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Chemical defense and herbivory: A case study of phenolics versus *Bactrocera dorsalis* (Hendel) infestation in mango

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ABSTRACT: Studies were undertaken to determine the defensive role played by phenolic acids present in peel and pulp of mango against fruit fly, *Bactrocera dorsalis* (Hendel) in different mango cultivars. The fruit fly infestation exhibited a significant negative relationship with total phenolics. Gallic acid, Proto-catechuic acid, p-OH benzoic acid, p-coumaric acid, ferulic acid, o- Coumaric acid and t- Cinnamic acid were the phenolic acids recorded in mango peel. Very high gallic acid was noticed in Langra (2569.43 µg/ml), followed by EC 95862 (7153.99 µg/ml). and Dusheri and Totapuri also showed considerably higher gallic acid conents while Banganapalli recorded the lowest content (193.0 µg/ml). Among the varieties, Banganapalli (69.20%) had significantly higher infestation whereas EC 95862 (1.40%) and Langra (0.00%) had lower infestation at 100 per cent maturity. The defensive mechanism exhibited by the phenolic acids in resistant varieties was evident from these results and confirmed through multiple regression.

Keywords: *Bactrocera dorsalis*, infestation, fruit fly, mango, penolic acids, secondary metabolites

INTRODUCTION

The Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) is a major pest of fruit crops with quarantine importance. Mango (*Mangifera indica* Linn.) is the most economically important host of *B. dorsalis*. Though India is the major mango producing country, share of Indian mangoes in the international market is very minimum as the export of fresh fruits is less than five per cent (Verghese *et al*., 2002) and *B. dorsalis* is reason behind trade restrictions in international trade of mango from India. Exploring host plant resistance helps in developing sustainable fruit fly management strategies. In this direction, biochemical components like phenols of host plants play a greater role.

Plant phenolics are secondary metabolites that encompass several classes of structurally diverse natural products biogenetically arising from the shikimate phenyl propanoids- flavonoids pathways (Berardini *et al.,* 2005; Ribeiro *et al.,* 2007). Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pests and pathogens and for many other functions. Generally, ripe peels contain higher total polyphenols than raw peels (Ajila *et al*., 2007a). The polyphenolic constituents of mango peel include mangiferin, quercetin, rhamnetin, ellagic acid, kaempferol, and their related conjugates. Polyphenolic compounds commonly serve as protective mechanisms in plants, warding off predator and microbiological attack. Many factors affect polyphenolic concentrations, including cultivar differences, growing conditions, maturity and postharvest handling of fruit (Lakshminarayana *et al.,* 1970; Selvaraj and Kumar, 1989; Wang and Lin, 2000). Verghese *et al*. (2012) reported that resistant varieties have high phenolic levels and they play a defensive role in preventing fruit fly herbivory in mango. With this background, present study was conducted on seven mango varieties representing resistant, moderately resistant and susceptible varieties to mango fruit fly with the following objectives. Comparative assessment of phenolics and estimation of phenolic acids contents in peel and pulp of mango varieties at different maturity levels and its influence on the infestation.

MATERIALS AND METHODS

Plant material

The present study was undertaken in the experimental

mango orchard at the Indian Institute of Horticultural Research, Bengaluru (12°58′N; 77°35′E) during march 2013-14. In the present study, seven mango varieties were selected and grouped into three groups as susceptible (cvs. Banganapalli, Alphonso and Totapuri), moderately resistant (cvs. Mylupilian and Dusheri) and resistant (cvs. Langra and EC-95862) to fruit fly based on the earlier studies of Verghese *et al*. (2012) and Jayanthi and Verghese, (2008). All the mango trees were approximately 15 years old.

Four trees of each cultivar were selected for study purpose; these selected trees were regularly monitored for flowering and fruiting. At fruit set more than 100 inflorescence were tagged on each selected tree. Once the fruit setting was stabilized nearly 50 marble sized fruits (~2-2.5cm). Pea sized fruits were considered as day -1 fruits and they were monitored for maturity. The tagged fruits were harvested at different maturity levels, namely 50% maturity, 75% maturity and 100% maturity in order to obtain variability in tannin levels in the fruits and variability was further increased by using fruits unknown to be susceptible, moderately resistant and resistant. Maturity was calculated based on the protocol standardized by Verghese *et al.* 2012.

