTANCE 1 (PYR1)/ PYR1-like (PYL)/ REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) receptors. This complex inhibits protein phosphatase 2C (PP2C) from dephosphorylating SUCROSE-NON-FERMENTING (SNF1)-related protein 2 (SnRK2) (Banerjee & Roychoudhury, 2017; Siemiatkowska *et al*., 2022). SnRK2 activates several kinases. In turn, kinases activate several transcription factors (Conti, 2019b; Conti *et al*., 2014; Hwang *et al*., 2019; Y. Wang *et al*., 2013, 2019; Xiong *et al*., 2019), which promote the expression of ABA-responsive FTGs- florigens and anti-florigens (Gawarecka & Ahn, 2021, Odgerel *et al*., 2022; Siemiatkowska et al., 2022; Xing *et al*., 2015; Zhu *et al*., 2020). In eggplant, we monitored florigen *SmSP3D* induction and anti-florigen *SmSP5G* reduction in the apical buds. *SmSP3D* was downregulated in leaves. It is possible that the mobile floral regulator *SmSP3D* moved from the leaves to the apical bud and promoted flowering. Another inducer, *SmCO2* was found to be induced in fruit post frugivory (fig. 3.12).

The FTGs regulate meristem identity genes *AP1* and *LFY* (Jin *et al*., 2021; Seibert et al., 2020; Serrano-Mislata *et al*., 2017; S. Wang *et al*., 2022) (fig. 3.12). In Lichi, ABA promoted flowering by indirectly inducing *AP1* (Cui *et al.*, 2013). Similarly, in eggplant, we monitored significant *SmAP1* induction at fruits on the third DPF and an insignificant two-time induction in apical buds. *At*AP1 overexpression suppressed the negative floral regulator *At*TFL1 in Arabidopsis (Andrés & Coupland, 2012). Similarly, here we monitored a suppression in transcript level of *SmSP5G*, the *AtTFL1*-ortholog, both in leaves and the apical buds (fig. 3.12). Taken together, all these results support the flowering induction phenotype.

Together, we showed that eggplant fruits respond to ESFB frugivory using a phytohormone ABA-mediated pathway. Unlike folivory, ESFB frugivory-mediated signaling is JA-independent and ABA-mediated. From an agricultural perspective, ABA applications can be used to increase eggplant yield. From an ecological perspective, such flowering induction may help compensate for the plant's fruit loss or ensure fruits for ESFB's next generation.

This study opens a new area of research on frugivory-mediated fruit and plant response. Several plant-frugivore interactions-related phenomena can be investigated. Outcomes can

be used to enrich the knowledge of plant-insect interacting systems and also in effective pest management.

