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Assessment of Mycorrhizal Colonization and Soil Biological Attributes in Healthy and Declining Khasi Mandarin (*Citrus reticulata* Blanco) Orchards in Acidic Inceptisols

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ABSTRACT

Background: Khasi mandarin (*Citrus reticulata* Blanco) a loose-skinned commercial cultivar is one of the premier citrus species native to sub-tropical hill zones of Northeast India. But this fruit crop suffers a serious threat due to the dieback condition in the present years.

Methods: Khasi mandarin orchards of different categories *viz*. young non-bearing orchards, bearing healthy orchards, bearing old orchards and declining orchards were selected for the study from the sub-tropical hill zones of acidic Inceptisols at varying altitudes of <150 m to >1400 m amsl. In those selected orchards, a series of soil physico-chemical, biological and microbiological attributes were characterized in the healthy as well as in the declining orchards.

Result: The results revealed that both the physico-chemical and biological parameters in the healthy orchards were found to be significantly ($P \leq 0.05$) higher than the declining orchards. Further, the mycorrhizal colonization effectively influenced the composition of microbial structure in the rhizospheric zone, affecting the health and vigour of the plant.

Key words: Acid soils, Khasi mandarin, Microbial biomass, Mycorrhizal colonization, Soil quality.

INTRODUCTION

Khasi mandarin (*Citrus reticulata* Blanco, family Rutaceae) a commercial cultivar with loose-skinned is one of the premier citrus species native to sub-tropical hill zones of Northeast India. This fruit crop occupies nearly 41.5% of the total citrus production, with 0.404 million hectares out of 0.923 million hectares citrus area and 5 million tonnes production in India (www.agricoop.nic.in 2018-19). The rich agro-climatic conditions in Northeast India, favour the Khasi mandarin to thrive in undulating topography with different altitudes ranging from low to high *i.e.* 50 to 5000 metre above mean sea levels (m amsl) (Sanabam *et al*. 2015). But the Khasi mandarin trees in the present years suffer a serious threat due to dieback/rapid decline condition, with typical characteristic symptoms *viz*. yellowing, interveinal chlorosis and mottling like that of plant nutritional disorders. At the advanced stage dieback of branches starts from tip to downward and branches turn yellow to brown and die rapidly. This dieback is often observed at any stage of growth of the affected tree.

In general, Khasi mandarin orchards flourish well in light soils with good drainage conditions and deep soils with pH range of 5.5 to 7.5 are considered ideal for its growth. However, they can also be grown in a pH range of 4.0 or below, but, high concentration of aluminum in soil can adversely affect the feeder root zone affecting the overall growth. In North Eastern Hill (NEH) region the soils are acidic with high phosphorus (P) fixing (as iron and aluminum phosphates) capacity, making it insoluble for plant absorption. So, solubilization of P carried out by P solubilizing (bacteria or fungi) and mobilizing microorganisms (vesicular arbuscular mycorrhiza-VAM) through symbiosis play an important role in acid soils (Balota *et al*. 2011) by increasing the pathway for the uptake of nutrients and increase the plants' resistance to abiotic and biotic stresses (Wu *et al*. 2006).

Besides the symbiotic mycorrhizal colonization, the soil physico-chemical and biological properties and density of the viable populations present in the rhizosphere, play an important role in maintaining the health and vigour of the Khasi mandarin trees. Organic carbon in soil (SOC) is the key element in determining the soil quality, productivity and sustenance of any agro-ecosystem and the biological pools

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viz. the microbial biomass and the microbial activity are the indicators to any changes in soil health due to management practices and their capacity to supply nutrients for both plants and microorganisms. So, a comprehensive study on soil type, chemical and biological activities in soil and presence or absence and infection percentage of VAM fungi, in bearing and non-bearing healthy and declining Khasi mandarin plants in acidic Inceptisols will give the idea to what soil conditions the healthy and declining plants are reacting to?