Five apparently healthy fruits were randomly selected and harvested along with a long stalk (3-4cm) to avoid oozing of the sap from fruits and prepared for further analysis on the same day. Each maturity class fruits were separately grated using grater/peeler to get exocarp (Peel) which is the greenest part of the fruit. Peeling was done till the whitish or yellowish pulp was visible. The remaining part except seed was considered as mesocarp (Pulp). Peel and pulp of each fruit was divided into four sections representing different portions of the fruit and sample was collected from all the sections and mixed together to avoid bias. Dry samples were prepared by drying 50g of peel and 50g of pulp of all varieties (five replications each) in glass Petri plates in hot air oven at 65^{o}C for 48 hours or the period was extended until the samples had completely dried. This was then blended in the mixer for 4-6 min to get a fine powder of the sample. Further two sub replications of the extract from each main replication were used for analysis (five main replications; ten sub replications). Powdered samples were stored in butter paper covers with proper labeling at room temperature till further analysis.

Measurements of biochemical parameters

Total phenols

Total phenols in the extracts were estimated following Singleton and Rossi (1965) using gallic acid as standard. 80 per cent methanolic extract (0.5mL) of tissue sample was mixed with distilled water (3.3mL). Folin-Ciocalteau phenol reagent (0.2 mL) was added to tubes and mixed thoroughly. After five min, Na2CO3 (20%; 1mL) solution was added and the mixture incubated at room temperature for 1 hour. Absorbance was measured at 700 nm using Beckman Model DU 64 UV-Visible spectrophotometer and the content of total phenols was expressed as mg Gallic acid equivalents (GAE) per gram of sample**.**

Table 1. Total phenolics content in peel and pulp of mango varieties at 50, 75, 100 per cent maturity

Variety	Total phenolics content (mg GAE /g FW) at maturity level							
	50%		75%		100%		Average	
	Peel	Pulp	Peel	Pulp	Peel	Pulp	Peel	Pulp
Totapuri	11.55^{d}	0.954b^{c}	5.04^{f}	0.885^{b}	5.86^{e}	0.361^{e}	7.481	0.733
Alphonso	13.38^{d}	1.135^{b}	18.51^{c}	0.388^{c}	12.46^{cd}	0.766^{c}	14.783	0.763
Banganapalli	12.71^{d}	0.917^{bc}	10.25^{e}	0.999^{ab}	9.24^{d}	0.944^{b}	10.731	0.953
EC 95862	25.55^{b}	1.538^{a}	37.14^{a}	1.109^{a}	18.57^{b}	1.085^{a}	27.087	1.244
Langra	42.07^{a}	1.078^{b}	26.24^{b}	0.871^{b}	35.43^{a}	1.093^{a}	34.579	1.014
Dusheri	21.21^{c}	0.519^{d}	14.46^{d}	0.933^{ab}	13.25^{c}	0.730^{c}	16.31	0.727
Mylupilian	21.00^{c}	0.753^{cd}	8.86^{e}	0.987^{ab}	10.10^{cd}	0.642^{d}	13.32	0.794
CD (p=0.01)	3.535	0.2406	3.779	0.1873	3.15	0.0721		

FW: Fresh weight For a particular maturity level, means followed by different alphabets in the same column are significantly different (ANOVA and Duncan's Multiple Range Test (DMRT), $p < 0.01$).

