Journal of Sugarcane Research (2020) **10**, 63-73
https://doi.org/10.37580/JSR.2020.1.10.63-73

RESEARCH ARTICLE

Induced resistance and differential allocation of herbivore defensive chemicals: a case study with internode borer *Chilo sacchariphagus indicus* (Kapur) in sugarcane

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(Received 19 June 2020; accepted 15 August 2020)

Abstract
Methyl jasmonate (MeJA), a known resistance inducer molecule, when applied was found to give protection against the attack of internode borer (INB) *Chilo sacchariphagus indicus* (Kapur) (Lepidoptera: Crambidae) in sugarcane to the extent of 86.67% as against 73.33% damage in control plants. Estimation of 12 phenolic compounds in leaf and stem tissues, following external application of 100 ppm MeJA, indicated wide variation with a 19-fold higher production in leaf than in stem tissue implying differential allocation of these chemicals within by the plant. Among all the phenolics, ferulic acid was expressed at the highest levels in both leaf and stem tissues. Such differential allocation of defensive chemicals is resorted to by the host plant to economize its resources by directing the chemicals to the site of initial attack, i.e. leaf tissue in the present case, rather than systemically deploying throughout the plant. From the observations, reduced INB attack and production of phenolics under the influence of MeJA, it is hypothesized that plant recognizes feeding by herbivores in their early stages and triggers differential production of defensive chemicals to target the early and vulnerable stage of the pest to limit losses.

Keywords: Sugarcane; Internode borer; Induced resistance; Methyl jasmonate; Phenolic compounds

Introduction

Plants emit a bouquet of volatile substances from fruiting bodies and vegetative tissues which enables them to communicate with their surroundings, attract specific pollinators and impart resistance to harmful insects (Pichersky and Gershenzon 2002). In addition, the synthesis of plant volatiles is induced by certain external challenges caused by pathogens, herbivores or adverse weather conditions (Paré and Tumlinson 1999). Moreover, such gaseous compounds act as airborne signals that mediate inter-plant communication thus affecting not only the challenged plant but also its neighbors (Arimura et al. 2000). Methyl jasmonate (MeJA), a fragrant volatile compound initially identified from flowers of *Jasminum grandiflorum*, has proven to be distributed ubiquitously in the plant kingdom. The volatile nature of MeJA led to the discovery of its role as a signal in plant cellular responses, plant–herbivore interactions and plant–plant interactions. MeJA and its free-acid jasmonic acid (JA), collectively referred to as jasmonates, are important cellular regulators involved in diverse developmental processes, such as seed germination, root growth, fertility, fruit ripening and senescence (Creelman and Rao 2002; Wasternack and Hause 2002). In addition, jasmonates activate plant defense mechanisms in response to insect-driven wounding, various pathogens, and environmental stresses, such as drought, low temperature, and salinity (Wasternack and Parthier 1997). External

application of MeJA on plants has been known to induce resistance against crop pests (Erb and Reymond 2019). For example, seed treatment of rice with MeJA induced resistance to rice water weevil but with costs to plant growth and fitness (Kraus and Stout 2019).

Plant phenolics comprise a diverse group of phytochemicals, ranging from small phenolic acids to complex polymers such as tannins and lignins (Dey and Harborne 1997). Phenolic compounds are derived from shikimic acid pathway and include simple phenols, phenolic acids, coumarins, flavonoids, isoflavonoids, quinines, tannins and lignins. Phenolic compounds are converted into several derivatives, including phytoalexins (antimicrobial), coumarins (oral anticoagulants), lignin (cell-wall strength), various flavonoids and condensed tannins (feeding deterrents) (Chan et al. 1978; Swain 1979; Salisbury and Ross 1992). They have been implicated in plant resistance to insects (Lege et al. 1995) or defense mechanisms because of their general accumulation near the wounded and infested tissues (Johnson and Schall 1957; Kuc 1966; Levin 1971). There are several examples of constitutive phenolics acting as feeding deterrents to herbivores and inhibitors of enzymes (Cheeke 1989). The role of phenolics in plant defense against herbivores has been particularly an intense area of study and has been the basis of several plant defense theories (Appel, 1993). The toxicity of many phenolics, from simple phenolic acids to complex polyphenols, has been attributed to their ability to function as pro-oxidants (Appel 1993; Summers and Felton 1994).

