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Foliar supplementation with micronutrients during early growth of bitter gourd fruit (*Momordica charantia*) augments resistance to melon fly (*Bactrocera cucurbitae*) infestation by activation of antioxidant system

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ABSTRACT

Melon fly is the most destructive pest of cucurbits all over the world. Although, the pest is controlled by the use of insecticides, the risks of development of resistance in the pest due to continued application of pesticide and accumulation of toxic residues in fruits have led to the search for alternative non-chemical control strategies which are eco-safe and environment friendly. In this study, an attempt was made to examine the effect of foliar mineral nutrition on pest incidence and fruit quality. The field study was laid out in a Randomized complete block design in which bitter gourd fruits during the early fruit development phase were sprayed with micronutrient solution containing Ca, Cu, Mg and Mn. Results showed that melon fly infestation was significantly reduced coupled with increased fruit yield and quality. Treated fruits showed significantly higher levels of antioxidants, phenolic acids and flavonoids compared to Control indicating that mineral nutrients protect bitter gourd through activation of antioxidant system in the host combined with their inhibitory effects on oviposition, adult emergence and increased mortality of melon fly larvae. The study established that resistance to melon fly could be induced by supplementing the levels of Ca, Cu, Mg and Mn in bitter gourd fruit by exogenous application at a time when the fruit is most vulnerable to infestation. It appears, therefore, possible to explore foliar micronutrient supplementation as a component of Integrated Pest Management (IPM) strategy for the successful control of melon fly infestation in bitter gourd coupled with higher yield and improved fruit quality.

***Keywords*:** Melon fly, *Bactrocera cucurbitae*, chayote, bitter gourd, host resistance, mineral elements, defence compounds

Introduction

Melon fly [*Bactrocera cucurbitae* (Coquillett)] is the most destructive insect pest of cucurbits in India (Narayanan, 1953). The pest severely infests members of the *cucurbitaceae* family (Dhillon *et al.,* 2005) including the bitter gourd fruit (*Momordica charantia* L.,) leading to dramatic reduction in yield ranging from 30-90% (Dhillon *et al.,* 2005). However, chayote (*Sechium edule* L,) is the lone exception showing resistance to the pest (Shivashankar *et al*., 2014). Repeated application of insecticides by farmers to control the insect pest is leading to increasing environmental pollution, higher cost of production, residual toxicity and development of resistance in the target insect (Aktar *et al.,* 2009). Hence, integrated pest management (IPM) strategies to control pest damage without the associated adverse effects on the ecosystem (Pretty and Bharucha, 2015) are desirable.

Mineral nutrients play a major role in controlling plant pests and diseases (Christos, 2008). Plants with an optimal nutritional status are known to show the highest resistance/tolerance to pests (Huber *et al.,* 2012). Since mineral micronutrients act as signalling molecules (Hossain *et al.,* 2012) and constitute an integral part of the antioxidant defence machinery of plants (Marschner, 2012) , they influence host metabolism to provide protection against pests by way of synthesis of natural defence compounds such as phytoalexins, antioxidants and flavonoids (Schumann *et al.,* 2010). Thus, considering the critical role of micronutrients in development of resistance against insect pests, foliar application of nutrients is being recommended as an IPM strategy to improve plant resistance coupled with long-lasting benefits to the environment (Gupta *et al.,* 2017). The key to using this IPM approach, however, is to maintain optimum nutrient levels in the target tissue, at a time when the crop

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is most vulnerable to insect attack based on crop type, plant life cycle, and cultural practices. Besides, mineral elements also alter the quality of the food source of herbivorous insect pests (Goncalves-Alvim *et al.,* 2004) and thus affect aphid population dynamics under field conditions (Silva *et al.,* 2005).

An exploratory study conducted in our lab on melon fly susceptible bitter gourd and the resistant chayote had revealed considerable differences in the levels of micronutrients in fruits among which Ca, Mg, Mn and Cu were significantly lower in bitter gourd compared to chayote.Assuming that resistance of chayote to melon fly is governed by the levels of mineral micronutrients,the present study was conducted to determine if application of the four deficient micronutrients on young developing fruits could induce resistance to melon fly infestation in susceptible bitter gourd. Experiments were, therefore, conducted by supplementing young developing bitter gourd fruits with these four critical mineral nutrients and evaluated for the rate of melon fly infestation and fruit yield.

Materials and Methods

Plant material

Experiments were conducted at the Indian Institute of Horticultural Research, Bengaluru during 2015 and 2016. Ten-day-old plantlets raised from seeds of bitter gourd were planted at a spacing of 2.5m×2.0m in pits measuring 0.5m length×0.4m width×0.5m depth during the first week of May. Vines were trained on poles to a height of 1.6 m above the ground to facilitate spreading on a raised structure made of bamboo canes. Fruits were harvested for studies from third week of July.

Field studies with mineral nutrient spray

The experimental plants laid out in a Randomized complete block design (RCBD) were supplied with the recommended dose of N, P and K fertilizers plus Farm yard manure and the mixture was incorporated into the top 15 cm of the ridge at soil preparation. Surface irrigation was given to plants twice a week. Field studies were performed by spraying leaves and young fruits from thirty plants each with mineral nutrient solutions, T_1, T_2 and T_3 (Table 1) mixed with the surfactant, All Purpose Spray Adjuvant formula (APSA 80, Amway India Enterprises Pvt. Ltd, New Delhi, India) (0.03%) (Treated) 10, 15 and 20 days after fruit set (DAF) while water mixed with APSA 80 (0.03%) was sprayed on 30 plants (Control) on corresponding days.