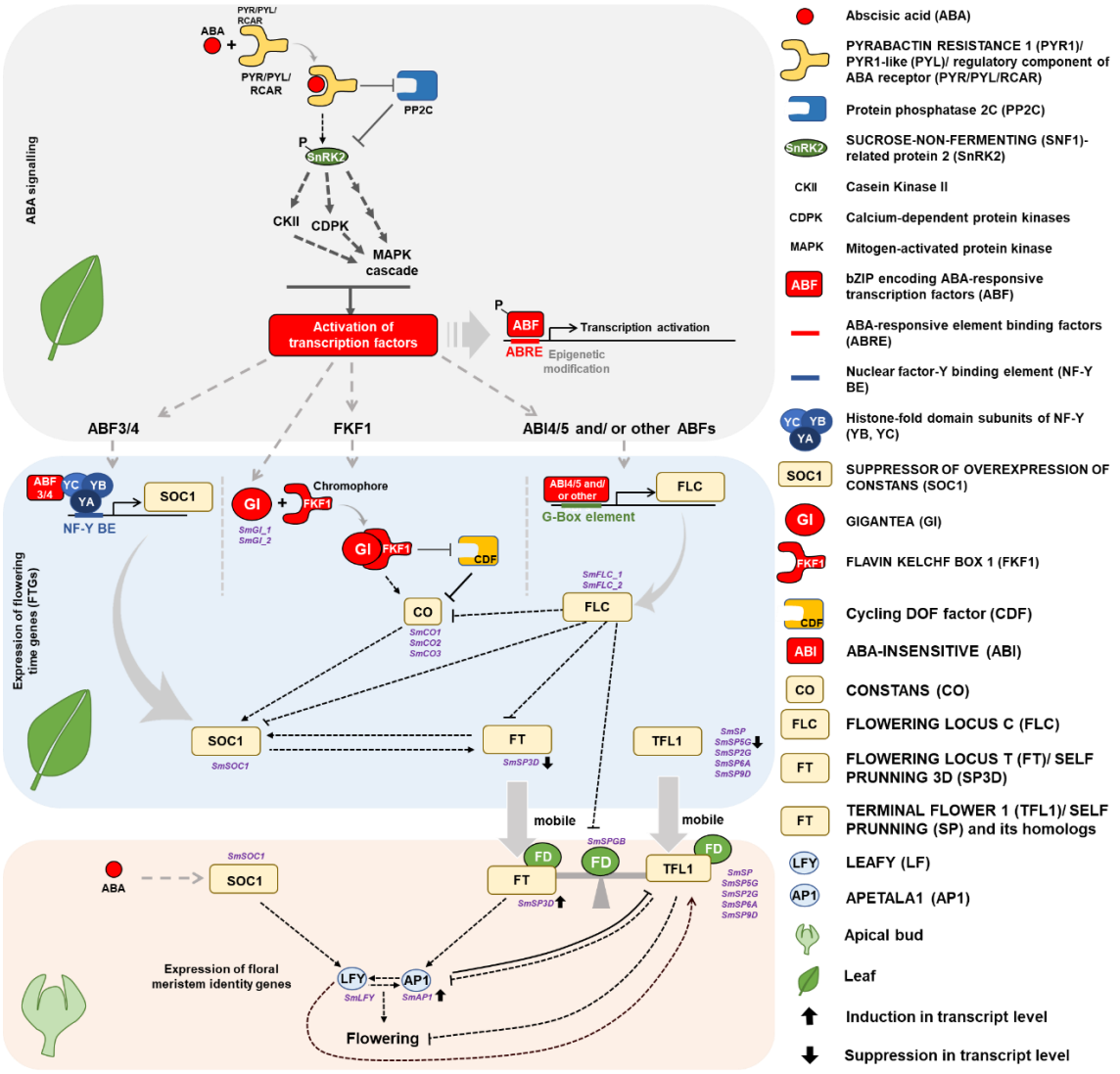

Fig. 3.12 Flowering induction by ABA-mediated signaling. The events mentioned in the top two rectangles represent the events in the leaves; bottom rectangle represents the events in the apical bud. ABA binding with PYR/PYLRCAR soluble receptors inactivate PP2C phosphatases after which, PP2C cannot dephosphorylate SnRK2. Phosphorylated SnRK2 activates several protein kinases to initiate their signaling cascades. This leads to the expression of transcription factors like ABFs and ABIs. These promote the transcription of several FTGs. FTGs include both florigens (promotes flowering; e.g., SOC, FT, CO) and anti-florigens (reduces flowering; e.g., FLC, TFL1). Interaction between FTGs regulates the expression of floral meristem identity genes (LFY, AP1). The eggplant orthologs of many Arabidopsis genes, identified in this study, are mentioned in purple font. Induction and suppression of eggplants' flowering-related genes' transcript levels are shown using black arrows beside the gene names. Frugivory was associated with the suppression of *SmSP3D*, and *SmSP5G* in the leaves, induction of *SmSP3D*, and suppression of *SmSP5G* in the apical buds. The floral marker *SmAP1* is also upregulated in the apical buds which confirm flowering induction.

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Chapter 4

Summary and future directions

4. Summary and future directions

4.1. Chapter 1: Introduction

The increasing need for food with the increasing population has changed agriculture dramatically after World War II (Reganold *et al.*, 1990; Brodth *et al.*, 2011). With the high demand and development of new technologies, farming has become mechanized. Synthetic pesticide usage increased crop production enormously and helped to bring a green revolution. However, conventional synthetic chemicals impart long-lasting negative impacts on society and the environment (Bernhardt *et al.*, 2017). A growing population has started to prefer crops cultivated without harmful chemicals (Reganold *et al.*, 1990). However, eco-friendly cultivation practices increase production costs and lower productivity (Abubakar & Attanda, 2013). Consequently, their usage remains limited in society. Therefore, the need for developing better alternatives persists (Dubey *et al.*, 2010).