The internode borer (INB) *Chilo sacchariphagus indicus* (Kapur) (Lepidoptera: Crambidae) is a major pest of sugarcane in peninsular India with distribution in Tamil Nadu, Andhra Pradesh, Karnataka, Maharashtra, Bihar, Uttar Pradesh and Haryana States (David et al. 1986; Srikanth et al. 2012). Under tropical conditions, the borer remains active throughout the year and heavy buildup of the borer occurs after the commencement of internode formation during July-December coinciding with monsoon season. The cryptic nature of feeding makes its management difficult and the most economical and ecologically sound way of sustainable management of this pest is through host plant resistance and biological control. The present investigation aims at examining induction of resistance against INB and understanding the expression of phenolic compounds involved in resistance mechanism consequent to the application of MeJA, thus exploring the possibility of using MeJA as an external resistance inducer for the control of the borer in sugarcane.

Materials and Methods

Host plant maintenance

Three single-bud setts of the popular variety in tropical India, i.e. Co 86032 were planted in pots (45 cm ht x 45cm dia.) and the plants were raised following standard agronomic practices (Sundara 1998). Healthy plants of 6 months age were used for the experiment.

Insect culture

INB larvae collected initially from nearby farmers' fields were reared in the laboratory on a standard artificial diet (Easwaramoorthy and Shanmugasundaram 1991). Pupae were collected from the diet vials after 20 days of rearing and kept in an adult emergence cage. Freshly collected sugarcane leaf bits placed in small plastic rearing boxes with water to keep them turgid were provided for oviposition. Egg masses turning black were collected and kept in clean tubes and the emerging neonate larvae were used for tests.

Tests with MeJA

MeJA obtained from Sigma-Aldrich, USA, was dissolved in HPLC grade acetone to make a

concentration of 100 ppm. Five six-month old healthy potted plants, each with three plants, were sprayed with MeJA @100 ppm with an atomizer. For control, two sets of potted plants were sprayed either with acetone or water. Freshly emerged neonate larvae were released @ 5 larvae / plant with the help of a fine camel hair brush and the pots were kept 2 m distance from one another. Treated and control plants were observed for insect feeding symptoms, namely leaf scraping, leaf sheath feeding and feeding on top internodes (Fig. 1). Counts of plants showing these symptoms were recorded for a period of 30 days after treatment. For the estimation of induced phenolic compounds, a separate set of plants treated with MeJA (100 ppm) was used. Leaf and stem samples were collected 1, 2, 3, 24 and 48 h after treatment for estimation of phenolics.

Sample preparation for HPLC analysis

Phenolics were estimated in leaf and stem tissues of MeJA treated plants. For stem tissue, the region 10 cm above the node of second fully opened leaf, housing the meristem and the region immediately below the meristem; and for leaf tissue, the third fully opened leaf from top were used. The samples were cut and chopped finely with a sharp knife and 5 gm tissue was powdered immediately with liquid nitrogen. The powdered tissue was ground again with 10 times w/v of deionized water (pH 2 adjusted with concentrated HCl) and centrifuged at 10000 g for 30 min. The supernatant was then vacuum filtered in Buchner funnel using Whatman filter paper no. 42. Next, the filtrate was mixed with 60 g of clean, swelled Amberlite XAD-2 resin (Supelco, Bellefonte, PA, USA, pore size 9 mm, particle size 0.3 - 1.2 mm) and stirred slowly with a magnetic stirrer for 60 min. The slurry of the Amberlite XAD-2 resin and sample was then packed (poured) in a glass column (50 cm L x 1.5 cm dia.) and the resin was washed with acidified water (pH 2 with HCl, 100 ml) followed by rinsing with deionized water (100 ml) to remove all sugars and other polar constituents and then eluted with methanol. This methanol fraction was concentrated to dryness in a flash rotary evaporator (Heidolph Laborota, 4011, Germany) at 40°C under reduced pressure. The residue was dissolved in 1 ml HPLC grade methanol and filtered through 0.2 µ, 13 mm GH Polypro membrane filter before injecting in HPLC.

