Virus Diagnostic and Planting Material Production

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Abstract: Disease caused by viruses in one of the important production constraints in many black pepper growing countries. Mosaic, mottling and small leaf conditions are the symptoms for identifying the disease. Association of two viruses namely, *Cucumber mosaic virus* (CMV) (genus: *Cucumovirs*) and *Piper yellow mottle virus* (PYMoV) (genus: *Badnavirus*) are reported with the disease. The major means of spread of the viruses is through the use of infected stem cuttings. PYMoV is also known to spread through mealybugs (*Ferrisia virgata*, *Planococcus, P. citri* and *P. elisae*), black pepper lace bug (*Diconocoris distanti*) and through seeds. In view of systemic nature and lack of chemicals to control viruses, use of virus-free planting materials is an important component in increasing the productivity of black pepper. Though external symptoms are the good criteria for detection of viruses, depending on the season, growth stage and other factors, the disease can be difficult to detect visually. Masking of symptoms during monsoon and winter months was seen in many of the affected black pepper vines. Hence external symptoms can not be used as the sole criterion to identify virus infected plants. There is a need to use sensitive techniques such as ELISA and PCR to detect the virus in mother plants so that such contaminated source can be avoided for vegetative multiplication. Both ELISA and PCR based methods are available for the detection of CMV and PYMoV in plants. In addition to viruses, pathogens such as fungi, nematodes and phytoplasma are also the important production constraints in black pepper. Of these, fungi and nematodes are known to survive in soil / planting material and are carried to the field inadvertently. Hence it is important to develop and use reliable diagnostics for early detection of both fungi and nematodes in soil and root tissues so as to avoid its spread especially to a new area. Thus ideally it is important to establish mother block using disease free planting materials collected from certified good bearing source plants of known elite variety. Disease free materials collected from mother block are then grown in a nursery under protected condition with regular monitoring. In order to avoid chance contamination of fungi and nematodes, the potting mixture is heat sterilized using steam or by soil solarisation. Then the mixture is fortified with beneficial micro organisms such as *Trichoderma harzianjum* /*Pseudomonas fluorescens*. The pathogen free stocks from nurseries are then multiplied in secondary nurseries or used for commercial planting. The future thrusts should include: (i) development of multiplex PCR/microarray for detection of all pathogens infecting black pepper (ii) Development of easy to use diagnostic kits such as lateral flow device and (iii) Development of certification programme to produce disease-free planting materials.

1.0 Introduction

Disease caused by viruses is becoming important production constraints in recent years among black pepper growing countries because of their systemic nature and difficulty in control. Virus induced symptoms referred differently like dog's ear, mosaic, little leaf, wrinkled leaf and stunted disease have been reported from black pepper growing countries such as Brazil, India, Indonesia, Malaysia, Philippines, Sri Lanka and Thailand (Paily *et al*., 1981; Randombage and Bandara, 1984; Kueh and Sim, 1991; Prakasam *et al*., 1990; Sitepu and Kasim, 1991). At the 1991 International workshop on black pepper diseases held at

Lampung, Indonesia, it was decided to use "stunted disease of black pepper" as a uniform terminology to include all viral diseases (Wahid *et al*., 1992). At present stunted disease is widespread in all black pepper growing countries (Lockhart et al., 1997; de Silva et al., 2002). In India, high incidence and severity of the disease was reported from black pepper plantations located especially at high altitudes (Bhat et al., 2005c).

2.0 Disease symptoms

A wide range of symptoms are observed on infected vines. Mosaic, mottling and small leaf conditions are most obvious symptoms for identifying the disease in the field. The symptoms on leaves include vein clearing, yellow specks, distortion, reduction in size, mottling and mosaic, along with stunting of the whole plant (Figure 1). The infected vines produce short spikes with poor filling leading to yield reduction. In severe cases, the leaves become abnormally narrow and give a sickle shaped appearance. The internodes of vines become abnormally short leading to stunting of plants and the affected branches give a typical witches broom appearance in advanced stages (Holliday, 1959; Eng *et al*., 1993; Lockhart *et al*., 1997; Sarma *et al*., 2001; de Silva *et al*., 2001; 2002).

Fig.1. Symptoms of viral disease affected black pepper vine showing mosaic mottling and deformation of leaves.