Sustainable agriculture is defined as environment-friendly cultivation practices with adequate crop yield (Dubey *et al.*, 2010), and at the same time cost-effective so that a large population can incorporate it into their traditional practices (Brodth *et al.*, 2011). Here chemical and molecular ecology give researchers a platform. It is the study of chemicals involved in interactions between organisms (Pickett *et al.*, 1997). It describes the origins, and functions of naturally produced chemicals used in communications between interacting organisms. With the advancement of technologies and the amalgamation of different science disciplines like genomics, transcriptomics, metabolomics, ecology, etc. it has been possible to develop novel techniques to elucidate the structure and quantity of chemicals produced, released, or perceived by organisms (Sylvie *et al.*, 2016). These studies can help to find novel botanicals and semiochemicals that can have a direct application in pest management, and productivity enhancement. Botanicals, which are used as biopesticides, and biofertilizers currently account for approximately 10 % of the global market (Ahmed *et al.*, 2022). Further chemical and molecular ecology-based studies of plants and other interacting organisms can contribute to sustainable agriculture.

I tried to understand the interaction between eggplant (*Solanum melongena* L., Solanaceae), an important crop of tropics and subtropics, and its major insect pest eggplant shoot and fruit borer (ESFB, *Leucinodes orbonalis* Guenee, Lepidoptera: Pyralidae) during two of ESFB's growth stages- adult and larva. A detailed review of common ESFB management practices is given in chapter 1 of this thesis.

Based on the eggplant-ESFB interaction, my two Ph.D. objectives were formulated.

1. Understanding the role of eggplant volatiles in ESFB adult's host location (chapter 2)

2. Understanding eggplant's response to ESFB frugivory (chapter 3)

The major concern associated with current eco-friendly ESFB management practices is that these are labor-, time-, and cost-intensive. As a result of which, their usage by farmers is still limited especially in developing and under-developed countries (Dara, 2019). Chemical ecology allows us to study the chemistry of interacting eggplant-ESFB systems and that knowledge can be translated into bringing effective IPM. In chapter 2, we explored the possibility of using eggplant leaf volatiles to deter ESFB adults and their use in ESFB management.

The eggplant-ESFB system also provides an opportunity to study the chemical basis of plant response to lepidopteran frugivory. From a human perspective, the quality and quantity of fruits are directly related to the economic well-being of our society. If we can explore the frugivore-ESFB system, we can use that knowledge in generating resistant varieties or in developing other sustainable agricultural techniques. In chapter 3, we have reported ESFB-frugivory-associated eggplant signaling and response.

4.2. Chapter 2: Understanding the role of eggplant volatiles in ESFB adult's host location

Our field observations of gravid ESFB females' behavior, that they (1) can locate and oviposit on solitary eggplants (susceptible varieties), present under the dense and aromatic tree canopy, and (2) oviposit predominantly on leaves, led us to hypothesize that ESFB follows contactless leaf olfactory cues for host location. We also observed that ESFB does not oviposit on the leaves of the Himalayan eggplant variety RL22, which is ESFB-resistant. This indicated the presence of repellants in RL22s' leaf-blend. We investigated leaf-volatiles of six susceptible varieties and RL22. The GCMS-based volatile profile showed the presence of geraniol, and two other volatiles uniquely released by RL22-leaves. Foliar geraniol application on susceptible varieties reduced oviposition (>90%) both in the controlled environment and the agricultural field. We also identified RL22's geraniol synthase gene (*SmGS*) and characterized (heterologous expression using *Escherichia coli* system, followed by *in-vitro* characterization) the protein. RNA interference (RNAi)-based (virus-induced gene silencing or VIGS) silencing of RL22's

SmGS rendered RL22 ESFB-susceptible. Loss of ESFB-deterrence phenotype could be recovered by foliar geraniol application. Following are the key discoveries from this work.

4.2.1. Geraniol as ESFB-deterrent

Geraniol, a monoterpene alcohol, is already known to have a role in lepidopteran herbivore management. Geraniol's role in lepidopteran herbivores' oviposition deterrence [*Epiphyas postvittana* (Lepidoptera: Tortricidae) (Suckling *et al.*, 1996); *Tineola bisselliella* (Lepidoptera: Tineidae) (Plarre *et al.*, 1997)], fecundity reduction [*Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Moawad & Ebadah, 2021)], modulation of egg biochemical properties [*Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Guedes *et al.*, 2020), egg hatching impairment [*Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Moawad & Ebadah, 2021)], larval feeding deterrence [*Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Moawad & Ebadah, 2021)], hampering larval growth [*Glyphodes pyloalis* (Lepidoptera: Pyralidae) (Yazdani *et al.*, 2013); [*Spodoptera exigua* (Lepidoptera: Noctuidae) (Nobsathian *et al.*, 2021), increasing larval mortality [*Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Moawad & Ebadah, 2021)]; [*Helicoverpa armigera* and *Spodoptera litura* (Lepidoptera: Noctuidae) (Chen & Viljoen, 2010; Kaur *et al.*, 2019); [*Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Diédhiou *et al.*, 2021)], and reducing adult longevity [*Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Moawad & Ebadah, 2021)] is well documented. Even though geraniol's use in insect management has been known, the eggplant genome with the potential of geraniol production was not known which is shown by this study for the first time.