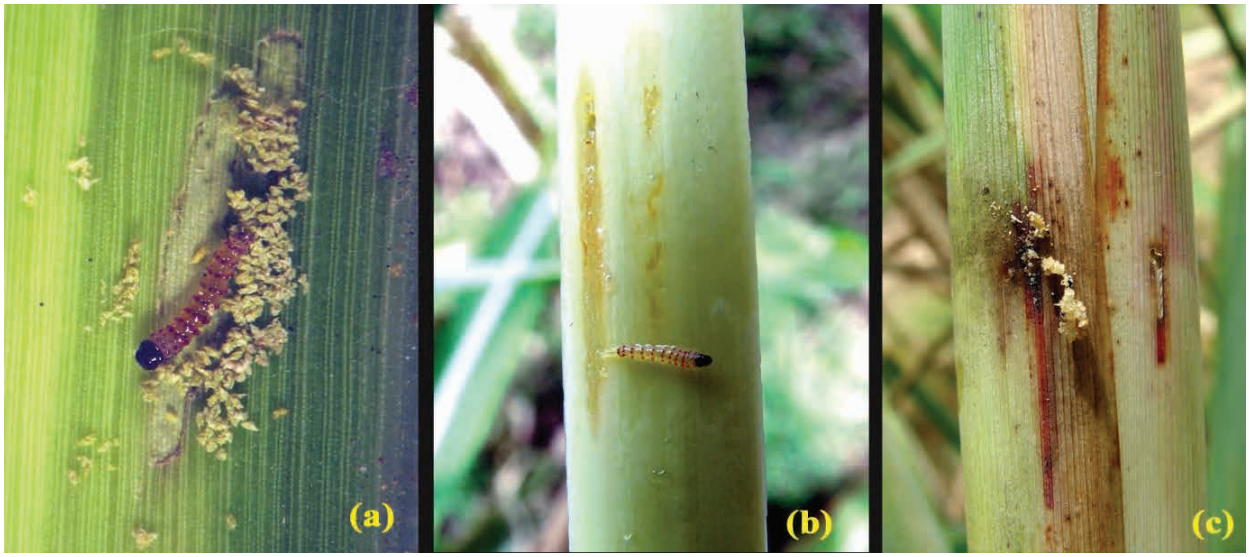

Figure 1. Internode borer damage in cane: (a) leaf scraping (b) leaf sheath feeding (c) bore hole with frass

HPLC-DAD conditions

HPLC analysis was performed with Shimadzu LC-8A Semi-preparative system equipped with SPD-M10A Diode Array Detector (DAD) and a column oven. Separations were performed on a Phenomenex Gemini-NX C18 column (250 mm x 4.60 mm), particle size 5µ with a solvent gradient of 0.25% formic acid and 2% methanol in deionized water (A): methanol (B). A Phenomenex KJ0-4282 column with RP-18 10 µ material in 4.0 mm x 3.0 mm cartridge was used as Guard Column. The gradient was: t = 0 min 90% A : 10% B; t = 15 min 90% A : 10% B; t = 20 min 60% A : 40% B; t = 30 min 55% A : 45% B; t = 50 min 40% A : 60% B; t = 52 min 20% A: 80% B; t = 60 min 20% A: 80% B; t = 65 min 10% A: 90% B; t = 68 min 10% A : 90% B; t = 75 min 90% A : 10% B. Between runs the column was equilibrated with 90% A for 5 min. The system was operated at a flow of 1 ml/min at 27^{0}C with sample injection volume of 25 µl. Chromatograms of standards and samples were recorded at 290 nm and DAD spectra 190 - 370 were stored for all peaks. The chromatogram data generated were analyzed using the software Shimadzu-ClassVP Ver.6.4SP1.

Statistical Analysis

The percent data of INB damage symptoms in inducer treated and untreated plants were subjected to ANOVA after arcsin transformation and means compared with Newman-Keuls test using StatSoft, Inc. (2004).