Table 1: Composition of treatment formulation used for spray application

Mineral nutrients	T_1	T_2	T_3
		g/Lit	
$CaCl_2$	12.15	16.2	20.250
$MgSO_4$	7.575	10.1	12.625
$ZnSO_4$	0.173	0.231	0.289
$MnSO_4$	0.187	0.249	0.311
$CuSO_4$	0.191	0.255	0.319
EDTA	0.075	0.10	0.125
APSA	0.3 mL	0.3 mL	0.3 mL

Rate of melon fly infestation

Fruits showing brown spot at the site of insect incursion was considered 'infested' while those remaining free of brown spot were considered 'healthy'. Harvested fruits in each batch were observed to record the number of infested fruits.

Measurement of life cycle of melon fly

Treated and Control fruits were compared for the number of oviposition punctures/dm^2, number of eggs laid, rate of pupation and the number of adults emerged.

Biochemical studies

Antioxidative enzymes

Catalase (CAT, E.C. 1.11.1.6) activity One g of fruit tissue was homogenized in 8.0 ml of 67 mM sodium phosphate buffer, pH 7.0 and centrifuged at 12,000 ×*g* for 10 min at 4ºC The supernatant was

used as the crude enzyme source. The assay mixture (3 ml) contained 100 mM Tris- HCl buffer, pH 7.0, 0.1 ml crude enzyme extract, and 200 mM H_2O_2. Specific activity of CAT was determined using the molar extinction coefficient of H_2O_2 at 240 nm (ϵ = 43.6 $M^{-1}cm^{-1}$) and expressed as nmoles mg $protein^{-1}$ min^{-1} (Luck, 1965).

Peroxidase (POD) activity POD (EC 1.11.1.7) enzyme was extracted by grinding 1 g fresh tissue in a chilled mortar and pestle in 100 mM sodium phosphate buffer (pH 7.0). The homogenate was filtered through four layers of muslin cloth and centrifuged at 18,000 ×*g* for 15 min at 4°C and the supernatant was used as enzyme source. The reaction mixture (5ml) for the assay of POD contained 50 mM sodium phosphate buffer (pH 7.0), 20 mM guaiacol and 100 µl enzyme extract. The reaction was initiated by the addition of 0.042% (v/v) H_2O_2, and the increase in absorbance at 470 nm was monitored for 3 min at 30 s intervals with a T80+ UV-Visible spectrophotometer (PG Instruments Inc., UK) and estimated according to Saroop *et al.* (2002). The specific activity of POD (nmoles mg^{-1} protein min^{-1}) was calculated using the molar extinction coefficient for guaiacol ($26.6M^{-1}$ cm^{-1}).

Superoxide dismutase (SOD) activity SOD (E.C.1.15.1.1) enzyme was extracted by homogenizing 1 g fresh fruit tissue in 50 mM sodium phosphate buffer, pH 7.8. The homogenate was centrifuged at 10,000 ×*g* for 10 min at 4ºC and the clear supernatant containing the enzyme was used to assay for SOD activity based on its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) following Beauchamp and Fridovich (1971). The reaction mixture (3 ml) contained 50 mM sodium phosphate buffer, pH 7.8, 100 mM EDTA, 130 mM methionine, 0.75 mM NBT, 20 µM riboflavin, and 0.1 ml crude enzyme extract. Reaction was initiated by illuminating the tubes under four 40 W fluorescent lamps for 8 min following which absorbance at 560 nm was recorded against a blank mixture maintained in dark. One Unit of SOD activity was defined as the amount of enzyme required to inhibit the photo-reduction of NBT by 50% compared to that caused by the superoxide generated in the blank by the reaction between photo-reduced riboflavin and oxygen under the assay conditions. SOD enzyme activity was expressed as Units mg^{-1} protein.

Antioxidant capacity

Ferric reducing anti-oxidant power (FRAP)

FRAP was assayed according to Benzie and Strain (1996) based on the increase in absorbance at 593 nm The anti-oxidant capacity of a sample which is related to its ability to reduce ferric ions, was expressed as ascorbic acid equivalent anti-oxidant capacity (AEAC; in mg ascorbic acid 100 g^{-1} FW of tissue), where the reducing power of a 1.0 mg sample was equivalent to the reducing power of 1 nmole ascorbic acid.

2, 2-diphenyl-l-picrylhydrazyl (DPPH) radical scavenging activity (DPPH)

DPPH radical scavenging activity was estimated following Brand-Williams *et al.* (1995) with some modifications. A 0.2 ml sample of 80% (v/v) methanolic extract of fruit tissue was mixed with 0.3 ml of 10 mM acetate buffer, pH 5.4 and 2.5 ml 0.008% (w/v) DPPH in 100% (v/v) methanol. The mixture was incubated at room temperature (30ºC) for 30 min, followed by measurement of absorbance at 517 nm. DPPH scavenging activity was expressed as mg ascorbic acid equivalents g^{-1} FW of sample tissue.

Total phenols and flavonoids

Phenolic acids and flavonoids were extracted in 80% (v/v) methanol. Phenolic acids were estimated following Singleton and Rossi (1965) using gallic acid as standard and expressed as mg gallic acid equivalents g^{-1} fresh weight (FW) of sample tissue. Total flavonoids were determined according to Kim *et al.* (2003) using catechin as standard and expressed as mg catechin equivalents g^{-1} FW of sample tissue.

LC-MS separation of phenolic acids and flavonoids

Phenolic acid standards namely ferulic acid, 2,4 dihydroxy benzoic acid, caffeic acid, gallic acid, gentisic acid, *o*-coumaric acid, *p*-coumaric acid, *p*-hydroxy benzoic acid, protocatechuic acid, salicylic acid, syringic acid, *t*-cinnamic acid, vanillic acid, chlorogenic acid and flavanoid standards namely catechin, hesperitin, apigenin, neringenin, myrcetin, rutin, luteoline, quercetin, umbelliferone

were prepared in 80% ethanol. The organic solvents used as mobile phase for liquid chromatography were of chromatographic/MS grade and all other reagents were of analytical grade. Water purified in Milli-Q (Millipore) system was used to prepare the mobile phases. All solvents used for mobile phase were filtered through membranes with a pore size of 0.45 µm. Standard curves for individual phenolic acids and flavonoids were prepared using different concentration of individual compounds.