MATERIALS AND METHODS

Description of soil sampling site and method of sampling

The study area consist of 16 (sixteen) no. of Khasi mandarin orchards located from 25°42.926′N to 25°47.475′N latitude and 93°48.213′E to 94°09.093′E longitude and altitude range of 141 m to 1442 m amsl in acidic Inceptisols of Northeast India (Table 1). These selected orchards were categorized into four different types, *viz*. (1) Young non-bearing orchards of 0-7 years old, (2) Bearing healthy orchards of 8-30 years old, (3) Bearing old orchards of 30-45 years old and (4) Declining orchards includes, any orchard age with nutrient deficient and infested unhealthy plants. For each orchard type, 4 (four) replication orchards were taken.

Soil samples collected were a random composite of 5 spots pooled in zigzag pattern and 3 composites were collected from each orchard. The collected soils were within the radius of 60 cm of tree trunk of Khasi mandarin plant at two soil depths of 0-15 cm and 15-30 cm and air dried and sieved for physico-chemical analysis. For biological parameters soil samples were kept in a refrigerator at 4°C until analysis. Core samplers were used for the root sampling and kept at 4°C until analysis. For the determination of percent root mycorrhizal colonization, root samples were collected from Khasi mandarin plants of five types, *i.e.* healthy non-bearing, healthy bearing, bearing old plants, disease plants and declining plants. The replications for root samples also follow the same way as that of soil samples.

All the laboratory analytical works were carried out in ICAR Research Complex for NEH Region, Nagaland Centre, Medziphema, India and the duration of the research work was from 2016 to 2020.

Soil chemical parameters

Gravimetric soil moisture content (MC) was determined by oven drying at 105°C to constant weight. The soil texture was analysed by the hydrometer method (Buoyoucous, 1927). Soil pH was analyzed using 1:2.5 soil/water suspension (Jackson, 1973). Soil organic carbon (SOC) was determined by the wet oxidation method as described by Walkley (1947). Available nitrogen (AvlN) was determined by Subbiah and Asija (1956) method. Available phosphorus (AvlP) was determined by the stannous chloride blue colour method (Bray and Kurtz, 1945). Available potassium (AvlK) was determined by Hanway and Heidel (1952) method in flame photometer.

Soil biological parameters

Freshly collected soil samples were used for microbial biomass carbon, nitrogen and phosphorus (MB-C, MB-N and MB-P) determination by the chloroform-fumigation-extraction method (Brookes and Joergensen, 2006). The difference in C, N and P content between fumigated and non-fumigated sub-samples was calculated using a conversion factor, K_{EC} = 0.25 (Jenkinson and Powlson, 1976), K_{EN} = 0.45 (Jenkinson, 1988) and K_{EP} = 0.40 (Brookes *et al*. 1982) for MB-C, MB-N and MB-P respectively. Basal respiration (BAS) was measured by using the standard base trap method in a NaOH solution (Pell *et al*. 2006). Dehydrogenase activity (DHA) was determined as per the method described by Casida *et al*. (1964). The intensity of the reddish colour concentration of triphenyl formazan (µgTPF g^{-1} (dw) soil h^{-1}) was measured in a spectrophotometer at a wavelength of 485 nm. Acid phosphomonoasterases activity (PHA) was determined in fresh soil samples as per the procedure described by Tabatabai and Bremner (1969). The intensity of the yellow colour (µg *p*-nitrophenol g^{-1}(dw) soil h^{-1}) was measured at 440 nm using a spectrophotometer. Metabolic quotient (MQ- qCO_2) is derived by taking the ratio of BAS and MB-C (Anderson and Domsch, 1990).

$$q\,(CO_2) = \left[(mg\ CO_2 - \frac{C}{mg}\frac{Cmic}{h}\right]$$

The enumeration of the total viable microbial population was carried out at two soil depths (0-15 cm and 15-30 cm), in two types of media- nutrient agar (NA) for general soil bacteria and Jensen agar medium (JA) for nitrogen fixing types. The population count was quantified by following the serial dilution plate technique and expressed as cfu ml^{-1}. Each colony that appeared on the plate was considered as one colony forming unit (cfu) (Waksman, 1927).