3.0 Causal viruses

In Brazil, it was suspected to be caused by *Cucumber mosaic virus* (CMV) and hence quarantine regulations were suggested for movement of stem cuttings from diseased to non-diseased areas (Duarte and Albuquerque, 1991). Lockhart *et al*. (1997) reported a new

mealybug transmitted badnavirus, namely *Piper yellow mottle virus* (PYMoV) as the cause of the disease in Malaysia, Thailand, The Philippines and Sri Lanka. PYMoV had non-enveloped bacilliform virions in size (30 X 125 nm) containing a ds DNA genome. The virus was serologically related to *Banana streak* and *Sugarcane bacilliform* badnaviruses. Genomic PYMoV sequence analysis comparisons of putative reverse transcriptase (RT) domain showed PYMoV to be closely related to other mealybug transmitted badnaviruses. Occurrence of PYMoV and CMV was reported on black pepper from Sri Lanka (de Silva *et al*., 2001; 2002) and India (Sarma *et al*., 2001; Bhat *et al*., 2003; Hareesh and Bhat, 2008). The CMV infecting black pepper in India was shown to belong to subgroup IB based on coat protein gene sequence studies (Bhat *et al*., 2005a). Based on sequencing of potion of open reading frame (ORF) I and ORF III of different isolates of PYMoV from India, Hareesh and Bhat (2008) reported highly conserved ORF III region among isolates while ORF I was variable.

4.0 Transmission and spread

The major means of spread of the viruses is through the use of infected stem cuttings. The disease can also be transmitted experimentally through grafting. One of the causal viruses, CMV could be easily transmitted mechanically to several cucurbitaceous and solanaceous hosts (de Silva *et al*., 2001; Sarma *et al*., 2001). In general, CMV is known to have a very wide host range infecting several plant species and spread in nature through aphids in a non-persistent manner. Although colonization of aphids on black pepper is seen, its role in the transmission of CMV is yet to be established. On the other hand, PYMoV is known to have a narrow host range and spread in nature through mealybugs. The PYMoV has been shown to be transmitted through mealybug, *Planococcus citri* in Malaysia, Philippines, Sri Lanka and Thailand (Lokhart *et al*., 1997) while it is suspected to be transmitted by *P. elisae* in Brazil (Duarte *et al*., 2001). In addition to *P. citri*, PYMoV particles were also shown to be transmitted through black pepper lace bug (*Diconocoris distanti*) in Sri Lanka (de Silva *et al*., 2002). In India it was shown to be transmitted through the striped mealybug, *Ferrisia virgata* and *P. citri* (Bhat *et al*., 2003; 2005b). Recently based on symptoms and PCR test on the seedlings raised from berries collected from infected plants Hareesh and Bhat (2010) have reported transmission of PYMoV through seeds.

5.0 Detection and diagnosis for viruses

Though external symptoms are the good criteria for detection, sometimes depending on the season, growth stage and other factors, the disease can be difficult to identify or detect visually. Masking of symptoms (especially in older leaves) during monsoon and winter months was seen in many of the affected black pepper vines. Symptoms were best exhibited in the affected plant during March to May months under Indian conditions (Bhat et al., 2005c). Thus external symptoms can not be used as the sole criterion to identify healthy plants. Hence there is a need to use sensitive techniques such as ELISA and PCR to detect the virus in mother plants so that such contaminated source can be avoided for vegetative multiplication. ELISA based methods are available for the detection of CMV (de Silva et al., 2001; Bhat *et al*., 2004) and PYMoV (Bhadramurthy et al., 2005) infecting black pepper. Similarly PCR (for PYMoV) and RT-PCR (for CMV) based methods are available for the detection of PYMoV and CMV infections (Lockhart et al., 1997; de Silva et al., 2002; Bhat et al., 2005a; 2009). Using PCR, Bhat et al. (2009) reported presence of PYMoV in apparently healthy plants of black pepper indicating the need to use sensitive methods to identify virus-

free plants (Fig. 2). A reliable method for RNA isolation from black pepper and sensitive detection of CMV through RT-PCR was reported (Siju et al., 2007). Recently, a protocol for isolation of total nucleic acids from black pepper tissue and a single tube multiplex RT-PCR (mRT-PCR) for the combined detection of both CMV and PYMoV has been reported (Bhat and Siju, 2007). The mRT-PCR is rapid, reliable and requires only small tissue sample for sensitive detection. The entire procedure can aid in the rapid screening of a large number of plants for both the viruses. Once healthy plants are identified, they can be protected and used as source for further multiplication either through cuttings or micropropagation.