Results and Discussion

MeJA-treated plants recorded lower levels of different symptoms produced by INB than those in acetone-treated and absolute control plants (Table 1). Percent of plants with leaf scraping was lowest in MeJA-treated plants but it did not differ significantly from that in acetone and control. On the other hand, percent of plants with internode bore hole damage was significantly lowest in MeJA-treated plants; however, the higher levels observed in acetone and control were not significantly different. Deadheart damage was observed only in control plants with no significant differences among treatments. Percent of plants showing larva was lowest in MeJA treated plants but the differences were not significant among the three treatments. While percent of plants showing no symptoms was significantly highest in MeJA-treated plants, there was no difference between acetone treatment and control.

Application of MeJA induced changes in important phenolic compounds in the leaf tissues (Table 2). Vanillic acid, syringic acid, cinnamic acid, flavone, gallic acid, catechin, phloroglucinol and caffeic acid were produced in very low quantities in both MeJA-treated and untreated plants at all intervals of observation. While most of these were below detectable limits (BDL), phloroglucinol showed slightly higher values in both treatment and control at all time interavals with the highest (4.93 ppm) being at 3 h after treatment. Although

Table 1. Effect of methyl jasmonate treatment on internode borer damage in sugarcane plants

Treatment	Percent of plants showing symptoms				
	Leaf scraping	Internode hole	Deadhearts	Larva present	No symptoms
Methyl jasmonate	6.67 a	6.67 a[#]	0.00 a	6.77 a	86.67 a
Acetone	20.00 a	66.67 b	0.00 a	13.33 a	13.33 b
Control	13.33 a	73.33 b	13.33 a	13.33 a	13.33 b

[#] Means followed by the same letter in a column are not significantly different (P>0.05) by ANOVA and Newman-Keuls test on arcsin data

Table 2. Phenolics in leaf tissue (ppm) of sugarcane at different time intervals after application of methyl jasmonate

Phenolics	Interval after treatment (h)									
	1		2		3		24		48	
	T	C	T	C	T	C	T	C	T	C
Vanillic acid	0.00	0.00	0.00	0.00	0.78	0.00	0.00	0.00	0.00	0.00
Coumarin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	149.24	0.00
Syringic acid	0.62	0.00	0.10	0.47	0.00	0.96	0.00	2.00	0.39	0.21
Cinnamic acid	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.65	0.00
Flavone	0.05	0.05	0.03	0.97	0.00	0.06	0.02	0.03	0.12	0.00
Gallic acid	0.28	0.19	1.42	1.52	0.36	0.29	0.00	0.00	0.00	0.00
Catechin	0.00	0.42	0.53	0.51	0.00	0.73	1.04	1.02	0.46	0.27
Phloroglucinol	1.32	0.95	1.11	0.99	4.93	0.18	0.90	2.80	2.17	1.79
Catechol	67.74	1.59	0.00	0.00	0.00	0.00	0.00	0.00	3.18	53.36
Caffeic acid	1.88	1.68	0.83	0.00	1.64	1.47	0.00	0.00	1.17	1.36
Orcinol	6.15	4.52	8.12	0.00	6.90	16.32	13.75	15.40	11.00	7.97
Ferulic acid	45.12	133.47	356.22	428.50	0.00	1189.55	343.75	0.00	778.27	299.65

***T**= Treatment; **C**= Control*

coumarin was BDL at all intervals, it expressed a very high level (149.24 ppm) 48 h after treatment. Orcinol and catechol were expressed at moderate levels, the former being more consistent at all time intervals and the latter showing the highest value in treatment in the initial stages. Of all the phenolic acids, expression of ferulic acid was the maximum with lower values in treatment in the first half of the observation period. However, the trend reversed in the second half in that treated plants showed higher values.

Mean leaf phenolic content in MeJA treatment and control showed some fluctuations (Fig. 2a). In both, phenolic content was more or less uniform up to 2 h after treatment. While the content decreased slightly in MeJA treatment (1.22 ppm), it showed a spurt in control (100.80 ppm) 3 h after treatment. In the next two intervals, phenolic content increased in MeJA-treated plants reaching its peak (78.89 ppm) at 48 h whereas it showed a decreasing trend in control (Table 3).