An Acquity UPLC-H class coupled with TQD-MS/MS (Waters, USA) and equipped with ESI source, degasser, quaternary pump, automatic injection system (0–10 µl), diode array detector and a temperature control compartment for the analytical column was used for determinations of phenolic acids and flavonoids. The system, controlled by Mass lynx software, allowed for simultaneous detection at multiple wavelengths, MRM for individual masses Table (2) and administration of data collection and treatment system. All the phenolic acids and flavonoids were identified and quantified by the molecular weight (parent mass m/z) of most abundant fragmented daughters**.**

Table 2: MRM data for phenolic acids

Compound	Formula/ Mass	Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode
Caffeic acid	180	178.90	30	135.05	16	ES-
2,4-Dihydroxybenzoic acid	154	152.90	28	65.02	18	ES-
Chlorogenic acid	354	352.97	22	191.10	18	ES-
Ferulic acid	194	192.90	26	134.02	14	ES-
Gallic acid	170	168.90	28	125.03	12	ES-
Gentisic acid	154	152.90	24	108.98	12	ES-
***o*-Coumaric acid**	164	162.90	22	119.06	12	ES-
***p*-Coumaric acid**	164	162.90	24	119.05	14	ES-
***p*-Hydroxybenzoic acid**	138	136.90	26	93.01	12	ES-
Protocatechuic acid	154	152.90	26	109.05	16	ES-
Salicylic acid	138	136.90	28	93.10	14	ES-
Syringic acid	198	196.97	26	182.07	10	ES-
***t*-Cinnamic acid**	148	146.90	26	103.05	10	ES-
Vanillic acid	168	166.97	26	108.01	20	ES-

LC and MS-MS conditions

Mobile phase consisted of (A) an aqueous component containing 0.1% formic acid in water and (B) organic phase containing 0.2% formic acid in methanol. The solvent gradient had 90% (A) and 10% (B) initially which was changed to 70% (A) and 30% (B) at 4.0 min. At 5.0 min, the gradient was changed to 60% (A) and 40% (B) followed by 80% (A) and 20% (B) at 10.0 min. The final gradient at 12.0 min was 90% (A) and 10% (B) for 2.0 min. At 14 min, the system was returned to initial conditions and held for 1 min for equilibration before the next injection. The flow rate was maintained at 0.3 ml/min. The analytical column, 2.1×50mm UPLC BEH- C18 column (Waters, USA) with 1.7µm particle size maintained at 25°C, was protected by a Vanguard BEH C-18, 1.7µm particle size guard column (Waters, USA). The sample injection volume was 2 µl for both phenolic acids and flavonoids. The eluted metabolites were monitored using the UPLC column. Effluent was pumped directly without split into the TQD-MS/MS (Waters, USA) system, optimized for analysis of phenolic acids and flavonoids.

Lignin content

Lignin content was estimated according to Doster and Bostock (1988) with modifications. Samples were extracted in methanol for 48 h after which residue was dried in a desiccator and ground to a fine powder. Five hundred milligrams of dry powder were mixed with 5 ml of 2M HCl and 0.5ml of thioglycolic acid. Samples were heated at 95°C for 4 h and centrifuged at 3,000 ×*g* for 20 min. Pellets were washed twice with deionised water, extracted with 2mL of 0.5M NaOH for 18 h followed by 2 ml of deionised water at room temperature. Sample was centrifuged at 15,000 ×*g*, supernatants collected, mixed, acidified with 1 ml of concentrated HCl and stored at 5°C overnight. Sample was centrifuged, residue dissolved in 10 ml of 0.5M NaOH and centrifuged at 15.000 ×*g*. The absorbance

of the clear supernatant was measured at 280 nm and the lignin content was expressed as $A_{280}.g^{-1}$ dry weight of sample.

Extraction of Lignin monomers

Lignin monomers were extracted from protein-free cell wall fraction following Lima *et al.* (2013). Fruit tissue (5 g) was homogenized with 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 10.000 *g* for 4 min. The pellet was washed sequentially with phosphate buffer (pH 7.0) followed by 1% (v/v) Triton X-100 in pH 7.0 buffer, 1M NaCl in pH 7.0 buffer, distilled water and finally acetone. The pellet was dried in an oven at 60˚C for 24 h and cooled in a vacuum desiccator. The dry mass containing protein-free cell wall fraction (100 mg) was transferred to a Pyrex glass ampoule containing 1ml of 2M NaOH and 1ml of nitrobenzene and sealed. The ampoules were heated at 170˚C for 90 min on a silicone oil bath with occasional stirring. The tubes were cooled to room temperature, washed twice with chloroform, acidified to pH 2.0 with 2M HCl and extracted twice with chloroform. The organic extracts were combined, dried and re-suspended in 1ml of a mixture of methanol and 4.5% acetic acid in water (20:80, v/v). The samples were filtered through a 0.2 µm nylon filter before analysis by high-performance liquid chromatography.