The sample was processed for root staining and mycorrhizal colonization following the method given by Koske and Gemma (1989), in which the roots (1cm) were dipped in 10% KOH (potassium hydroxide- at room temperature) for the cellulose destruction, after which roots were acidified with 1% HCl (hydrochloric acid) followed by 10% H_2O_2 (hydrogen peroxide) for bleaching and better staining. Finally, they were stained with 0.01% trypan blue in lacto-glycerol (Brundrett *et al*. 1996) and mounted lengthwise on a glass slide for observing under a microscope for arbuscules, vesicles and internal hyphae in the root cortex. The percent root colonization was calculated by following the formula:

% Root colonization =

$$\frac{\text{Total no. of root segments colonized}}{\text{Total no. of roots examined}} \times 100$$

Data analysis

Data generated from the laboratory analysis were subjected to the statistical analyses of variance appropriate to the experimental design. Data were assessed by Duncan's

multiple range tests (Duncan, 1955) with a probability of P≤0.05. The least significant difference (LSD) between means was calculated using the SPSS program (SPSS version 21.0). The pair-wise correlation matrix was also developed irrespective of orchard type to find out the relationship between various parameters. The result obtained for the microbiological parameters (VAM and population count) were subjected to statistical analysis for mean and standard deviation by following the method for one-way ANOVA.

RESULTS AND DISCUSSION

The healthy orchards in the selected study areas were mostly located in the high altitude regions with clay and clay loam type soil textures and the declining orchards on the other hand were mostly found in the low altitude areas and have light texture soils (Table 1). Soil pH, SOC, AvlN, AvlP and AvlK content in those healthy orchard soils were significantly (P≤0.05) higher than the other orchards (Table 2). Soil pH in the declining orchards were very low (4.69 pH), AvlN, AvlP and AvlK in declining orchards were also found to be low to moderate range. Similar observations were also reported by various researchers, where the health and productivity of the crop, whether field or fruit crop depends on the nutritional status of the soil (Obreza *et al*. 2008; Han *et al*. 2008).

Soil biological attributes in different Khasi mandarin orchard types

Analysis of the microbial biomass directly or indirectly determines the active nutrient status of the soil, mobilization

Table 1: Khasi mandarin orchard location with soil texture.

Orchard type	Age	Latitude (N)	Longitude (E)	Altitude (m amsl)	Soil texture
Young non-bearing orchards	0-7 years old	25°47.461′	94°07.351′	1000, 1300, 1313, 1315	Clay loam, clay loam,
		25°47.475′	94°07.366′		clay loam, clay loam
		25°47.475′	94°07.360′		
		25°47.470′	94°07.356′		
Bearing healthy orchards	8-30 years old	25°42.843′	94°09.093′	1204, 1433, 1436, 1442	Clay loam, clay, clay, clay
		25°42.945′	94°08.598′		
		25°42.940′	94°08.590′		
		25°42.926′	94°08.573′		
Bearing old orchards	30-45 years old	25°43.234′	94°08.552′	1245, 1240, 1311, 1313	Clay, clay, clay
		25°43.230′	94°08.540′		loam, clay loam
		25°43.230′	94°08.550′		
		25°43.340′	94°08.522′		
Declining orchards	Any age-infested	25°45.321′	93°50.720′	284, 255, 274, 141	Sandy clay loam, sandy clay
	un-healthy plants	25°45.248′	93°48.436′		loam, clay, sandy clay loam
		25°45.198′	93°48.213′		
		25°45.240′	93°50.380′		

m amsl: metre above mean sea level.

In a column, figures with same letters do not differ significantly at *P*≤0.05.

Table 2: Basic soil parameters and soil microbial biomass -C, -N and -P for different Khasi mandarin orchard types.