Table 2. *Bactrocera dorsalis* infestation at different maturity levels

Variety	Infestation (%) at maturity stage			Reaction to fruit fly
	50%	75%	100%	
Totapuri	0.48	24.9	62.5	Susceptible
Alphonso	0.55	20.8	55.8	Susceptible
Banganapalli	0.6	34.5	69.2	Susceptible
EC 95862	0	0.1	1.4	Resistant
Langra	0	1.88	0	Resistant
Dusheri	0.1	29.8	42.8	Moderately Resistant
Mylupilian	0.2	21.8	24	Moderately Resistant

N=50

Phenolic acids

Phenolic acids were extracted and estimated as described by Tuzen and Ozdemir (2003). Tissue sample (50g) was extracted with petroleum ether (b.p.40-60°C) and the extract was acidified using 2 M HCl. The liberated free acids were extracted with ethyl acetate from aqueous solution. Phenolic acids were recovered from the extract by washing with two per cent NaHCO3 (w/v) using a separatory funnel. The aqueous NaHCO3 layer was acidified and hydrolysed separately using eight per cent HCl and 2N NaOH solutions for 4 h under an atmosphere of nitrogen. The mixture was filtered, acidified and extracted three times with ethyl acetate, evaporated under vacuum at 40°C and dried at 8°C for 10 min. The residues were re-dissolved in 10 ml methanol (HPLC grade). Solutions were stored at -20°C for analysis of phenolic compounds. One milliliter of freeze-dried sample was re-dissolved in mobile phase and filtered through RanDisc PVDF (0.22 μm). Filtered methanolic solution was (20μL) analyzed by HPLC. The HPLC analyses were carried out on a Shimadzu LC-10A system (Shimadzu, Kyoto, Japan) consisting of a liquid chromatograph (Plate 19) connected to a UV-VIS detector (10A), binary pump and controlled by Shimadzu class VP workstation software. The column used was Synergi, 250 × 4.6mm, 4μm Hydro-RP, C18 (Phenomenex, USA) with security guard column having C18 cartridge (cat no. 7511, Phenomenex, CA, USA). Samples were injected using a 20μL loop (Rheodyne, Rohnert Park, CA, USA). The column and guard column were thermostatically controlled at 35°C. The flow rate was 1mL/min and mobile phase consisted of 4.5% acetic acid (solvent A) and methanol (solvent B). The detection was monitored at 280nm.

Per cent infestation

Extent of herbivory or infestation status of the seven varieties was recorded at the three maturity levels. Randomly selected fruits (n=50) from insecticide-free orchards of the seven varieties were harvested at three maturity levels and brought to laboratory and kept in cages for ripening. They were dissected on full ripening and those with maggots were recorded as infested.

Statistical Analysis

The data on biochemical components analysed at three different maturity levels were subjected to one way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) to examine if the treatment means were significantly different (Microsoft corporation version 12.0). Further to study the possible influence of biochemical factors of selected varieties on infestation levels by fruit fly, the data were subjected to correlation and linear regression analysis (Minitab Inc. version 16.1.1).

RESULTS AND DISCUSSION

Among secondary metabolites, phenolic compounds play a major role in defence mechanisms. Phenolics, commonly found in fruits, have been reported to exhibit antioxidant activity, due to the reactivity of the phenol moiety, and have the ability to scavenge free radicals, *via* hydrogen donation or electron donation (Shahidi *et al*., 1992). In the present study, the infestation levels were highly influenced by the total phenolics content where, the total phenolic content was very high in resistant varieties Langra's and EC 95862's peels and the infestation was zero. Whereas, the moderately resistant varieties Dusheri and Mylupilian also had considerably high amount of total phenolics in their peels and the infestation percentages was 0.10 and 0.20, respectively. In case of the susceptible varieties Totapuri, Banganapalli and Alphons's, peels had lower phenolic content with 0.48, 0.60 and 0.55 percentage infestation at 50 per cent maturity (Table 1 and 2).

Lattanzio *et al*. (2006) also reported that the phenolic compounds play a vital role in plant resistance and protect

Table 3a. Phenolic acids (µg/ml) in peel of different mango varieties at 50 per cent maturity

