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Influence of Nitrogen Fertilisation on Red Spider Mites (*Oligonychus coffeae* Nietner) and Overhead Volatile Organic Compounds in Tea (*Camellia sinensis)*

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ABSTRACT

Nitrogen fertilisation influences tea yields, quality and pests infestation levels. Red spider mites reduce tea production in western Kenya during prolonged droughts. Nitrogen fertiliser use maybe an agronomic practice that may influence infestation levels by mites. Overhead volatile compounds (OVOCs) composition also influences infestations of tea by mites. Influence of nitrogenous fertiliser rates on red spider mite infestations and OVOCS levels was determined. Mites populations varied (pd$\leq$0.05) with nitrogenous fertiliser rates. High infestations were at 0 and 300 kg N/ha/year, and sharp decline between 150 and 225 kg N/ha/year. Green leaf volatiles increased while some aromatic and terpenoid compounds decreased with increasing nitrogenous fertiliser rates. Significant (pd$\leq$0.05) direct linear regressions between 1-penten-3-ol, 3-penten-2-ol, E-2-hexenal, Z-3-hexenyl acetate and inverse relationship between 2-phenyl ethanol, ethyl benzene, α-methyl styrene, longifolene and ß-cedrene and nitrogenous fertiliser rates were observed. Most aromatic and terpenoid compounds, which are repellents of mites, were highest between 150 and 225 kg N/ha/year where infestations by mites were lowest. The recommended fertiliser rates of 100 to 225 kg N/ha/year in Kenya also produce most repellents of mites, protecting tea plants against infestations. Use of recommended nitrogen rates can protect tea from infestations by mites.

INTRODUCTION

Tea *Camellia sinensis* (L) O. Kuntze is an important player in the economy of Kenya, which is the third leading world producer and the largest exporter of tea. In Kenya, tea is grown in the east and west of Rift Valley (*1, 2*). Being a perennial monoculture, the crop provides stable microclimate and continuous supply of food for pests. Indeed, pest infestations are a major problem in tea growing areas causing yield losses, and losses up to 100% have been reported (*3*). Several species of mites attack tea. Red spider (*Oligonychus coffeae* Nietner) and red crevice or scarlet (*Brevipalpus phoenicis* Geijskes) are of economic importance in tea causing a great deal of crop loss in many countries (*4, 5*). These two mites species threaten tea production in Kenya especially during prolonged drought, in which yield losses have been estimated to be up to about 50% (*6*). Normally, the mites attack the upper surface of mature leaves, feeding on chlorophyll of the maintenance foliage but in severe cases young leaves are also attacked (*4*) leading to defoliation and occasional death of the tea bushes (*7*). Although the mites can be controlled through pesticide sprays, in the Kenya tea industry, use of pesticides is prohibited and control of pests is strictly through use of cultural practices including use of resistant/tolerant tea cultivars and agronomic inputs that deter attack by pests (*5, 6, 8*).

Fertiliser is one of the major agro-inputs in tea production. Due to continuous tea cropping (*9*), high nutrient leaching (*10*) and surface run-off in the high rainfall areas (*11*) where tea is grown, soil nutrients diminish, making it necessary to replenish nutrients through inorganic fertilisers applications to maintain high yields. Nitrogen, phosphorus and potassium are the most critical nutrients in the fertilisation programme of tea (*12, 13*). Nitrogen is the key element in tea nutrition as it promotes vegetative growth, improves shoot succulence, shoot size and leaf size (*14*) thus increasing leaf yield (*15-17*). However, nitrogen nutrition in tea affects the levels of fatty acids (*18-20*), plain black tea quality parameters (*21, 22*) as well as volatile flavour compounds composition (*23, 24*). The unsaturated fatty acids are precursors of green leaf volatile compounds in tea (*25, 26*). Excessive use of nitrogenous fertilisers may reduce plain black tea quality (*23*), and yields (*16, 27*), but increase in unsaturated fatty acid levels (*18-*

