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Variation in the concentration and indexing black pepper plants for PYMoV and CMV through DAS-ELISA

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Abstract

Variation in concentration of Piper yellow mottle virus (PYMoV) and Cucumber mosaic virus (CMV) in stunted disease infected black pepper plants from different varieties/cultivars during different months of a year was studied by DAS-ELISA. Higher A405 values were recorded from October to February in four varieties/cultivars of black pepper tested and Panniyur-1 had the highest concentration of both the viruses. Results on distribution of the viruses within a plant from three varieties/cultivars showed that young leaf contained highest concentration of both the viruses while root region recorded the least. A total of 2186 black pepper nursery plants from eleven varieties were indexed by DAS-ELISA for the presence of viruses. Of these, 714 plants were found infected with either of the two viruses. Among these, IISR-Sreekara had the highest number of infected plants followed by IISR-Subhakara while Panniyur-5 had the least number of infected plants. Of the 714 plants tested positive, 390 plants exhibited no visible external symptoms indicating the necessity for sensitive diagnosis in identifying virus-free planting material.

Key words: black pepper, Cucumber mosaic virus, DAS-ELISA, indexing, Piper yellow mottle virus

Black pepper (*Piper nigrum* L.), the "King of Spices" is native to Western Ghats of South India. The dried berries obtained from the vines have a commercial value as an important spice condiment used allover the world (Ravindran, 2000). In India the crop is mainly grown in the states of Karnataka and Kerala. Fungi, nematodes and viruses are the major pathogens affecting black pepper plantations causing economic loss to the black pepper industry. Stunted disease of black pepper was the first identified viral disease, reported from a nursery at District Agricultural Farm, Neriamangalam, Idukki, Kerala, during 1975 (Pailey *et al*. 1981). Mosaic, mottling, small leaf condition, incomplete filling of spikes and stunting of the whole plant are the major symptoms of the disease. Disease of similar nature was also reported from Brazil, Indonesia, Malaysia, Sri Lanka and Thailand (Lockhart *et al*. 1997; Duarte *et al* 2001; de Silva *et al*. 2002). Association of Cucumber mosaic virus (CMV) (Sarma *et al*. 2001; Bhat *et al*. 2005) and Piper yellow mottle virus (PYMoV) (Bhat *et al*. 2003; Hareesh and Bhat, unpublished) with stunted disease of black pepper was reported from India. The major spread of the disease

is by infected stem cuttings used as planting material.

Proper detection of causal pathogens and use of healthy planting material are the prerequisites for integrated management of diseases. Seasonal variation, genotype, viral load, growth stage and other factors influence the expression of symptoms in a plant. Masking of visible external symptoms especially during monsoon months was observed in black pepper indicating that symptomatology cannot be the sole criterion in identifying infected plants. Sero-diagnosis is currently the method of choice for detecting viral infections in plants and vectors as it is cost effective, easy and a large number of field samples can be tested in relatively less time. Direct Antigen Coating Enzyme Linked Immunosorbent Assay (DAC-ELISA) and Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) are the two preferred methods of sero-diagnosis of large number of plant samples. Standard DAS-ELISA based detection of CMV (Bhat *et al*., 2004) and PYMoV (Bhadramuthy *et al*., 2005) infecting black pepper were reported. This paper reports variation in concentration, distribution within a plant of CMV and PYMoV in black pepper varieties and indexing nursery plants for these viruses by DAS-ELISA.

Black pepper plants collected from Indian Institute of Spices Research (IISR), Calicut and IISR Experimental Farm, Peruvannamuzhi were used in the study. Varieties/ cultivars such as Karimunda, IISR-Malabar Excel, IISR-Panchami and Panniyur-1 were used to study variations in concentration of the viruses by DAS-ELISA. Three plants from each variety/cultivar were selected and young leaf collected from each plant during the first week of every month from April to March was subjected to DAS-ELISA. Three plants each from Karimunda, IISR-Panchami and Panniyur-1 were used to study the distribution of viruses in different parts of a plant for a period of one year from April to March. Young leaf, old leaf, stem, spike and root from each plant were subjected to DAS-ELISA. A total of 2186 black pepper nursery plants from eleven varieties from IISR Experimental Farm were indexed for the presence of CMV and PYMoV by DAS-ELISA during September to December. The varieties include: IISR-Panchami, Panniyur-1, Panniyur-2, Panniyur-3, Panniyur-5, Panniyur-6, Panniyur-7, IISR-Pournami, IISR-Sreekara, IISR-Subhakara and IISR-Thevam. Indexing was followed by individual examination of ELISA positive plants for any visible external symptoms to correlate with the results.