The trend in MeJA induced phenolic content in stem tissues was somewhat similar to that observed in leaf tissue for most phenolic acids (Table 4) but with lower quantities than in leaf tissue. Vanillic acid, syringic acid, cinnamic acid, flavone, gallic acid, catechin and phloroglucinol and caffeic acid were produced in very low quantities as in the case

Table 3. Dynamics of leaf phenolic content in sugarcane plants treated with methyl jasmonate

Treatment	Mean phenolic content (ppm) at different intervals (h)					
	1	2	3	24	48	Mean
Methyl jasmonate	10.35	30.70	1.22	29.96	78.89	30.22
Control	11.91	36.08	100.80	1.77	30.38	36.19
Mean	11.13	33.39	51.01	15.86	54.64	

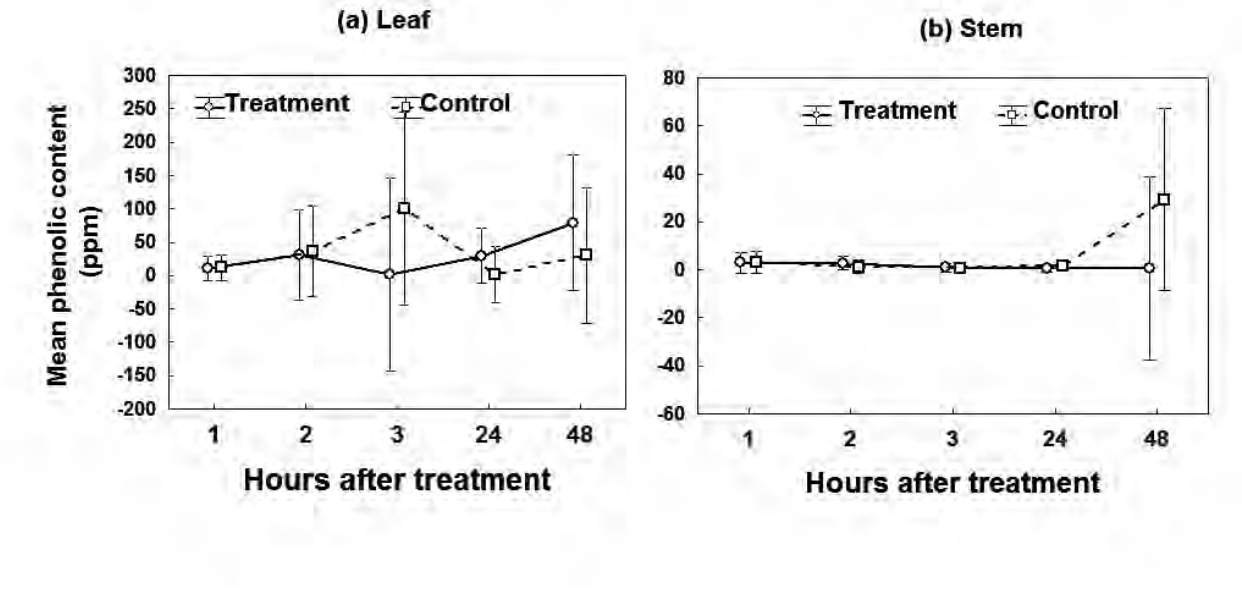

Figure. 2. Dynamics of phenolics in leaf (a) and stem (b) of sugarcane plants treated with methyl jasmonate

of leaf tissue. While coumarin produced a spurt in leaf tissue of MeJA-treated plants at 48 h, it was moderate throughout in stem tissue. Catechol and orcinol were also expressed in moderate quantities but were slightly lower than those in leaf tissue. Ferulic acid, despite some fluctuations over time, was the highest among all phenolics in stem but lower than those in leaf tissue.

Mean phenolic content of stem tissues in MeJA treatment and control plants was lower than that of leaf tissue. Stem phenolic content in treated and control plants was more or less similar in the first two observations (Fig. 2b). While it continued to decrease in treated plants until the last observation, it showed a slight increase reaching a peak (29.27 ppm) at 48 h in control plants (Table 5).