HPLC separation and determination of lignin monomers

HPLC analyses were carried out on a Shimadzu LC-10A system (Shimadzu, Kyoto, Japan) consisting of a liquid chromatograph equipped with UV–VIS detector (10A), binary pump and controlled by Shimadzu class *VP* workstation software. The column used was Synergi, 250 × 4.6mm, 4 µm Hydro-RP, C 18 (Phenomenex, Torrance, CA, USA) with guard column having C18 cartridge (cat no. 7511, Phenomenex). Samples were injected using a 20 µL loop (Rheodyne, Rohnert Park, CA, USA), and the absorption of separated compounds was monitored at 280 nm. The temperatures of both the sample column and guard column were thermostatically controlled at 35˚C. The flow rate of the mobile phase consisting of 4.5% acetic acid (solvent A) and methanol (solvent B) was maintained at 1 ml min^{-1}. The instrument was run in gradient mode for which conditions were maintained as follows, 0–20 min, 10% B; 20–25min, 10–15% B; 25–40min, 15–20% B; 40–60min, 20–45% B; 60–65min, 45–75% B, 65–75min, 75–100% B. The concentration of phenolic acid was expressed as µg 100 g^{-1} FW of sample. Estimation of lignin monomers in protein-free cell wall fractions was carried out by HPLC on a Shimadzu Series LC-10A system consisting of a liquid chromatograph connected to a UV–VIS detector, binary pump and controlled by Shimadzu Class *VP* Workstation software. The column used was Luna HST C_{18} (2) (100 × 0.3mm, 2.5 µm, Phenomenex) with security guard column having C 18 cartridge (cat no: 4287, Phenomenex). The temperatures of both the columns were thermostatically controlled at 30˚C. Samples were injected using a 20 µl loop (Rheodyne). Methanol (B) and 4.5% acetic acid in water (A) in 20:80 ratio were used as the mobile phase at a flow rate of 0.3 ml min^{-1}. The instrument was run in isocratic mode and the peak absorption was monitored at 290 nm. Aldehyde standards, *p*-hydroxybenzaldehyde, vanillin and syringaldehyde (Sigma-Aldrich Corp., St Louis, MO, USA) dissolved in methanol and filtered through 0.2 µm nylon filter were also analysed simultaneously for which the retention times were 7.76, 9.72 and 11.02 min, respectively.

Protein content

Protein content was determined using Folin-phenol reagent (Lowry *et al.,* 1951). One gram of tissue was homogenized in 5ml of 0.1M Na-phosphate buffer (pH 7.6) and centrifuged at 2,500 ×*g* for 10 min at room temperature. One hundred microliters of supernatant were mixed with 900 µl water and mixed with 5ml alkaline copper reagent [50ml Reagent A plus 1ml Reagent B; where Reagent A=2% (w/v) sodium carbonate in 0.1M NaOH and Reagent B=0.5% (w/w) copper sulphate in 1% (w/w) potassium sodium tartrate]. The reaction mixture was allowed to stand at room temperature for 10 min. Then, 0.5ml of Folin-phenol reagent was added, mixed and incubated for 30 min in the dark. The amount of protein was calculated from the absorbance at 660 nm and expressed as mg g^{-1} FW.

Mineral nutrients

Tissue was dried to constant weight at 70˚C in an hot air oven and ground to a fine powder. Nitrogen was determined by titrimetry after Kjeldahl digestion (Jackson, 1973). For all the other

nutrient elements, 1 g of dry tissue was digested with nitric acid–perchloric acid (9:4) mixture. Phosphorous, potassium and the micro-nutrient elements, Fe, Mn, Cu, and Zn were estimated according to Jackson (1973). Ca and Mg were estimated following Jones *et al.* (1991). Perkin ElmerA-Analyst-200 model atomic absorption spectrophotometer (Perkin Elmer, Waltham, MA, USA) was used to estimate the microelements.

Histochemical staining

Lignin histochemistry was studied using Wiesner reagent (Pomar *et al.,* 2004). Hand-cut cross-sections of Control and Treated bitter gourd fruits were incubated in phloroglucinol solution (2% in 95% ethanol) or 95% ethanol (staining control) for 10 min followed by treatment with 18% HCl for 5 min, and directly observed under bright-field using a DM2500 Fluorescence microscope (Leica Instruments, Wetzlar, Germany).

Statistical analysis

Field experiments: Experiments were laid out in a Randomized Complete Block Design (RCBD) with five replications. Each replication consisted of six plants of bitter gourd. Data on the rate of infestation, number of oviposition punctures, number of eggs laid, rate of pupation and the number of adults emerged during 2015 and 2016 were pooled and subjected to analysis of variance (ANOVA) using the MSTAT statistical program (Michigan State University, East Lansing, MI, USA) and were presented as means ± standard errors (SE).

Laboratory experiments: Differences between Control and Treatment were tested by applying Student's *t*-test with three replications and were presented as means ± standard errors (SE) (Panse and Sukhatme, 1978).

Results

Levels of mineral elements in bitter gourd and chayote fruits

The levels of mineral elements in bitter gourd and chayote fruits showed statistically significant differences for the levels of microelements among which Ca, Mg, Mn and Cu were found to be significantly lower in bitter gourd Table (3).

Table 3: Concentration of micronutrient elements in healthy chayote (CC) and bitter gourd (BG) fruits

Minerals	Healthy	
	CC	BG
Ca %	1.62±0.13a	0.45±0.02b
Mg %	1.01±0.09a	0.46±0.02b
Fe ppm	61.2±2.56b	92.2±5.32a
Zn ppm	23.1±1.03b	26.2±1.43a
Mn ppm	24.9±0.88a	16.6±1.09b
Cu ppm	25.5±1.21a	12.9±0.87b

Means with different letters represent statistically significant differences between CC and BG according to Student's t test (P≤.05).

Field studies with mineral supplements

The rate of melon fly infestation in control was very high at 76.49% in which the fruit pulp showed rotting symptoms and turned brown (Fig.1) and fruit yield was the lowest at 131.35 kg from a total of six harvests while plants treated with T_1, T_2 and T_3 showed significantly lower rate of infestation and higher cumulative fruit yield compared to Control Table (4). Among the three treatments, T_2 gave the lowest rate of melon fly infestation combined with a significantly higher fruit yield than T_1 or T_3 Table (4). Based on the results, further experiments on the mode of action of foliar application of mineral nutrients on melon fly infestation was limited to T_2 treatment only.