20) which reduce black tea aroma (*23, 24, 28*). Additionally use of high rates of nitrogen fertilisers degrade soil quality (*13*) and increase insect pest incidences (*29, 30*). The nitrogenous fertiliser use on tea varies from country to country (*12*) but in Kenya for most tea cultivars, use of 100 to 200 kg N/ha/year is recommended (*11, 31*). In the eastern highlands of Kenya, nitrogen fertiliser application was note to increase plant vigour and induce tolerance to attack by the red crevice mite (*30*). Indeed, application of nitrogenous fertilisers (NPKS 25:5:5:5) at rates between 150 and 200 Kg N/ha/year, induced tolerance to red crevice mites infestations, while rates above 400 Kg N/ha/year encouraged the build-up of mites on tea (*30*). Also, the levels of nitrogen in the leaf tissues increased with soil applied nitrogen and were related to the mite infestation levels (*32*).

Plants use indirect defences such as overhead volatile organic compounds (OVOCs) to repel herbivores or attract predators and parasitoids of the herbivores feeding on them (*33*). Tea produces OVOCs belonging to classes; monoterpenes, sesquiterpenes, homoterpenes, green leaf volatiles (GLVs) and aromatics (*34, 35*) which have been implicated in plants defense mechanism against pests and diseases (*36-39*). Nitrogen fertilisation rate had a significant effect on the emissions of OVOCs and the overall odour blend of the compounds in corn (*40*). Indeed nitrogen availability affects both the direct and indirect defence system of plants (*41*). Increasing nitrogen fertiliser application rates increased the concentrations of GLVs in *Brassiaca napus* (*42*) and processed black tea (*23, 24, 28*) and their precursors, unsaturated fatty acid levels (*18-20*) but decreased the levels of aromatic and terpenoid compounds in black tea (*23, 28, 43*). However, such studies were conducted on black tea that had undergone many biochemical transformations during processing. Levels of the OVOCs in tea have not been evaluated, although there appears to be a relationship between nitrogen fertiliser application rates, OVOCs emissions and insect pest incidences. This study evaluated the effects of nitrogenous fertiliser rates on level of infestations by red spider mites and OVOCs composition of clonal tea, and the relationship between nitrogenous fertiliser rates and OVOCs levels and clonal tea infestation by red spider mites.

Materials and methods

Plant materials for use

The experiment was conducted in Timbilil Estate (Tea Research Foundation of Kenya, TRFK) in upper Kericho, (Latitude $0^0$22'S, Longitude 35^0 21'E, Altitude 2180m amsl), in the west of the Great Rift Valley. Red spider mites sampling and OVOCs collection were superimposed on the on-going nitrogenous fertiliser rates trial on clone TRFK 6/8 (*27, 44*). The trial was set up as a randomized complete block design replicated three times. Nitrogen fertiliser as NPKS 25:5:5:5 was applied at 0, 75, 150, 225 and 300 kg N/ha/year. Prior to setting up the experiment, the field was uniformly managed using recommended agronomic and cultural practices (*11, 31*) and received 150Kg N/ha/year.

Determination of mites infestations levels

Ten mature leaves per bush were plucked randomly then the mites were brushed using mite brushing machine (Model–Leedom Engineering, USA) and the number counted under dissecting microscope (*5, 6, 8*).

Volatile Collection in the field

The OVOCs collection was carried out according to the method of Chen *et al* (*45*). Shoots of clone TRFK 6/8 from different fertiliser treatments were individually enclosed in large oven bags (355mm x 508mm) (that had been conditioned in an oven at 170°C, then cooled to ambient temperature) together with two Teflon pipes and tied with elastic bands. Activated charcoal-purified air generated by a pump entered the bag through one pipe flowing at 100ml min^{-1}. The air exiting the bag passed through an adsorbent trap consisting of a 200 × 7-mm glass tube containing 30 mg Alltech Super-Q, 80/100 mesh (Alltech Associates Inc., Deerpeld, IL) adsorbent material. Collected volatiles were eluted from the Super-Q adsorbent with 250 ìl dichlomethane (HPLC grade; purity: 99%) containing 10 ìl of cumene internal standard and then concentrated to 20 ìl with a stream of nitrogen gas while cooling under ice.