DAS-ELISA for detecting CMV and PYMoV was carried out separately in polystyrene plates (Co-Star) using the protocol described by Bhat *et al*. (2004) for CMV and Bhadramuthy *et al*. (2005) for PYMoV. Wells initially were coated with virus specific IgG at 1µg/ml in coating buffer (pH 9.6). Antigen preparation included homogenization of leaf tissue in five volumes of PBS-T (pH 7.2) containing 2% polyvinyl pyrrolidone (PVP) and 0.2% BSA followed by centrifugation at 8000 rpm for 1 min. The supernatant obtained was loaded into ELISA plates. CMV and PYMoV specific IgG-alkaline phosphatase-conjugate was used at 1:1000 and 1:2000 dilutions respectively. The colour reaction was read at 405 nm using ELISA plate reader (µQuant, Bio Tek Instruments Inc., USA), 1 h after addition of substrate (p-nitro phenyl phosphate, Genei, Bangalore). Buffer and healthy black pepper samples served as negative control in all tests. Each sample was loaded in duplicate wells and the average A405 values obtained for three samples from each variety/cultivar was used in the analysis. The A405 values obtained for the samples were deducted from A405 values obtained for healthy sample. The resulting A405 values were then ranked as: 0.01 to 0.20 (+); 0.21 to 0.40 (+=); 0.41 to 0.60 (+++); 0.61 to 0.80 (++++) and > 0.81 (+++++).

Results of DAS-ELISA from four varieties/ cultivars of black pepper showed variation in the concentration of CMV and PYMoV during different months of a year (Table 1). In general higher titres of both the viruses

Table 1. Variation in the concentration of CMV and PYMoV in black pepper varieties/cultivars during different months of a year as detected by DAS-ELISA*

Month	IISR Malabar Excel		Karimunda		Panniyur-1		IISR-Panchami	
	PYMoV	CMV	PYMoV	CMV	PYMoV	CMV	PYMoV	CMV
April	+	+	+	++	++	++	+	++
May	+	+	++	+	+	+	+	+
June	+	+	+	+	++	+	++++	++++
July	+	+	++	++	++	+	++	++
August	+	+	++	+	+	+	++	+++
September	++	+	+	+	++	+	+	+
October	+++	+++	++	+++	+++++	++++	++	+++
November	+	+	++	++	++++	+++	++	++
December	++	+	++	++	++++	+++	+++	++
January	++	+	+	+	+++	++	++++	+
February	++	++	++	++	+++	++	++	++
March	-	-	+	+	++	+	+	+

* The average of A405 values obtained for three samples from each variety/cultivar after 1h of substrate addition was deducted from A405. values obtained with healthy controls. The resulting A405 values were then ranked as follows: 0.01 to 0.20 (+); 0.21 to 0.40 (++); 0.41 to 0.60 (+++); 0.61 to 0.80 (++++); > 0.81 (+++++).

were recorded from October to February. Among the varieties/cultivars tested, var Panniyur-1 recorded higher A405 values for CMV and PYMoV followed by IISR-Panchami. Cultivar Karimunda showed a steady trend in the concentration of both CMV and PYMoV in all the months tested. Lower A405 values for both the viruses was recorded in the case of var IISR-Malabar Excel compared to other varieties/cultivars. During the month of March, ELISA could detect no measurable virus in var IISR-Malabar Excel. This kind of variation in virus concentration may be attributed to the genotype or effect of seasonal variations on plants. Similar kind of variation in the concentration of Leek yellow stripe virus infecting garlic during a crop cycle was reported (Conci *et al*. 2002).