If the plant can defend itself from the herbivore at the latter's most vulnerable stage, say first instar, the resources required for its defence would be minimum and economical, in light of the small quantities of tissue lost. Plants have the ability to differentially allocate secondary metabolites to different tissues (McKey 1974; Zangerl and Bazzaz 1992) and can change this allocation through time (Barton and Boege 2017). For herbivores, this means that an individual plant presents a mosaic of food sources that may vary in spatial and temporal quality and availability. If an insect herbivore, for example, hatches upon a plant, it may encounter several tissues, i.e. from leaves to flowers to fruits that contain different concentrations and compositions of secondary metabolites (Matthias and Reymond 2019). As the insect develops, it may also encounter changes in secondary metabolites due to up- or down-regulation of their production (Gershenzon et al. 2000) or changes in movement of metabolites within the plant (van Dam et al. 1995) throughout it's growing season. In the subtropical sugarcane top borer *Scirpophaga excerptalis* Wlk. (Lepidoptera: Crambidae), which has the potential to cause severe losses to farmers and sugar industry (Avasthy 1981; Srikanth et al. 2012), resistance mechanism operates at the level of midrib which neonate larvae tunnel through (Mukunthan and Mohanasundaram 1988). Similarly, neonate larva of INB, after encountering the leaf tissue,

Table 4. Phenolics in stem tissue (ppm) of sugarcane at different time intervals after application of methyl jasmonate

Phenolics	Interval after treatment (h)									
	1		2		3		24		48	
	T	C	T	C	T	C	T	C	T	C
Vanillic acid	0.09	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Coumarin	7.14	8.51	19.78	9.49	10.27	9.25	3.69	7.84	5.16	35.41
Syringic acid	0.00	0.05	0.00	0.04	0.42	0.00	0.04	0.08	0.03	0.24
Cinnamic acid	0.03	0.23	0.15	0.03	0.00	0.01	0.02	0.00	0.00	0.09
Flavone	0.00	0.01	0.04	0.01	0.02	0.02	0.00	0.00	0.05	0.06
Gallic acid	0.00	0.10	0.00	0.00	0.49	0.26	0.28	0.00	0.00	0.00
Catechin	0.12	0.00	0.03	0.00	0.20	0.14	0.19	0.39	0.00	0.22
Phloroglucinol	0.10	0.15	0.53	0.35	0.38	0.34	2.10	0.24	1.58	2.54
Catechol	6.22	0.35	3.29	3.16	0.00	0.00	0.00	10.01	0.00	0.00
Caffeic acid	0.29	0.00	0.00	0.02	0.00	0.00	0.00	0.22	0.14	0.00
Orcinol	0.54	0.22	9.03	1.03	0.00	0.00	0.17	0.00	0.00	0.00
Ferulic acid	20.93	28.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	312.65

***T**= Treatment; **C**= Control*

scrapes the surface of leaf blade first followed by feeding on the ligular region and scraping the inner side of leaf sheath for 7 - 8 d (David et al. 1986; Srikanth et al. 2012) before boring in to the preferred tender top internodes for further feeding and development. The differential content of 12 phenolic compounds in leaf and stem under MeJA treatment, known to have the same effect as larval feeding in triggering defensive mechanisms in plants (Wasternack and Parthier 1997; Cheong and Do Choi 2003) observed in the present study points to higher allocation of defensive chemicals to the former than the latter. In these two cases of herbivore feeding, the plant appears to employ its defensive mechanism at the leaf tissue level itself since the most vulnerable stage of the herbivore can be defended successfully in the most economical way by losing very small quantities of the leaf tissue. If the herbivore successfully overcomes this defensive mechanism at the leaf tissue level, further growth and development of the insect on the stem tissue are ensured.