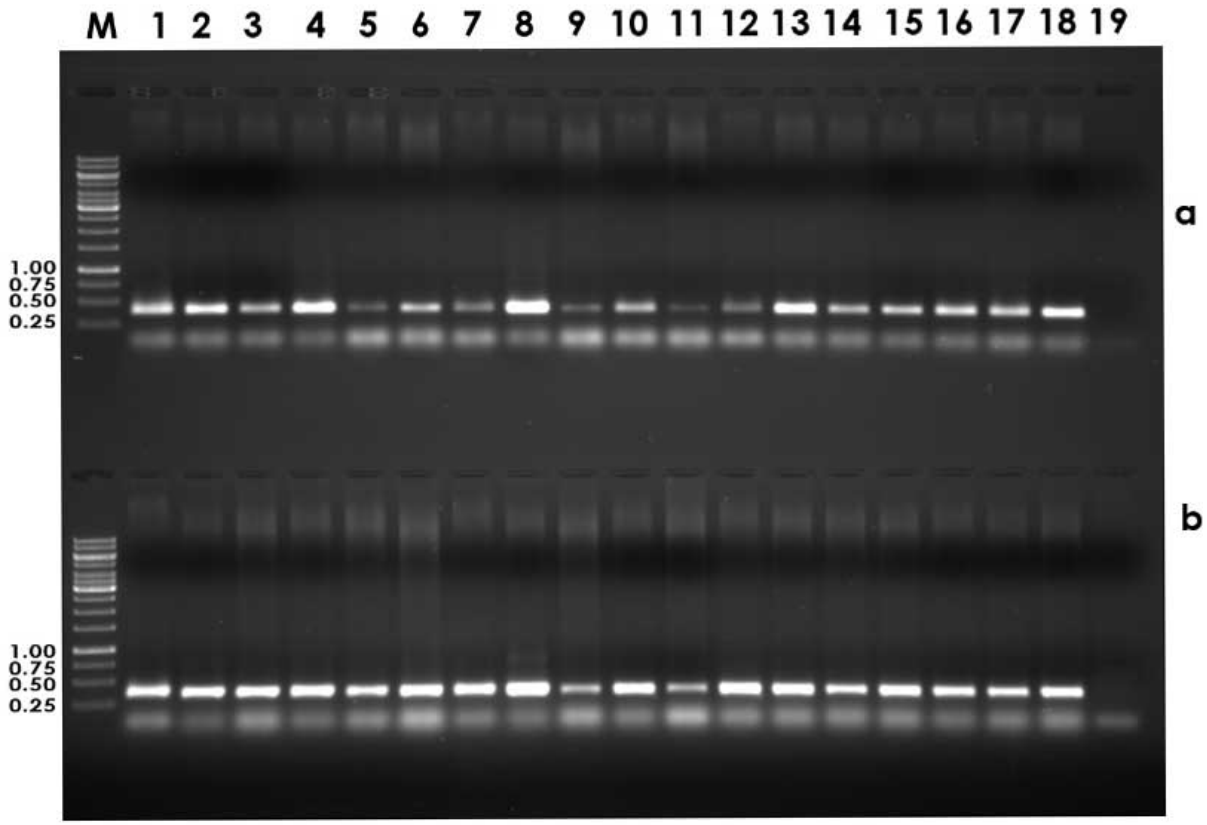

Fig. 2. Indexing black pepper plants for *Piper yellow mottle virus* (PYMoV) through PCR. Lane M: 1 Kb ladder; Lane 1: Positive control (known infected plant); Lanes 2-18: test plants of var. IISR Panchami; Lane 19: Negative control (known healthy black pepper plant) (a) PCR performed with 1.0 µl template; (b) PCR performed with 0.5 µl template

6.0 Other pathogens infecting black pepper and need for their diagnostics

Pathogens such as fungi, nematodes, phytoplasma and viruses are the important production constraints in black pepper (Table 1) (Anandaraj, 2000). Of these pathogens, fungi and nematodes are known to survive in soil / planting material and are carried to the field inadvertently. Thus major spread of these pathogens take place through nursery planting materials and multiply on plant roots and soil. Since these pathogens infect underground parts of plant, infection generally goes unnoticed until substantial portions of roots are damaged. External symptoms such as foliar yellowing /wilting are seen only after extensive root damage. Taking up any control measures at this stage will be futile as they cannot revive the crop. Hence it is important to check nursery planting material for the presence of pathogens so as to avoid its spread especially to a new area. Similarly in the case of viruses and

phytoplasma as they are systemic in nature, primary spread occurs through use of infected planting material. As these pathogens once infected cannot be eradicated from plant, selection of pathogen-free mother plant for further propagation is very important. Hence it is important to use sensitive technique to detect all the above mentioned pathogens early in the soil /plant system so that they can be effectively controlled (Anandaraj et al., 2008). Choice of planting material to be sampled is very important for successful detection and diagnosis. Sensitivity (how small an amount of pathogen can be detected), accuracy, reproducibility, number of samples that can be processed in a given time, adaptability to field conditions, cost and degree of operator training required are some of the factors one need to consider while selecting a method for detection. Diagnostics developed for various pathogens infecting different black pepper is listed in Table 1.