Variety	Phenolic acids								
	Gallic acid	Proto catechuic acid	P-OH benzoic acid	Vanillic acid	Syringic acid	P-coumaric acid	Ferulic acid	O-Coumaric acid	T-Cinnamic acid
Totapuri	1021.17	23.11	161.23	25.61	A	12.74	33.74	5.55	4.12
Alphonso	930.91	31.23	483.23	14.68	A	14.96	29.03	6.94	2.16
Banganapalli	193	17.42	447.28	32.32	A	9.32	21.92	7	1.4
EC 95862	1753.99	37.35	66.43	11.28	A	33.91	40.24	8.24	1.67
Langra	2569.43	29.33	11.47	21.68	A	25.21	16.66	13.92	14.2
Dusheri	1097.45	15.37	95.11	10.13	A	11.02	10.64	9.45	4.83
Mylupilian	487.26	12.26	67.2	9.92	A	10.07	22.89	6.08	0.9

(A=Peak Absent; T=Traces)

Table 3b. Phenolic acids (µg/ml) in pulp of different mango varieties at 50 per cent maturity

Variety	Phenolic acids								
	Gallic acid	Proto catechuic acid	P-OH benzoic acid	Vanillic acid	Syringic acid	P-coumaric acid	Ferulic acid	O-Coumaric acid	T-cinnamic acid
Totapuri	147.6	7.92	9.7	A	A	3.15	4.33	5.27	A
Alphonso	55.7	4.04	6.75	A	8.59	3.88	A	10.34	T
Banganapalli	40.71	3.35	6.14	A	2.16	A	A	7.74	A
EC 95862	207.3	13.73	14.11	A	A	3.63	4.42	6.28	4.64
Langra	134.04	5.16	4.37	A	A	3.13	3.82	4.19	T
Dusheri	19.03	3.59	10.08	A	17.17	3.11	A	7.16	T
Mylupilian	10.11	3.98	6.29	A	A	3.09	3.81	A	A

(A=Peak Absent; T=Traces)

fruits and vegetables against pests. Likewise Abu-Goukh and Abu-Sarra, (2003) and Al-Ogaidi and Mutlak (1986) too reported role of phenolics in resistance of dates (*Phoenix dactylifera*) to insect infestation during storage. A well-known example of simple phenolics being feeding barriers to insect herbivores is represented by salicylates in *Salix* leaves and their role in the feeding and growth of the polyphagous larvae of *Operophtera brumata*. It has been observed that the levels of salicylates correlated negatively with growth (Simmonds, 2003). Salicylic acid induces changes in mango fruit that affect ovipositional behavior and development of *Bactrocera dorsalis* (Jayanthi *et al*., 2015). At 75 per cent maturity, there was a decrease in the total phenolic content in peels of all the varieties except for EC 95862 where there was an increase in phenolics from 25.55 (50% maturity) to 37.14 mg GAE /g FW. The extremely low percentage infestation (0.10) in EC 95862 may be attributed to increase in phenolics. Zero per cent infestation in Langra even at 100 per cent maturity maybe due to the increase in total phenolic content in its peel (fig 2). The pulps of all the mango varieties had low total phenolic content compared to the peels at all stages and there was a significant negative relation with total phenolics and infestation levels. These results are consistent with previously reported result. In other crops and in mango this is confirmatory of earlier study (Verghese *et al*., 2012).

The phenol in mango resin might have exhibited toxicity to eggs and larvae of *B. dorsalis* as observed in the present study. Similar effect was reported in case of *Rhagoletis pomonella* in crab apple (Walsh) (Pree, 1977). Total polyphenolic content in unripe mango peel was 92.62 mg GAE/g. which was approximately three-fold higher than unripe mango flesh and ripe mango pulp. Results showed that mango peel contained more phenolics than mango pulp, regardless of ripeness (Kim *et al*., 2010) and total polyphenolic content in extract of raw mango peels ranged from 90 to 110 mg/g peel, and 55-100 mg/g in ripe peel, depending on the variety (Ajila *et al*., 2007b). Peel constitutes of about 15–20% of the fruit. Higher amount of phenolics is reported in peel than in pulp of mango (Abu-Goukh and Abu-Sarra, 1993) and guava (Bashir and Abu-Goukh, 2003). As *B. dorsalis* is a pest on guava, perhaps the same principle can be applied, provided peels don't alter the taste as of table guava are consumed with peel. However, for processed guava this may not apply.