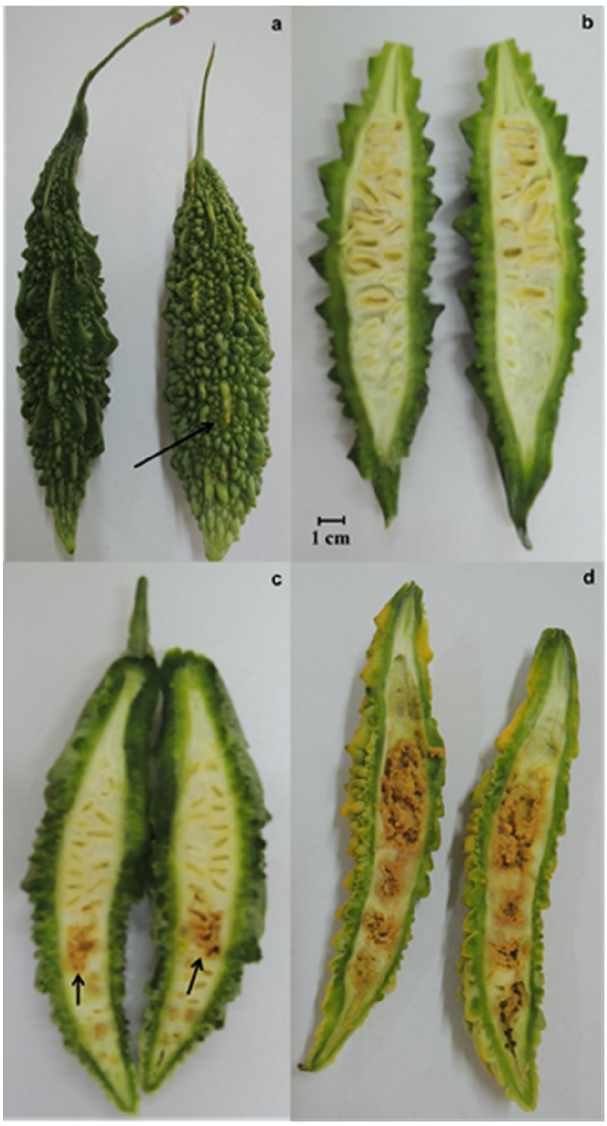

Fig. 1: Healthy (Panels a and b) and melon fly- infested (Panels c and d) bitter gourd fruits. Arrow indicates the site of infestation by melon fly. Scale bar =1.0 cm

Table 4: Melon fly infestation and fruit yield in control and treated fruits of bittergourd

Treatment	No of harvests	No.of harvested fruits	Fruit yield (kg)	No.of infested fruits	% infested fruits
Control	6	604±15.02	131.35±5.30	462±6.79	76.49±4.23a
T_1	6	648±7.89	146.24±6.74	84±3.86	12.96±1.54b
T_2	6	819±17.00	238.40±14.15	39±1.76	4.77±0.51d
T_3	6	684±9.92	160.58±8.05	42±2.08	6.14±0.83c

Means with different letters represent statistically significant differences between Control and Treatments according to Student's *t* test (P≤.05).

The field study using mineral supplementation showed that T_2 treated fruits of bitter gourd had attained nearly the same levels of Ca and Cu as in Chayote while the levels of Mg and Mn were about 50% of their levels compared to Chayote Table (5). Data on the number of oviposition punctures, number of eggs laid, rate of pupation and the rate of adult emergence were significantly lower in T_2 treated plants compared to Control. It was clear that oviposition was strongly inhibited in T_2 treated

fruits compared to Control and the slower rates of pupation and adult emergence led to increased mortality Table (6).

Table 5: Concentration of mineral elements in control and treated fruits of bittergourd

Minerals	Control	Treated (T_2)
N %	2.0 ± 0.095b	2.31 ± 0.054a
P%	0.48 ± 0.032b	1.70 ± 0.158a
K%	3.8 ± 0.131b	6.45 ± 0.226a
Ca%	0.76 ± 0.061b	1.37 ± 0.073a
Mg %	0.39 ± 0.042a	0.48 ± 0.036a
Fe ppm	73 ± 1.190b	119 ± 2.455a
Mn ppm	15 ± 0.208b	31 ± 0.708a
Cu ppm	10 ± 0.147b	12 ± 0.326a
Zn ppm	24 ± 1.372b	33 ±1.871a

Means with different letters represent statistically significant differences between Control and Treated according to Student's t test (P≤.05).

Table 6: Life cycle of melon fly in control and treated (T_2) fruits of bitter gourd

Treatment	No. of oviposition punctures/dm^2	No. of eggs / puncture	No. of days for pupation	No. of days for adult emergence	No. of adults emerged (%)
Control	12.0±1.73 a	8.0±0.33 a	5.92±0.35 b	6.69±0.23 b	85.6±2.72 a
Treated (T_2)	3.0±0.32 b	2.0±0.12 b	7.88±0.09 a	9.23±0.12 a	11.4±0.23 b

Means with different letters represent statistically significant differences between Control and Treated according to Student's t test (P≤.05).

Changes in antioxidant enzymes, antioxidant capacity, phenolic acids and flavonoids in T_2 Treated fruits

The study showed that the levels of total phenols, flavonoids, DPPH, FRAP, activities of POD and SOD were significantly higher in T_2 treated fruits compared to Control while CAT activity was lower in T_2 treated fruits (Fig. 2). T_2 treated fruits showed higher concentrations of flavonoids compared to control fruits Table (7). The data profiles of phenolic acids separated and identified based on MRM analysis showed significantly higher values in T_2 treated fruits compared to control Table (8) with the exception of vanillic acid, 2,4-dihydroxy benzoic acid, gentisic acid and protocatechuic acid.