Geraniol is naturally emitted by many aromatic plants which have been used for intercropping with eggplants traditionally, like fennel, marigold, coriander, etc. (Chanthai *et al.*, 2012; Sujayanand *et al.*, 2015; Elmassry *et al.*, 2018; Iamba & Yaubi, 2021). The fact that this age-old intercropping technique is still successful in reducing the ESFB population, suggests the possibility of geraniol-based methods being a susceptible solution.

4.2.2. Use of geraniol in IPM

Geraniol can be synthesized chemically from other monoterpenes like pinene and citral, or isolated from natural resources like citronella oil by distillation (Gerke *et al.*, 2020). Industrial production is as high as 1000 tons/ year (Lapczynski *et al.*, 2008). As a result of the high production rate, the cost has been reduced to as low as 1000 ₹ kg^{-1} or 12 $ kg^{-1}

(IndiaMART, 2022). Low cost and high availability make geraniol application on agricultural lands an easy solution to be used in pest management. Several studies have documented the use of nanoparticles made of dextran (Nee *et al.*, 2019), pluronic f-127 (Yegin *et al.*, 2015), zinc oxide (Farokhcheh *et al.*, 2021), β-cyclodextrin (Hadian *et al.*, 2017), zein (Oliveira *et al.*, 2018), chitosan/ gum Arabic (Sampathkumar *et al.*, 2020; Shakiba *et al.*, 2020), etc. for geraniol application. Together, geraniol-based ESFB repellants can be a cheap and effective strategy to push ESFB adults away from the eggplant field. In IPM, geraniol-based repellants can be implemented. For better push-pull strategy-based repellants with geraniol along with ESFB traps can be used together. More such studies will help to identify more crops' antixenosis factors and those also can be used in agriculture.

4.2.3. *SmGS* as a selection marker

Plant breeding programs select desirable traits. These traits can be qualitative (traits controlled by few genes having distinguishable effects) or quantitative (traits controlled by quantitative trait loci containing a large number of genes having minute effects). Dependent on the nature of the trait, the difficulty in plant breeding techniques varies (Merrick *et al.*, 2021). Therefore, the discovery of new traits, which bring disease resistance, is always in demand. This trait of geraniol production can be included in breeding programs. RL22 variety can be used for hybridization with susceptible, and high-yield varieties. We also identified RL22's *GS*, which can be used as a selection marker or overexpressed in vegetative tissues of susceptible varieties to deter ESFB.

4.2.4. Future directions

i. A future study on finding differences in *SmGS* sequences, transcriptional rates, post-transcriptional modifications, protein activities, etc. between RL22 and other susceptible varieties, will help to incorporate geraniol-production traits effectively into breeding programs.

ii. To find whether the geraniol emission by RL22 is season-, photoperiod- or circadian rhythm-dependent and most importantly, whether it is tuned to the ESFB oviposition timing will require further studies.

iii. Effective geraniol formulation preparation to be used in pest management is a part of future investigation. A large-scale field survey of geraniol spraying on susceptible

eggplant varieties can be done soon which will help to understand the actual potential of geraniol usage in agriculture. Simultaneously, it is essential to look into the effect of geraniol spray on insects' beneficial microbes and insects, natural enemies, and other trophic levels.