Different phenolic acids in peel and pulp of mango varieties

Gallic acid, Proto-catechuic acid, p-OH benzoic acid, p-coumaric acid, ferulic acid, o- Coumaric acid and t- Cinnamic acid were the phenolic acids recorded in mango peel. Very high gallic acid was noticed in Langra (2569.43 µg/ml), next highest was EC 95862 (7153.99 µg/ml) and Dusheri and Totapuri also showed considerably higher gallic acid contents but Banganapalli (193.0 µg/ml) recorded the lowest content. Based on the present data it is evident that the resistance factor due to phenolics might be because of the gallic acid levels (Fig 1 & 2).

Gallic acid, chemically denoted as 3, 4, 5-trihydroxybenzoic acid, is thought to be derived from the dehydrogenation of 5-dehydroshikimic acid, an early intermediate of the shikimic acid pathway (Grundhöfer *et al*., 2001). Gallic acid has been associated with phytotoxicity and antifungal activity and has been of great interest in the potential prevention of atherosclerosis (Abella *et al*., 1997). Gorinstein *et al*. (1999) reported that the gallic acid content in the tropical fruits (litchi, guava, and ripe mango (cv. Keaw)) was in the range of 147.2 to 397.4 g/100 g of fresh fruit. The lowest content of gallic acid was recorded in pineapple and the highest in ripe mango (cv. Keaw). Same trend was seen in pulp, higher gallic acid was recorded in EC 95862 (207.3 µg/ml) and Langra (134.04 µg/ml) where the infestations were low and lowest was seen in Mylupilian with 10.11 µg/ml. Syringic acid was found to be absent in Totapuri, EC 95862, Langra and Mylupilian, whereas, p-coumaric acid was absent in Banganapalli and ferulic acid was not found in Alphonso, Banganapalli and Dusheri pulp during 50 percent maturity (Fig 1). As the function of each phenolic acid or combination of phenolic acids is unknown it is difficult to interpret the effect of absence or presence of these phenolic acids (Table 3a to 3b).

At 75 per cent maturity, there was a reduction in gallic acid content in all varieties except for Langra and EC 95862. There was an increase in the gallic acid levels compared to 50 per cent maturity. P-coumaric acid was absent in Banganapalli peel. Higher levels of proto catechuic acid and p-OH benzoic acid was seen in Langra and EC 95862 peel compared to other varieties. Further detailed studies on the phenolic acids are essential to confirm its function and it would be very useful in implementing it in IPM for *B. dorsalis*.

CONCLUSION

Among secondary metabolites, phenolic compounds play a major role in defence mechanisms. It was observed that phenolics content was more in resistant varieties like Langra and EC 95862. As fruits matured from 50-100% phenolics and tannins also increased in resistant varieties while in susceptible it was decreased. Therefore, the

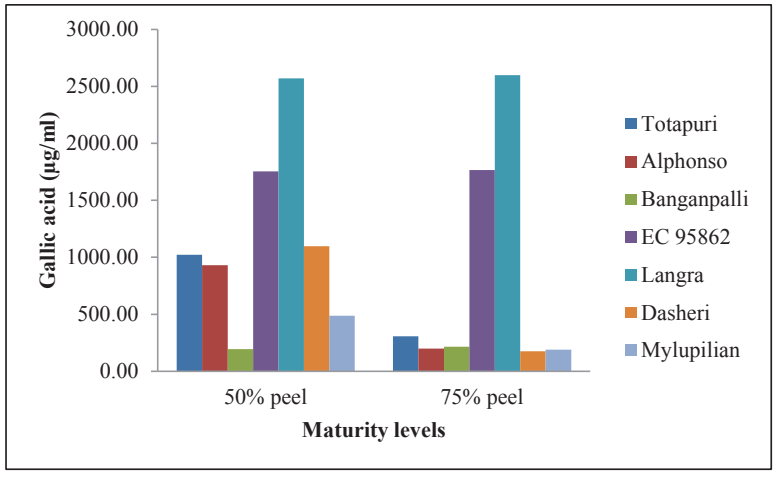

Fig 1. Gallic acid content (µg/ml) at different maturity levels in mango varieties