Table 1: Diseases, their causal organism and diagnostics available for various pathogens infecting black pepper

Disease	Causal organism	Diagnostics available
Foot rot	*Phytophthora capsici*	Baiting, Microscopy, ITS-PCR-RFLP, SCAR, Species specific primers
Slow decline	*Radopholus similis, Meloidogyne* spp.	Microscopy, rDNA-PCR
Stunted disease	*Cucumber mosaic virus, Piper yellow mottle virus*	ELISA, RT-PCR, mRT-PCR
Anthracnose	*Colletotrichum gleosporioides*	Microscopy, PCR
Phyllody	Phytoplasma (aster yellows group)	Nested PCR, PCR-RFLP

7.0 Production of disease-free planting materials of black pepper

Black pepper is infected by fungal, viral, phytoplasmal and nematode pathogens (Table 1). Of these, viruses and phytoplasma are systemic in nature and primarily spread by use of infected cuttings as source of propagation. As masking of symptoms are seen in infected vines, presence or absence of symptoms can not be used as the sole criterion to identify infected or healthy plants. There is a need to use sensitive techniques such as ELISA and/ or PCR to detect the virus in mother plants so that such contaminated source can be avoided for vegetative multiplication. Fungal and nematode pathogens survive in soil / planting material and are carried to the field inadvertently. In order to prevent chance contamination, the potting mixture is heat sterilized using steam or by soil solarization. Then the mixture is fortified with beneficial micro organisms such as *Trichoderma harzianum* / *Pseudomonas fluorescens*. The benefits of these organisms are twofold as it prevents infection by pathogens and also enhance growth by mobilizing nutrients and producing growth hormones. Proper diagnostics are in place for detection of all pathogens of black pepper. The important steps in the production of disease-free planting material include:

7.1 Establishment of Mother garden

(i) Good bearing and disease free vines of known variety should be indexed for viruses
(ii) Only cuttings from virus-free plants should be planted in mother garden

(iii) It is advisable to maintain these mother plants under insect-proof conditions

(iv) They should be periodically (at least once in a year) indexed for viruses and other pathogens

(v) Regular monitoring and rouging of diseased plants should be done whenever noticed

(vi) Whenever insects (aphids, mealybugs) are seen, spraying with insecticides is necessary

7.2 Multiplication of pathogen-free planting material in nurseries

(i) Cuttings obtained from bearing pathogen-free mother vines are raised on a large scale in a nursery under insect-proof conditions (Fig. 3, 4).

(ii) The potting mixture is heat sterilized using steam or by soil solarization. Then the mixture is fortified with beneficial micro organisms such as *Trichoderma harzianum* / *Pseudomonas fluorescens*.

(iii) Nursery plants also have to be checked for pathogens periodically (depending on the lot /batch size of plants produced)

Lot/batch size	**Number of plants to be sampled**
Up to 1000 Nos	1% plants subject to a minimum of 10 Nos
1001 to 10000 Nos	0.5% of plants subject to a minimum of 10 Nos
10001 to 100000 Nos	0.1% of plants subject to a minimum of 50 Nos

(iv) Regular monitoring and rouging of diseased plants should be done whenever noticed

(v) Whenever insects (aphids, mealybugs) are seen, spraying with insecticides is necessary

(vi) The pathogen-free stocks from the nurseries are then multiplied in secondary nurseries or used for commercial planting.

Fig. 3. Insect-proof shed used for multiplication of virus indexed black pepper

Fig. 4 Multiplication of black pepper through serpentine method in an insect-proof shed.

Conclusions and Future strategies

Diseases caused by viruses and other pathogens impose serious limitations on cultivation of black in all producing countries. The key factors in any efficient disease management program are reliable identification of pathogens and understanding their natural dissemination mechanism. Imuno assay and nucleic acid based techniques occupy the leading positions as methods of diagnostics in the present plant pathology. Among them, PCR and its modifications as well as various ELISA formats are most popular. They are highly sensitive, specific, rapid, user-friendly, and generally excel the conventional methods in these parameters. Immuno and PCR based diagnostics have been developed for the pathogens infecting black pepper . While some of the diagnostics were being used in routine large scale indexing, a few of them still needs validation. Parameters for production of disease-free planting materials of different spices have been developed. If used properly, this would lead in the production of disease-free certified planting material. There is also a need to establish planting material production chain involving selection of parent material, initial testing and periodic testing of sub samples during multiplication and at the time of distribution of planting material. There is also a need for awareness creation on the importance of disease free planting material and capacity building of all stake holders. The future thrusts should include: (i) development of multiplex PCR/microarray for detection of all pathogens infecting black pepper (ii) Development of easy to use diagnostic kits such as lateral flow device and (iii) Development of certification programme to produce disease-free planting materials

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