Volatile analysis and identification

Both GC and GC-MS were used for analysis. The GC analysis was carried on a Shimadzu model GC-2010 equipped with flame ionization detector. A 50 m silica gel capillary column (film thickness 0.20 µm, 0.25 mm inner diameter) was used. Oven temperature was programmed from 35 to 230°C with the initial temperature maintained for 5 min then a gradient if 5 °C/min to 190 °C, followed by increase at 50°C/min to 230 °C, and finally held at that temperature for 5 min. The flow rate for the carrier gas (N_2) was 3.0 ml/min and for detector gases 40.0 ml/min, hydrogen and 400.0 ml/min air, respectively. The detector temperature was set at 230.0°C. A sample volume of 1µl was injected in splittless mode. The compounds were quantified as ratio of the peak area of each compound to that of cumene (internal standard). Analysis on GC-MS was conducted using an Agilent 7890A Series gas chromatograph coupled to a mass spectrometer

Table1: Effect of nitrogenous fertilizer rates on the emissions of green leaf volatile organic compounds in relation to mites levels

N-rates	Green leaf volatile organic compounds								Mites	[Log(x+1) mite]
	1-Penten-3-ol	3-Penten-2-ol	Hexanal	E-2-Hexenal	Z-3-Hexenol	Heptanal	Z-3-Hexenyl acetate	Sum GLVS		
0	0.076	0.032	0.148	0.445	0.826	0.148	1.423	2.677	12	2.56
75	0.100	0.037	0.260	0.521	0.394	0.260	1.703	3.275	9	2.30
150	0.111	0.060	0.254	0.606	0.516	0.254	1.910	3.711	6	1.95
225	0.132	0.131	0.272	0.878	0.471	0.272	2.005	4.161	1	0.69
300	0.138	0.214	0.282	0.949	0.527	0.282	2.225	4.647	10	2.40
Mean	0.111	0.095	0.243	0.680	0.463	0.243	1.859	3.694	8	2.20
STDEV	0.025	0.077	0.000	0.222	0.061	0.000	0.314	-		
CV (%)	22.28	81.80	0.00	32.69	13.29	0.00	16.91	-		
R	0.983*	0.934*	0.815	0.971*	0.826	0.815	0.988*	0.658		
r^2	0.967*	0.873	0.664	0.943*	0.682	0.664	0.976*	0.433		
CV (%)										23.83
LSD ($p\leq0.05$)										2.91 (1.36)

(Agilent 5973 quadruple detector). The gas chromatographic conditions were as follows:- helium was used as the carrier gas at a flow rate of 1.25ml/min; in-let temperature 270°C, transfer line temperature of 280°C, and column (HP-5MS 30 m×0.25 mm×0.25 ìm film) oven temperature was programmed from 35 to 280°C with the initial temperature maintained for 5 min then 10 °C/min to 280 °C for 10.5 min and increased to final temperature 50 °C/min to 285 °C. Parameters for electron impact sample ionization were as follow: mass selective detector maintained at an ion source temperature of 250°C and quadruple temperature of 180°C, electron energy, 70 eV; source temperature, 250°C. Fragment ions were analyzed over 40-550 m/z mass range in the full scan. Identification of compounds was by comparing the fragmentation patterns with mass spectral data in mass spectral library and literature.

Statistical Analysis

The red crevice mites data were transformed, log e (x+1) and then subjected to analysis of variance (ANOVA) as randomised complete block design using MSTAT-C statistical package. The means were separated using least significant difference (LSD) at 5% level of probability and Excel Microsoft Office was used to perform regression analyses to establish inter-related factors.