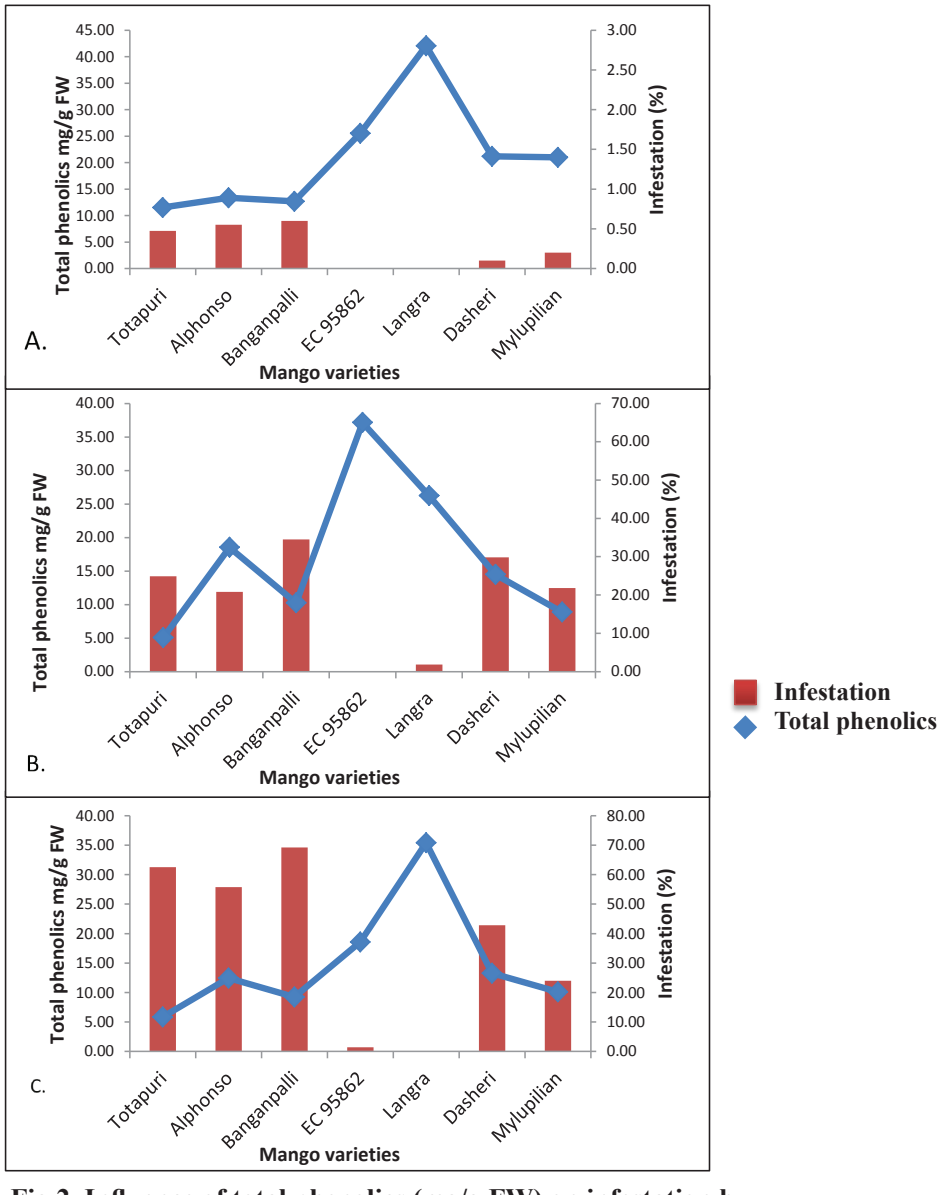

Fig 2. Influence of total phenolics (mg/g FW) on infestation by *B. dorsalis* at 50% maturity (A), 75% (B) maturity and 100% maturity (C)

data on the phenolics in different mango varieties in the present study it can be concluded that these are of importance in host plant resistance in mango and in developing resistant varieties to *B. dorsalis*. This will pave way to a stronger IPM which now involves methyl eugenol traps, bait sprays and sanitation.

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