Orchard type	Soil depth	pH	SOC (%)	AvlN	AvlP	AvlK	MB-C	MB-N	MB-P
				kg ha^{-1}			µg g^{-1} (DW) soil		
Young non-bearing orchards	0-15	5.41^{ab}	1.17	380.5^{a}	6.30^{c}	161.3^{b}	131.4^{abc}	214.2^{b}	18.7^{ab}
	15-30	5.01^{bc}	1.02	287.4^{abc}	5.57^{c}	129.5^{b}	122.6^{bc}	33.0^{d}	12.6^{cd}
Bearing healthy orchards	0-15	5.40^{ab}	1.58	422.0^{a}	33.22^{ab}	518.1^{a}	193.3^{ab}	392.7^{a}	18.9^{ab}
	15-30	5.02^{bc}	1.15	345.0^{ab}	9.35^{c}	306.7^{b}	96.2^{cd}	151.1^{bc}	14.6^{bc}
Bearing old orchards	0-15	5.82^{a}	1.72	340.8^{abc}	38.21^{a}	184.8^{b}	214.2^{a}	105.8^{cd}	22.0^{a}
	15-30	5.41^{ab}	1.54	316.7^{bc}	19.48^{bc}	261.0^{b}	32.8^{cd}	63.9^{cd}	18.3^{ab}
Declining orchards	0-15	4.69^{c}	1.32	224.6^{bc}	7.03^{c}	154.3^{b}	65.6^{d}	48.1^{d}	9.3^{d}
	15-30	4.15^{d}	0.85	209.1^{c}	6.06^{c}	140.3^{b}	14.7^{d}	33.8^{d}	9.5^{d}
CD (*P*≤0.05)		0.25	NS	48.5	6.29	73.4	30.8	31.5	1.7

[SOC: Soil organic carbon, AvlN: Available nitrogen, AvlP: Available phosphorus, AvlK: Available potassium, MB-C: Microbial biomass carbon, MB-N: Microbial biomass nitrogen, MB-P: Microbial biomass phosphorus].

In a column, figures with same letters do not differ significantly at *P*≤0.05.

and availability to the crops determining the quality of the soil (Deng *et al*., 2000). MB-C, -N and –P in the present study were also significantly higher ($P \leq 0.05$) in bearing old orchards, bearing healthy orchards and bearing old orchards respectively (Table 2), than the declining orchards. The higher microbial biomass in the study signifies the productivity of the soil, which is reflected in the growth, development and performance of the Khasi mandarin under study.

The enzyme activities are used as the index to microbial activity and are often considered to be the sensitive indicators for management-induced changes in soil fertility and stress (Wlodarczyk *et al*. 2002). Enzyme DHA is an index for total microbial activity and PHA, a key enzyme for hydrolyzing the organic phosphorus compounds (Pascual *et al*. 2002). The median values and the distribution of data on the microbial activities *viz*. dehydrogenase activity (DHA) and acid phosphatase activity (PHA), basal respiration (BAS) and metabolic quotient (MQ-qCO$_2$) for the study areas were illustrated in Fig 1. The enzyme activities in the present study corroborated with the earlier reports and higher activities were observed in those orchard types where the soil nutritional statuses and microbial biomass were higher (Zhang *et al*. 2007). The MQ show the stress condition in soil and higher amount of MQ in declining orchards, indicated that imbalance nutrient and disturbance in soil has negative effect on the efficiency of microbial activities and show high MQ. Laik *et al*. (2009) showed that higher soil respiration was observed in higher microbial biomass with enhanced soil microbial activities; identical results were also observed in the present study.