Phenols in general are known to be toxic to lepidopteran larvae (Hedin 1977). However, in sugarcane, no significant relationship was found between total phenol content in some of the commercially cultivated hybrid varieties and INB susceptibility (Anonymous 1985). This

Table 5. Dynamics of stem phenolic content in sugarcane plants treated with methyl jasmonate

Treatment	Mean phenolic content (ppm) at different intervals (h)					Mean
	1	2	3	24	48	
Methyl Jasmonate	2.96	2.74	0.98	0.54	0.58	1.56
Control	3.21	1.18	0.84	1.57	29.27	7.21
Mean	3.09	1.96	0.91	1.06	14.93	

observation might have been due to the fact that the phenols were estimated from the stem tissue of the plant, where the borer feeds in the later stage, and not from leaf tissue where borer defense mechanism manifests to a greater degree as the present study pointed out. In the case of sugarcane top borer, *Scirpophaga excerptalis* Wlk., resistance was observed to operate to a greater extent in the leaf midrib, where the young larvae feed by mining, than in the spindle, the site of feeding of grown-up larvae (Mukunthan and Mohanasundaram 1998). Such differential resistance was not related to total phenol content in midrib (Mukunthan 1990) which suggested the role of other defence mechanisms against top borer. In the case of top borer (Mukunthan 1990) and INB in the present study, by allocating defensive chemicals to the leaf tissue, the primary site of feeding by young larvae in both cases, the plant economizes its resources to defend itself efficiently and prevents occurrence of severe damage at a later stage.

Phenolics seem to play an important role against non-lepidopterans pests also in sugarcane. For example, in studies on white grub resistance in sugarcane, both soluble and cell-wall phenolics were found constitutively in the roots of 15 clones dominated by ferulic acid and p-coumaric acid (Nutt et al. 2004). The general higher content of ferulic acid in both leaf and stem tissue of sugarcane observed in the present study indicated that this phenolic is distributed throughout the sugarcane plant with a yet-to-be deciphered function. Although white grub feeding induced significant changes in the type and amount of phenolics in all clones, there was no relationship between phenolic type and quantity, and the antibiosis displayed by these clones (Nutt et al. 2004). This indicated the possible role of other defensive factors in white grub resistance. Phenolic acid content in sugarcane genotypes was related to antibiosis against woolly aphid *Ceratovacuna lanigera* Zehntner (Homoptera: Aphididae) (Hunsigi et al. 2006). However, of the 12 phenolics examined, only catechol was significantly related to aphid incidence at 8 months age (Srikanth et al. 2009). Despite the variation in concentrations of individual phenolic compounds in leaf and stem tissues following MeJA application observed in the present study, they may have a role in the defense mechanism of the plant as is evident from the protection MeJA application offered in treated plants against INB. Role of individual compounds needs to be studied to confirm their effect on different stages of herbivore development to ascertain how these phenols contribute individually and in combination towards the defense mechanism. It is also possible that MeJA application may trigger induction of other resistant factors such as polyphenol oxidases (Constabel and Ryan 1998) and proteinase inhibitors (Farmer and Ryan 1990). Further studies on these defensive factors in both resistant and susceptible varieties under insect feeding regimes would reveal how the plant overcomes insect feeding by allocating their resources in the early stages of attack. Also, molecular studies would reveal whether plants recognize insect attack much before actual feeding through stimuli from herbivore stages like eggs and / or damage-associated molecular patterns that would shed light on the mechanism behind secondary metabolite production (Erb and Reymond 2019).

In this preliminary study, MeJA application to sugarcane plants has been found to reduce damage caused by INB. Of all the damage parameters, only internode holes were significantly lower in MeJA-treated plants probably due to greater expression of defensive factors. The lower levels of leaf scraping, the initial symptom caused by INB larva, despite not being significant, indicated

operation of defensive mechanisms at leaf level. Further studies with a range of concentrations and application at different plant stages with observations over an extended period under INB feeding regimes would serve as the forerunner for field level application of inducer molecules. While pursuing this objective, the aspect of costs to plant growth and fitness under MeJA application, in addition to resistance induction against target pest (Kraus and Stout 2019), needs to be addressed in long-term studies.

Acknowledgements

The authors thank Dr. Bakshi Ram, Director, ICAR-SBI, for logistic support and academic encouragement.

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