Table 7: Levels of flavonoids in control and treated fruits of bittergourd

Flavonoids mg 100g^{-1} FW	Control	Treated (T_2)
Umbelliferone	8.72±0.25 b	21.80±1.11 a
Apigenin	19.37±0.61 b	58.11±1.28 a
Neringenin	18.84±0.42 b	32.97±1.19 a
Luteoline	150.80±2.53 b	339.30±1.62 a
Catechin	473.94±1.43 b	1026.87±3.61 a
Hesperitin	79.04±1.72 b	118.56±2.56 a
Quercetin	78.40±1.27 b	470.40±3.68 a
Myrcetin	24.12±0.68 b	48.24±1.21 a
Rutin	17.52±0.77 b	43.80±1.38 a
Kaempferol	11.24±0.48 b	28.32±1.14 a
Epicatechin	10.25±0.25 b	16.47±0.56 a
Epigallo catechin	19.28±0.53 b	31.22±1.20 a

Means with different letters represent statistically significant differences between Control and Treated according to Student's t test (P≤.05).

Table 8: Levels of phenolic acids in Control and Treated fruits of bittergourd

Phenolic acids mg 100g^{-1} FW	Control	Treated (T_2)
Chlorogenic acid	0.33±0.004 b	0.98±0.018a
Vanillic acid	2.92±0.061a	2.76±0.116 a
Syringic acid	0.15±0.008 b	0.73±0.020 a
Ferulic acid	3.99±0.121 b	6.03±0.220 a
Caffeic acid	0.03±0.002 b	0.08±0.009 a
Gallic acid	0.01±0.003 b	0.06±0.007 a
***p*-coumaric acid**	0.22±0.006 b	0.69±0.022 a
***o*-coumaric acid**	0.14±0.011 b	0.49±0.021 a
2,4-dihydroxy benzoic acid	0.06±0.006 a	0.01±0.002 b
Gentisic acid	0.006±0.0004 a	0.004±0.001 b
Protocatechuic acid	0.26±0.015 a	0.17±0.004 b
***t*-cinnamic acid**	0.025±0.0002 b	0.09±0.007 a
***p*-OH benzoic acid**	17.99±0.219a	19.58±0.547 a
Salicylic acid	35.39±0.622 b	67.54±1.213 a

Means with different letters represent statistically significant differences between Control and Treated according to Student's t test (P≤.05).

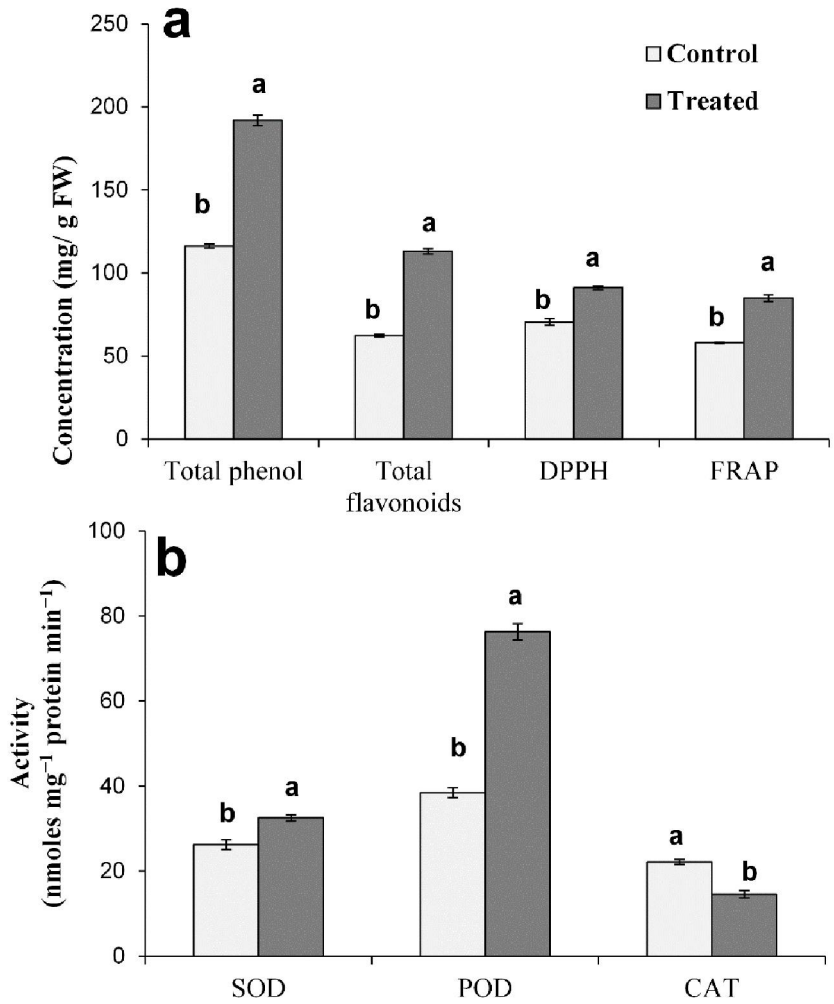

Fig. 2: Levels of antioxidants and scavenging enzymes in Control and Treated fruits of bittergourd. Means with different letters represent statistically significant differences between Control and Treated according to Student's t test (P≤.05).

Histochemical staining

Histochemical staining of fruit cross-sections following infestation showed formation of highly cross linked guaiacyl (G) units (Brown) at the site of infestation in T_2 treated fruit while Control fruit showed formation of syringyl (S) units (Red) (Fig. 3). Lignin content and the levels of monomers of *p*-hydroxycinnamyl (H), S and G moieties were significantly higher in T_2 treated fruits compared to Control fruits (Fig. 4).