Results and discussions

The variations in the OVOCs separated into classes and red spider mites levels with the rate of nitrogenous fertiliser rates are presented in Tables 1- 4. There were variations ($p\leq0.05$) in population of mites due to nitrogen fertiliser rates. The 300 Kg N/ha/year and control N (0 Kg N/ha/year) produced high population of red spider mites. There was decline in the population of mites from 0 to between 150 and 225 Kg N/ha/year followed by a rise at 300 kg N/ha/yea. The lowest ($p\leq0.05$) infestation by red spider mites were recorded at 150 and 225 kg N/ha/year fertiliser rates. The results are similar previous results on red crevice mites in the eastern highlands of Kenya (*30, 32*). Thus the recommended nitrogenous fertiliser rates in Kenya of 100-225 kg N/ha/year (*11, 31*) are not only leading to increased production (*16, 24, 27*) and being a compromise between yields and quality (*23, 28, 43*) but also helpful in reducing the mites infestations of tea plants. Application of correct nitrogenous fertiliser rate is therefore a viable pest control mechanism in tea, as had also been observed on other plants (*39, 46*).

All the seven green leaf volatiles and the sum of GLVs (Table 1) increased linearly with increase in rate of nitrogen fertiliser. The GLVs are products of oxidative unsaturated fatty acids breakdown in tea leaves (*25, 26, 47*). The pattern of response was similar to that of their precursor fatty acids (*18-20*) and levels in black tea (*20, 23, 26, 28*). The levels of GLVs released overhead plants are therefore directly related to the levels of the precursor compounds in the leaf. The correlation coefficients (r) of the linear regression between 1-penten-3-ol (r = 0.983), 3-penten-2-ol (r = 0.934), E-2-

Table 2: Influence of nitrogenous fertilizer rates on the emissions of aromatic compounds in relation to mites levels

N- rates	Aromatic compounds											Mites	[Log (x+1) mite]
	2-Phenyl ethanol	Ethyl benzene	1,4-xylene (p-Xylene)	1,2-xylene (o-Xylene)	Phenylmethanal (Benzaldehyde)	2-Phenylpropene (α-methylstyrene)	2-phenylethanal (Phenyl acetaldehyde)	Phenylmethanol (Benzyl alcohol)	1pOhenylethanone (acetophenone)	1,3-Benzothiazole (Benzothiozole)	Sum Aromatic compounds		
0	0.151	0.224	ND	ND	0.123	0.315	0.055	0.060	0.071	0.047	1.046	12	2.56
75	0.159	0.234	0.135	ND	0.131	0.260	0.077	0.077	0.100	0.073	1.246	10	2.30
150	0.141	0.206	0.133	0.133	0.157	0.231	0.056	0.086	0.114	0.071	1.328	6	1.95
225	0.116	0.169	0.119	0.027	0.153	0.127	0.202	0.084	ND	0.076	1.073	1	0.69
300	0.115	0.156	0.115	0.126	0.144	0.102	0.071	0.072	0.101	0.077	1.079	11	2.40
Mean	0.137	0.198	0.125	0.095	0.142	0.207	0.092	0.076	0.097	0.069	1.238	8	2.20
STDEV	0.020	0.034	0.010	0.059	0.014	0.090	0.062	0.010	0.018	0.012	-	-	-
CV (%)	14.71	17.28	7.71	62.30	10.11	43.63	67.58	13.79	18.82	18.09	-	-	-
R	-0.903*	-0.932*	0.584	0.638	0.701	-0.981*	0.399	0.469	-0.134	0.802	-0.126		
r^2	0.816	0.870	0.341	0.407	0.462	0.962	0.159	0.22	0.018	0.643	0.016		
CV (%)												23.83	
	LSD ($p \leq 0.05$)											2.91 (1.36)	

hexenal (r = 0.971) and Z-3-hexenyl acetate (r = 0.988) and nitrogenous fertilisers were direct and significant (pd”0.05)

Generally, the total levels of aromatic and terpenoid compounds declined with an increasing nitrogenous fertiliser rates (Tables 2-4). Nevertheless, the individual compounds (Tables 2-4) showed mixed responses to increasing nitrogen application rates, with some directly and other inversely correlating with nitrogen fertiliser rates.