4.3. Chapter 3: Understanding eggplant's response to ESFB frugivory

We investigated phenotypic variations between ESFB-infested and healthy fruit-bearing eggplant branches. Fruit infestation induced flowering and fruiting. Ultra-performance liquid chromatography coupled to electrospray ionization, and quadrupole-time of flight mass spectrometry (UPLC-ESI-QTOF)-based phytohormone profile and quantitative reverse transcription polymerase chain reaction (RT-qPCR)-based gene expression profile of fruits, apical part of the shoots, and leaves showed one particular phytohormone-abscisic acid (ABA) biogenesis in infested fruits and its transport from fruits to leaves through shoots. Also, frugivory led to a change in the expression of flowering-related genes in leaves, apical buds, and fruits, which explained flowering induction. Simulated frugivory by exogenous ABA applications both on fruits, and sink leaves induced flowering at the apical bud. The key discoveries of this chapter are-

4.3.1. Lepidopteran frugivory-associated, and ABA-mediated plant response

The role of ABA in abiotic stress is predominantly studied (Zhang *et al.*, 2006; Yoshida *et al.*, 2019). Few studies have shown the role of ABA in biotic stress, especially during plant-pathogen interactions (Cao *et al.*, 2011; Chan, 2012). When plant and chewing insect interaction is concerned, in most cases ABA is found to synergistically act with JA, and ethylene (ET) (Erb *et al.*, 2012). However, in this study, for the first time, we documented ABA playing the main role in frugivory-associated plant response.

4.3.2. Fruit-to-sink leaf systemic signaling: Sink-sink signaling

Leaf-leaf systemic signal transductions are well-documented in many systems (Nguyen *et al.*, 2018; Parmagnani & Maffei, 2022). Few studies have documented root-shoot, root-leaf, leaf-root, and leaf-flower signaling as well (Kessler & Baldwin, 2007; Kessler & Halitschke, 2007; Gil *et al.*, 2008; Tegeder & Masclaux-Daubresse, 2018). However, knowledge of fruit-to-shoot signaling is scarce. Fruit is known to be a sink organ as the photoassimilates are known to be one-way traffic towards it (Tegeder & Masclaux-Daubresse, 2018). Our observation that flowering is induced at the apical nodes upon

frugivory indicated that fruits are capable of generating systemic signals. ABA is found to be a major signaling molecule here, which is induced in the fruits after two days, followed by in the pedicels and shoots after four days, and in the young leaves after five days of frugivory. Therefore, ABA participated in long-distance signaling. As fruits, and young leaves both function as sink organs, this signaling can also be termed sink-sink signaling.

4.3.3. ABA sprays can increase eggplant yield

Previous studies reported that the exogenous ABA application promotes flowering (Cui *et al.*, 2013; Conti *et al.*, 2014). Similarly, in this study, we report that frugivory induces ABA, which promotes flowering at the apical bud of eggplant. Also, ABA spraying on sink leaves induced flowering. This observation opens up the possibility of ABA usage in agricultural fields to increase yield. Also, high ABA-producing varieties can be selected for cultivation, and incorporated into breeding programs. ABA biosynthetic and catabolism genes- *9-cis-epoxycarotenoid dioxygenase* (*SmNCED*), and *ABA 8' hydroxylase* can be used as markers to select varieties.

4.3.4. Ecological aspects

From an ecological perspective, it can be perceived as frugivory-induced flowering is a plant's adaptive response in anticipation of fruit loss to recover or compensate for the fruit/ seed loss which is similar to vegetative/ reproductive compensation after mammalian grazing (Paige, 1992). On the other hand, induced flowering and fruiting can ensure a sustained food supply for ESFB. From a human perspective, the fruit-frugivore interaction is understood as an antagonistic relationship. However, this interacting system can also share a mutualistic relationship to maintain coexistence.

4.3.5. Future directions

i. A future study on frugivory-associated plant responses using ABA deplete eggplant will validate the role of ABA in fruit-frugivore interaction.

ii. ABA-induced flowering is a multi-step process, as discussed in section 3.4 and fig. 3.12. It is poorly understood how ABA signaling leads to the flowering induction. Various proposed models suggest different interactions between these two. Which one or more of these are involved in the frugivory-associated ABA-mediated flowering will have to be studied by separately silencing each one of them. To better understand which genes

promoted flowering, the transcript-dynamics study of multiple genes is required. A high-throughput method like next-generation sequencing (NGS) will be required to shortlist the flowering-related gene targets. Upon ABA silencing, the levels of those genes can be monitored to better understand the frugivory-associated ABA-flowering pathway.

iii. A study of ESFBs' oral secretion components is required to find the effector molecule that triggers eggplants' response.

iv. The usage of ABA-based formulation in increasing eggplant fruit yield will be an applied aspect.

Together, this thesis reiterates the importance of the integration of metabolomics and biotechnological methods with ecological and conventional agricultural ones for discovering new pest management solutions as well as for increasing our knowledge of plant-insect interactions.

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