VAM colonization pattern in roots and viable microbial population in rhizospheric soils of Khasi mandarin plants

VAM infection type (Fig 2) and percent infection in 5 types of Khasi mandarin plants were presented in Table 3. The root examination showed that the vesicular (VI) and hyphal (HI) types of root infections were found in all plant types considered. But the arbuscular root infections (AI) were not found in some plant types. Percent infection was observed highest in healthy bearing plants (92.16%) and lowest in declining plants (42.66%). This showed that the clay and clay loam soil textures with higher soil pH levels (5.40 to 5.82 pH- Table 2) favoured the percent colonization more than the sandy clay loam texture soils with lower soil pH (4.69 pH).

The colonization of VAM in the roots (intracellularly) of the citrus plant influences the morphology of the root system, thereby affecting the nutrient uptake and growth and development of the plant (Wu *et al*. 2013) and fruit quality (Nzanza *et al*. 2012). The percent colonization of 19-51% was reported to be satisfactory in citrus crops (Wu and Zou, 2010). This showed that the present study corroborated with the earlier studies and the high colonization in the roots of the healthy bearing plants no doubt had high nutrient content even though the external chemical nutrients were not added and the colonization of the VAM also depends on the land disturbances and soil pH conditions (Lingfei *et al*. 2005). The infection observed in the Khasi mandarin roots were usually of vesicular and hyphal types and very few arbuscular infections were observed in few plants. The main reason for the absence of arbuscules (in the root cortex) might be due to the short study

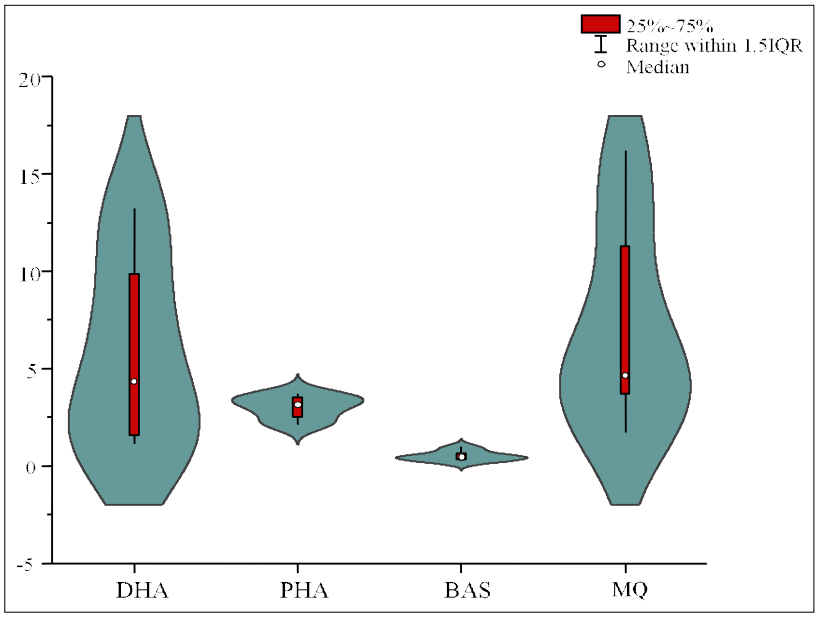

Fig 1: Violin with box-plot of dehydrogenase activity (DHA), phosphatise activity (PHA) and basal respiration (BAS). The microbial activities are expressed generally in graph as µg g^{-1} hr^{-1} whereas specifically the units are for DHA (µg TPF g^{-1}DW soil h^{-1}), PHA (µg p-nitrophenol g^{-1}DW soil h^{-1}), BAS (µgCO2 g^{-1}DW h^{-1}) and MQ (mg CO$_2$-Cg^{-1} Cmic h^{-1}). The box indicates the interquarter range, while the whiskers show non-outlier range.

period and degeneration of arbuscules within 14 days, another reason might be due to the infection of roots of non-host species, which produced intercellular hyphae and form vesicles only (Giovannetti and Sbrana, 1998).