Table 3: Effect of nitrogenous fertilizer rates on the emissions of monoterpenes in relation to mites levels

N- rates	1-Isopropyl-2-methylbenzene (O- Cymene)	3-methylidene-6-propan-2-ylcyclohexene (β – Phellandrene)	(*E*)-3,7-dimethylocta-1,3,6-triene (E-β-Ocimene)	2-[(2S,5S)-5-Methyl-5-vinyltetrahydro-2-furanyl]-2-propanol (Linalool oxide -Z- (furanoid))	3,7-Dimethyl-1,6-octadien-3-ol (Linalool)	(1S)-4-methyl-1-propan-2-ylcyclohex-3-en-1-ol (?-Terpinen-4-ol)	Sum monoterpenes	Mites	[Log (x+1) mite]
0	0.138	0.296	0.716	0.076	0.279	0.138	1.643	12	2.56
75	0.108	0.276	0.600	0.075	0.329	0.150	1.538	10	2.30
150	ND	0.297	0.761	0.113	0.358	0.155	1.684	6	1.95
225	0.099	0.299	0.763	0.094	0.346	0.146	1.747	1	0.69
300	0.057	0.278	0.756	0.099	0.351	0.144	1.685	11	2.40
Mean	0.101	0.289	0.719	0.091	0.333	0.146	1.6798	8	2.20
STDEV	0.033	0.011	0.069	0.016	0.032	0.006	-	-	-
CV (%)	33.22	3.89	9.660	17.44	9.57	4.310	-	-	-
R	-0.498	-0.182	0.553	0.638	0.800	0.197	0.594		
r^2	0.248	0.033	0.306	0.407	0.640	0.039	0.353		
			CV (%)					23.83	
			LSD ($p \leq 0.05$)					2.91 (1.36)	

Table 4: Effect of nitrogenous fertilizer rates on the emissions of sesquiterpenes in relation to mites levels

N- rates	(1R,2R,7S,9S)-3,3,7-Trimethyl-8-methylenetricyclo[5.4.0.0~2,9~]undecane (Longifolene)	Cedr-8-ene (α-Cedrene)	(1R,4E,9S)-4,11,11-Trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene (E-Caryophyllene)	7,11-Dimethyl-3-methylenedodeca-1,6,10-triene ((E)-β-Fernesene)	1H-3a,7-Methanoazulene (β-Cedrene)	(1S-cis)-; (Z)-Calamenene; Cadina-1,3,5-triene (Z-Calamenene)	3,3,7-Trimethyltricyclo[5.4.0.0^{2,9}]undecan-8-one (Longicamphenylone)	(8α)-Cedran-8-ol (α Cedrol)	Sum Sesquiterpenes	Mites	[Log (x+1) mite]
0	0.093	1.072	0.441	0.719	0.302	0.241	0.115	1.471	4.454	12	2.56
75	0.093	1.198	0.487	0.779	0.299	0.236	0.125	1.587	4.804	10	2.30
150	0.065	1.241	0.531	0.828	0.187	0.256	0.097	1.583	4.788	6	1.95
225	0.055	1.237	0.528	0.796	0.000	0.247	0.011	1.634	4.508	1	0.69
300	0.057	1.159	0.515	0.836	0.000	0.241	0.253	1.504	4.565	11	2.40
Mean	0.073	1.181	0.500	0.792	0.263	0.244	0.120	1.556	4.729	8	2.20
STDEV	0.019	0.070	0.038	0.047	0.065	0.008	0.087	0.067		-	-
CV (%)	26.30	5.90	7.50	5.92	24.86	3.12	72.08	4.28		-	-
R	-0.915*	0.484	0.797	0.849	-0.900*	0.226	0.295	0.268	-0.063		
r^2	0.838	0.234	0.635	0.720	0.810	0.051	0.087	0.072	0.004		
				CV (%)						23.83	
				LSD ($p \leq 0.05$)						2.91 (1.36)	