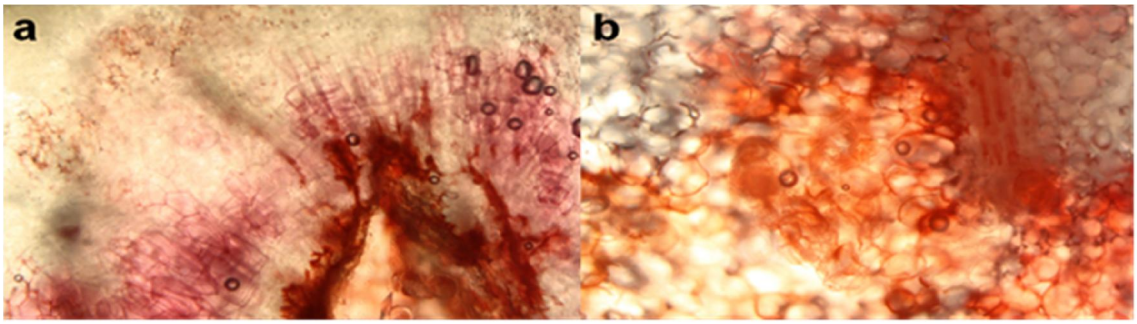

Fig. 3: Histochemical staining of lignin in cross-sections of bitter gourd fruit following melon fly infestation. Hand-cut cross sections of fruit were stained with Wiesner reagent for detecting lignin. Pink staining indicates the presence of *p*-hydroxycinnamyl aldehyde end groups in lignin while brown staining indicates formation of G units (Panel a). Red staining (Panel b) shows the presence of syringyl units. Adjustments to magnification and illumination were made to allow optimal viewing of sections.

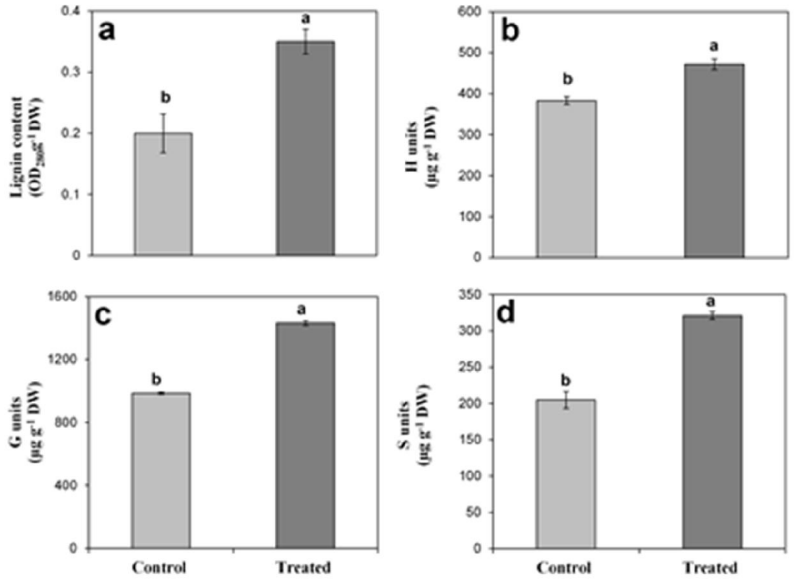

Fig. 4: Levels of lignins and their monomers in Control and Treated fruits of bitter gourd.
Means with different letters represent statistically significant differences between Control and Treated according to Student's t test (P≤.05).

Discussion

It is an established fact that well-nourished plants show better resistance/tolerance to pests and diseases compared to nutrient deficient plants since mineral elements constitute an integral part of their antioxidant defence machinery (Marschner, 2012). The melon fly susceptible bitter gourd showed significantly lower levels of Ca, Mg, Mn and Cu compared to the resistant chayote fruit Table (3). Hence, experiments conducted in the field by spraying bitter gourd plants with three concentrations of micronutrient solutions Table (1) containing the above four nutrients during the period of early fruit growth showed significantly lower rates of melon fly infestation coupled with

higher cumulative fruit yield compared to control plants Table (4). From a nutritional perspective, micro nutrient deficiency leads to impaired production of defense compounds, accumulation of soluble carbohydrates and reduced lignification, all of which contribute to lowered pest resistance. Among the three levels of nutrient sprays employed in this study, it was evident that T_2 treatment was the best in terms of lower infestation and higher yield compared to control and the other two treatments. Hence, further experiments were carried out with only spraying T_2 treatment solution. It was evident from the study that the number of oviposition punctures, number of eggs laid, rate of pupation and the rate of adult emergence were significantly lower in T_2 treated plants compared to control Table (6). A notable aspect of the study was that treatment of bitter gourd fruits with T_2 helped to restore the levels of Ca and Cu to the same level as in the resistant chayote while the tissue levels of Mg and Mn were also higher than that in control Table (5). This suggested a possible role for micronutrients in the development of host resistance against melon fly.

Mineral elements enhance the levels of defence compounds in host plant

The mode of interaction of mineral nutrients with insect pests is complex and depends on a number of external factors (Schumann *et al.,* 2010). In a previous study, we had shown that susceptibility to melon fly in bitter gourd was the result of an imbalance between the rate of production of nsert Reactive oxygen species (ROS) and its elimination by antioxidant enzymes (Shivashankar *et al.,* 2012). The present study revealed that mineral treatment with T_2 significantly enhanced the levels of the two antioxidant enzymes, SOD and POD, coupled with a significant reduction in CAT activity (Fig. 2). Since SOD enzyme has a role in detoxifying the excess H_2O_2, higher SOD activity in T_2 Treated fruits indicated its ability to more rapidly neutralize the excess H_2O_2 compared to control. POD is known to promote covalent linking between lignin and cell wall polymers helping to increase the rate of lignification. Direct proof for the increased rate of lignification in T_2 Treated fruits was obtained in this study from histochemical staining of fruit tissues (Fig. 3) in which T_2 Treated plants displayed higher number of G units (brown) around the site of infestation suggesting the formation of highly cross-linked lignin while on the contrary, control fruits showed formation of S units (Red) showing its susceptible nature. Similar results were reported in pepper and tomato by Pomar *et al.* (2004) after *V. dahliae* inoculation. CAT activity is known to be crucial for maintaining the redox balance during oxidative stress (Willekens *et al.,* 1997). Shim *et al.* (2003) showed that a fall in CAT activity under oxidative stress was related to the accumulation of salicylic acid which is believed to play a role in signalling plant resistance to herbivores (Ollerstam and Larsson, 2003). In a previous paper (Shivashankar *et al.,* 2012), we had shown that bitter gourd accumulated lower level of salicylic acid compared to the melon fly resistant chayote. Hence, it was obvious that the reduced CAT activity in T_2 Treated bitter gourd fruits indicated its increased resistance to melon fly attack compared to control.