The rhizospheric zone is the region influenced by plant roots and microbial activity and is considered to be the dynamic region of plants and microbial interaction (Kennedy and de Luna, 2004). Viable microbial population enumeration in nutrient agar (NA) media and Jensen agar media are presented in Table 3. The general viable population was highest in the disease infected plants with several types of microbes comparing among the other plant types showed that there was a competition among the microorganisms and might have invaded the beneficial type of population (Johansson *et al*. 2004). Similarly, the VAM symbiosis and the quantity and quality of plant root exudates in the rhizospheric zone can effectively influence the microbial structure, composition and activity to a great extent (Johansson *et al*. 2004).

Correlation study among different soil attributes observed in Khasi mandarin orchards

Chemical and biological attributes obtained were subjected to pair-wise correlations among themselves, irrespective of Khasi mandarin orchard types (Fig 3). Soil pH had a positive correlation with AvlN, AvlP, MBC, MBP and PHA (P<0.05). Similar observations were reported by Paul *et al*. (2001), where they showed that soil pH can affect the availability of nutrient elements through microbial activity as well as the decomposition in the soil. MBC had a direct correlation with AvlN, PHA and MQ (P<0.05). MBN maintain a positive correlation with AvlN, AvlK, DHA, PHA and BAS (P<0.05) and MBP also positively correlated with AvlP (r=0.76) and PHA (r=0.74). MQ in our study negatively correlated with MBC (r= 0.79, P<0.05). It was previously reported that labile fractions and their relationships are highly dependent on the land use types, microbial biomass present and the C inputs in the rhizosphere (Haynes, 2005). DHA also had a positive correlation with PHA (r=0.82, P<0.05) and BAS

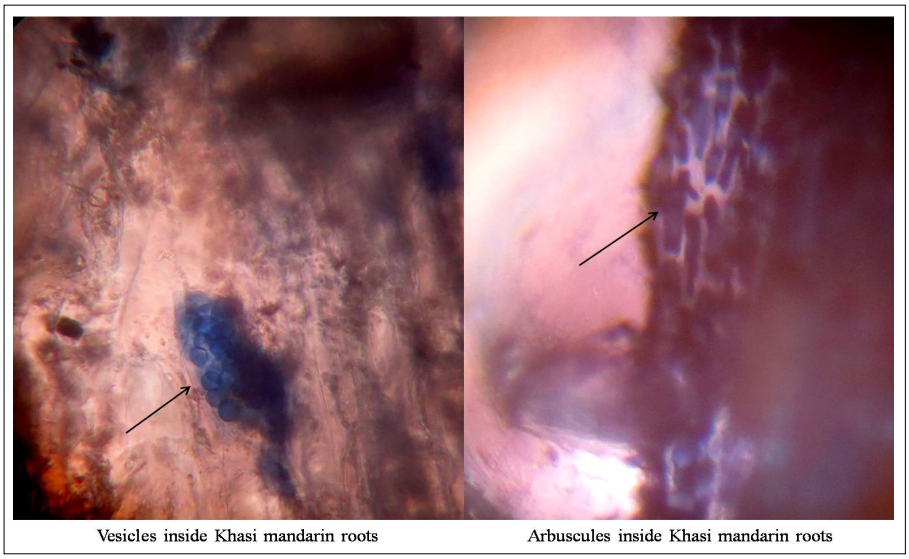

Fig 2: Vesicles and arbuscules of VAM fungi in the roots of the Khasi mandarin plant.

Table 3: VAM infection type and per cent infection and viable population and number of types of micro-organisms (mean±SE) in different types of Khasi mandarin plants.