None of the direct relationships was significant. However, only phenyl ethyl alcohol (r=-0.903), ethyl benzene (r=-0.932), α-methyl styrene (r=-0.981) longifolene (r=-0.915) and ß-cedrene were significantly ($p \leq 0.05$) inversely correlated with nitrogen fertiliser rates. In previous studies (*23, 28, 43*) the sum of the terpenoid compounds in black tea were decreased with increase in nitrogen fertiliser rates, although no regressions were performed to establish if the relationships were significant. However, the compositions of the terpenoid compounds detected in black tea (*23, 28*) were different from those in the overhead composition in live tea reported herein. Most of the terpenoids in the OVOCs composition were olefins and it is likely they were volatilised/lost during black tea processing. It may be necessary to establish the agronomic and cultural practices that influence the terpenoid compounds composition in the OVOCs, especially longifolene and ß-cedrene, as these may be used to deter attack by red spider mites. Similarly, cultural and agronomic factors that influence production of the volatile aromatic overhead compounds have not been established. For the first time influence of nitrogen fertiliser rates on the overhead aromatic compounds are reported. Phenyl ethyl alcohol, ethyl benzene and α-methyl styrene were demonstrated to decline with increasing nitrogenous fertiliser rates. Out of the 24 compounds released belonging to aromatics, monoterpenes and sesquiterpenes, 15 (63%) reached maximum level between 150 and 225 kg N/ha/year fertiliser application rates. This was the rate at which minimum mites levels were recorded. These three classes of OVOCs have been reported to defend plants against insect pests attack. In chilli (*47*) and tomato (*48*) high levels of monoterpenes repelled insects. The repellence was attributed to the monoterpenes being toxins and feeding deterrents (*49*) that interfered with acetyl cholinesterase enzyme activity in the insects (*50*). A number of aromatic compounds have been implicated in plant defence against insect pests (*39, 40, 42, 46*). These results may explain the low number of mites observed when their levels were high (between 150 and 225 kg N/ha/year fertiliser rates).

Several volatile compounds have been implicated in plant defence mechanism against insect attack. For example acetophenone causes acute insecticidal activities (*51*) while benzothiazole exhibit a wide range of biological properties including antimicrobial activities (*52*). Sesquiterpenes not only defend plants against pest attack by attracting natural enemies but also possess repellency and toxicity properties (*53*). Indeed, aromatic compounds, monoterpenes and sesquiterpenes are repellents to the two species of mites (Table 5). The high population of mites at 0 Kg N/ha/year may be attributed in part to the low levels of the repellent defence compounds. The high levels of mites at 300 Kg N/ha/year, may be due to the high levels of GLVs which are attractants to the mites (Table 1) and low amounts of aromatics, monoterpenes and sesquiterpenes (Tables 2-4). These findings corroborate earlier findings that mites infestation levels were high in plots receiving 0 and above 400 Kg N/ha/year and application of rates between 150 and 200 kg N/ha/year induced tolerance to mites attack (*30, 54*). The high levels of GLVs at high fertiliser rate may be contributing to the abundance of the mites, while the high levels of mites at 0 kg N/ha/year may be due to the low levels of aromatic and terpenoid compounds.

Preferences of insect infestation of plants that emit high levels of GLVs have been reported. For example, flea beetles (*Epitrix hirtipennis*) were more abundant on GLV-producing wild type plants compared to plants (*55*). *Uschistus heros* preferred soybean pods that released high amounts of GLVs for feeding and oviposition over deficient cultivars (*56*). The GLVs serve as feeding stimulants to pests (*57*). Highest yields had been reported in this trial at 225 kg N/ha/year (*27*). The low levels of aromatics, monoterpenes and sesquiterpenes at 300 Kg N/ha/year may be due to increased growth rates due to fertilisation which trade-off with carbon allocation to secondary metabolites, leading to reduced concentration of chemical defences hence reduced resistance against herbivores (*58*). A delicate balance between yield, nitrogen fertiliser application and formation of chemical defences in plant can protect plants against pests, a strategy that is superior to chemical control that sometimes makes tea rejected in international markets due to pesticide residues chemical contamination. Results presented herein confirm that the recommended rates of nitrogen fertiliser for tea in Kenya (*11, 31*) also induce protection to mites infestations.

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