Further evidence for the higher rate of lignification in T_2 Treated fruits came from data on the content of lignin and its monomers (Fig. 4). T_2 Treated fruits showed significantly higher contents of lignin and monomers of H, G and S compared to control fruits (Fig. 4). The S/G ratio in control was 0.207 while in T_2 Treated fruits, it was 0.224. The increased ratio of syringyl to guaiacyl moieties is related to higher tissue maturity representing a higher frequency of cross-linking in lignin structure (Kishimoto *et al.,* 2010). Thus, it was evident that the increased rate of lignification and cross-linking in T_2 Treated fruits aided in providing a more effective barrier to the entry of melon fly by inhibiting the penetration of insect stylets (Ingham *et al.,* 1998) through the reinforcement of cell walls and thus contributed to development of increased resistance to melon fly infestation.

The levels of flavonoids Table (7) and phenolic acids Table (8) were significantly higher in T_2 Treated fruits compared to control. Past studies had shown that mineral elements influence host resistance through metabolic changes by way of synthesis of natural defense compounds such as, phytoalexins, antioxidants and flavonoids which provide protection against pests (Schumann *et al.,* 2010). It was clear from Table (6) that there was a significantly higher rate of insect mortality in T_2 Treated fruits (88.6%) compared to control (14.4%) which was the result of a significantly lower oviposition, lower rate of pupation and slower rate of adult emergence. Phenolic acids are known to act as antifeedants, toxicants, ovicides and mitochondrial oxidation inhibitors in insects (Nishida, 2002) while flavonoids protect the plant against insect pests by influencing their growth, development and behaviour (Simmonds, 2003). Data shown in Table (7) showed that rutin and quercetin increased

rapidly in T_2 Treated fruits both of which are known to increase mortality of *Lymantria dispar* (Treutter, 2005) and tobacco armyworm (*Spodoptera litura*) in peanuts (Mallikarjuna *et al.,* 2004). A number of insect species have been shown to be sensitive to flavonoid compounds in feeding tests (Treutter, 2005). Flavonoids are reported to reduce nutritive value and digestibility of plants and even act as toxins altering their palatability (Sosa *et al.,* 2004). Among the phenolic acids, the rapid surge of gallic acid and syringic acid by 3-5 times in T_2 Treated fruits compared to control Table (8) was helpful in suppressing the development of melon fly larvae while the upregulation of caffeic acid might act as feeding deterrent as reported by Bhattacharya and Chenchaiah (2007) in red gram against *Spodoptera litura*. Increased catechin content is known to increase antifeedancy through complexation. The 3-fold increase of chlorogenic acid in T_2 Treated fruits compared to control was also helpful as it is a naturally occurring phenolic compound well known for its role in plant defence (Kirsten *et al.,* 2009). The two-fold increase of salicylic acid (SA) in T_2 Treated fruit was significant as SA generates a wide range of responses involved in regulation of plant defence against biotic and abiotic stresses (Shah, 2003 and Shivashankar *et al.,* 2015) through morphological, physiological and biochemical mechanisms (War *et al.,* 2012). SA is, particularly known to be effective against piercing and sucking type of insect pests such as the melon fly. The rapid increase of antioxidant activity in T_2 Treated fruits compared to control (Fig. 2) was also in tune with the earlier reports wherein phenols and flavonoids were found to confer resistance to infestation through production of antioxidants (War *et al.,* 2012). Thus, it was clearly evident from the present study that the resistance induced against melon fly by mineral supplementation during early growth of bitter gourd fruit was operating through the activation of antioxidant system involving synthesis of flavonoids, phenolic acids and antioxidant enzymes resulting in strengthening of the host plant to fight the infestation coupled with inhibition of oviposition, reduced pupation and increased mortality of melon fly insect.

Conclusion

The study established that resistance to melon fly in bitter gourd could be induced by micronutrient application although it is an inherited character. It was evident that expression of resistance to melon fly in bitter gourd was linked to the optimal levels of micronutrients. Supplementation of leaves and young developing bitter gourd fruits at 10,15 and 20 days after fruit set (DAF) with micronutrient formulation (T_2) consisting of Ca, Cu, Mg and Mn by spray application enhanced the levels of elements in fruit to optimum and significantly reduced the rate of melon fly infestation. The study proved that supplementation with mineral nutrients enhanced resistance to melon fly by inhibition of oviposition and increased mortality of melon fly insect through the activation of host antioxidant system involving synthesis of flavonoids, phenolic acids and antioxidant enzymes. Spray application of micronutrients to bitter gourd plants at the time of early fruit growth is a simple, safe, effective and eco-friendly approach to overcome melon fly infestation coupled with a significantly higher fruit yield. This approach could, therefore, be exploited for the successful field management of melon fly infestation in bitter gourd either singly or as a component of IPM strategy. This would not only minimize the use of hazardous plant protection chemicals but also aid in enhancing fruit yield, quality, food safety and nutritive value of fruits for human consumption.

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