Uneven distribution of the viruses in various parts of the plant was observed in three varieties/cultivars tested during different months of a year. From the various parts tested, young leaf as well as old leaf contained high concentrations of PYMoV but CMV was mainly confined to young leaf followed by old leaf (Fig.1). The var Panniyur-1 recorded high absorbance values for both the viruses compared to other varieties/cultivars tested. During October and November all parts of this variety recorded high A405 values (Fig. 1). In remaining months, root region showed relatively low concentrations of both the viruses. Though concentration of both the viruses was less in the remaining varieties, the same trend of virus distribution in different parts of the plant was observed (not shown).

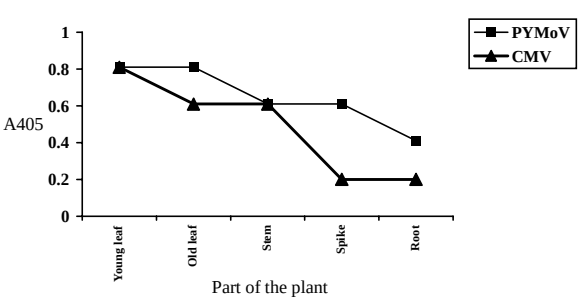

Fig. 1. Distribution of CMV and PYMoV in different part of the plant in variety. Panniyur-1 during October as determined by DAS-ELISA

Table 2. Indexing black pepper nursery plants for the presence of CMV and PYMoV in different varieties

Variety	Total number of plants tested	No. of plants infected	PYMoV positive	CMV CMV positive	PYMoV and positive
IISR-Sreekara	878	249	121	128	20
IISR-Subhakara	547	238	122	116	27
Panniyur-1	68	29	18	11	-
Panniyur-2	81	22	12	10	-
Panniyur-3	31	16	03	13	-
Panniyur-5	81	12	05	07	-
Panniyur-6	69	24	13	11	-
Panniyur-7	76	23	13	10	-
IISR-Thevam	60	16	06	10	-
IISR-Panchami	151	62	46	16	-
IISR-Pournami	144	23	15	08	-
Total	2186	714	374	340	47

Variations in visible external symptoms from severe to complete absence of symptoms in stunted disease infected black pepper plants were seen during different months of a year. This may be attributed to the influence of environmental factors on symptom expression. The effect of temperature on symptom expression in virus-infected potato plants was reported (DeBokx & Piron 1977). When the symptoms are masked it is very difficult to distinguish between healthy and infected plants. Masking of symptoms on black pepper plants infected with stunted disease during monsoon season makes it difficult to identify infected plants and hence sensitive diagnosis is necessary to identify healthy black pepper planting material. When 2186 black pepper nursery plants were indexed by DAS-ELISA for CMV and PYMoV, 714 vines were found infected with at least one of the two viruses (Table 2). Among the varieties tested, IISR-Sreekara had 249 plants infected, IISR-Subhakara had 238 plants infected and Panniyur-5 had 12 plants with least infection. Twenty plants from IISR-Subhakara and 27 plants from IISR-Sreekara

Table 3. Symptomatology of indexed black pepper nursery plants

Variety	Total plants tested	Plants positive in ELISA	Symptomatic plants	Asymptomatic plants
IISR-Sreekara	878	249	101	148
IISR-Subhakara	547	238	109	129
Panniyur-1	68	29	5	24
Panniyur -2	81	22	10	12
Panniyur -3	31	16	11	5
Panniyur -5	81	12	9	3
Panniyur -6	69	24	20	4
Panniyur -7	76	23	13	10
IISR-Thevam	60	16	10	6
IISR-Panchami	151	62	18	44
IISR-Pournami	144	23	18	5

were infected with both the viruses. When indexed DAS-ELISA positive vines were individually checked for the presence of any external visible symptoms, 390 plants out of 714 showed no visible external symptoms. (Table.3). This kind of results was observed on hosts, which did not show any visible external symptoms but were still positive for Apple stem grooving virus in ELISA during pear and apple certification program (Batlle et al. 2004). These results clearly indicate that a plant cannot be judged as healthy solely on the visible external symptoms but a much sensitive detection technique like ELISA proves to be useful in identifying healthy and infected black pepper plants.

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