Khasi mandarin plant type	VI	AI	HI	Percent (%) infection	Viable population in NA and types		Viable population in JA and types	
					cfu ml^{-1}	Type (no.)	cfu ml^{-1}	Type(no.)
Healthy young non-bearing plants	+	-	+	67.63±6.42	3.48×10^{7}±7.12	7.0±2.0	5.22×10^{6}±29.84	9.6±1.7
Healthy bearing plants	+	+	+	92.16±8.04	3.52×10^{7}±10.43	6.6±2.9	5.32×10^{6}±16.13	8.4±3.9
Bearing old plants	+	-	+	66.72±7.18	4.48×10^{7}±11.43	6.4±2.1	3.72×10^{6}±10.16	8.4±1.5
Disease infected plants	+	+/-	+	75.35±14.89	4.54×10^{7}±7.89	2.2±0.8	2.00×10^{6}±9.82	6.6±1.5
Declining plants	+/-	+/-	+	42.66±15.68	2.78×10^{7}±6.53	4.6±0.9	1.32×10^{6}±1.92	5.6±1.8

[VI: Vesicular infection, AI: Arbuscular infection, HI: Hyphal infection, +: positive or present and -: negative or absent].

[NA: Nutrient agar for general microbial population and JA: Jensen agar for nitrogen fixing types].

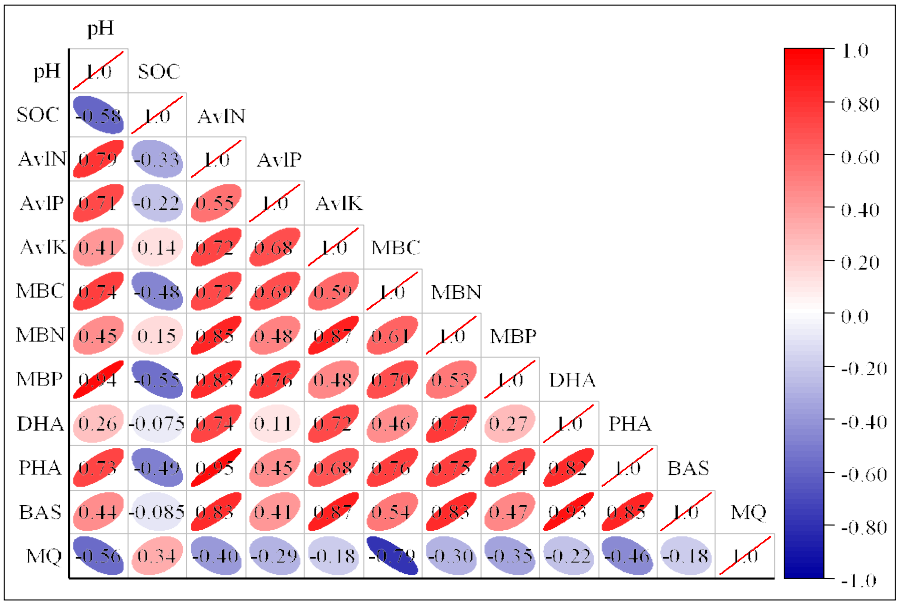

Fig 3: Pair-wise relationship between chemical and biological attributes in Khasi mandarin orchards

[SOC- Soil organic carbon, AvlN- Available nitrogen, AvlP- Available phosphorus, MBC- Microbial biomass carbon, MBN- Microbial biomasss nitrogen, MBP- Microbial biomass phosphorus, DHA- Dehydrogenase activity, PHA- Phosphatase activity, BAS- Basal respiration, MQ- Metabolic quotient]

* Significant at $r_{0.05}$= 0.71 and **Significant at $r_{0.01}$= 0.85.

(r=0.93, P<0.01) and a similar relationships were previously reported by many authors (Dilly and Nannipieri, 2001; Sangma *et al*. 2016).

CONCLUSION

The characteristics of the soil *viz*. type of soil, the nutritional status and biological and microbial activities are the factors greatly influencing the growth and development of the Khasi mandarin in the present study. Besides the soil related factors, the orchard age and altitude of the place also plays a great role in the longevity or economic lifespan of the Khasi mandarin trees in acid soils. The higher microbial biomass, enzyme activities and mycorrhizal colonization in healthy bearing Khasi mandarin orchards signifies the productivity of the soil, whereas the high metabolic quotient in the declining orchards indicated the stress related factors finally leading to the dieback condition in the Khasi mandarin trees.

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