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Review

Immunological and molecular diagnostic methods for detection of viruses infecting cowpea (*Vigna unquiculata)*

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Cowpea viruses are difficult to identify using morphological criteria which can be time consuming, challenging, and require extensive knowledge in taxonomy. In order to improve the quality and quantity of the germplasms and to significantly reduce the infection and transmission of virus to different cultivars of cowpea, proper diagnosis and control is essential. The immuno-diagnostic and molecular-diagnostic methods have shown great potential as far as specificity and sensitivity are concerned and can generate accurate results rapidly. The aim of this overview is to discuss the various immuno-diagnostic and molecular diagnostic methods such as enzymes linked immunosorbent assay (ELISA), immunosorbent electron microscopy (ISEM), polymerase chain reaction (PCR), nucleic acid hybridization, dot immunoblotting assay (DTBIA) found suitable for diagnosis of Cowpea aphid-borne mosaic virus (CABMV), Cucumber mosaic virus (CMV) and Cowpea mottle virus (CMeV) infecting cowpea. These techniques do not only provide information for epidemiological purposes, but also help to develop disease free stock of cowpeas. Therefore, these various techniques with symptoms and history are of immense value to diagnose cowpea viruses and are the cornerstone of the management of cowpea cultivars.

Key words: Immuno-diagnostic, molecular-diagnostic, *Vigna Unquiculata,* Cowpea aphid-borne mosaic virus, Cowpea mottle virus and Cucumber mosaic virus.

INTRODUCTION

Cowpea (*Vigna unquiculata* L. Walp) is one of the world's dicotyledonous leguminous food crops and a major food crop of millions of people in the developing countries (Summerfield et al., 1974). Cowpea belongs to the kingdom plantae, division (Magnoliophyta), class (Magnolliopsida), order (Fabales), family fabaceae formally (Leguminiseae), Sub-family (Faboideae), genus (Vigna) and species (unquiculata). Cowpea has probably been used as a crop plant since Neolithic time. A lack of archaeological evidence has resulted in contradicting views supporting Africa, Asia and South America as its origin (Summerfield et al., 1974; Tindall, 1983; Coetzee, 1995). One view is that cowpea was introduced from Africa to the Indian subcontinent approximately 2000 to 3500 years. Cowpea provides an extremely significant portion of the dietary protein of the people and plays an important nutritional role in developing countries of the tropics and subtropics especially in sub-Saharan Africa (Rachie, 1985; Singh et al., 1997). Cowpea young leaves, pods and pea contain vitamins and minerals which have fuelled its usage for human consumption and animal feeding (Rachie et al., 1985; Nelson, 1997). In the United States, green seeds are sometimes roasted like peanuts and consumed. The roots of the cowpeas are eaten in Sudan and Ethiopia and the scorched seeds are occasionally used as a coffee substitute (Duke, 1981). In Nigeria, cowpeas are used to make soups and bean mixes such as "moi-moi" and beans cakes. The leaves of cowpea may be boiled, drained, sun-dried and then stored for later use (Duke, 1981).

This world's dicotyledonous crop is highly variable crop

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cultivated around the world essentially for the seeds and also as vegetables in which there are about fifteen varieties of cowpea in common cultivation (Kipps, 1970). Nigeria is reputed to be the highest producer of cowpea in the world (Steele, 1976; Taiwo and Akinjogunla, 2006). Some other countries like Niger, Mail, Brazil and Australia produce significant amount. This leguminous food crop is greatly attacked by wide array of diseases of biological origin especially viruses which cause devastating effects and are a really constraint to increased yield of cowpea in several countries (Kaisser et al., 1965; Ladipo and Allen, 1979; Thottappilly and Rossel, 1992). The majority of the viral diseases of cowpea lead to overall stunting, reducetion in leaf size, mottling, mosaic, leaf chlorosis, leaf distortion, leaf curling, vein clearing, necrotic local lesion and death (Akinjogunla, 2005). Viral diseases have become serious due to extensive cultivation of cowpeas and also viral diseases have significant status because they do not only cause direct damage to the host, but they equally predispose the plants to secondary invader.

Over 140 viruses have been reported worldwide to infect cowpea cultivars, but only nine have been reported in Nigeria, and these are Cowpea aphid-borne mosaic virus (CABMV), genus *Potyvirus*; Cowpea golden mosaic virus (CPGMV), genus *Bigeminivirus*; Southern been mosaic virus (SBMV), genus *Sobemovirus*; Sunhemp mosaic virus (SHMV), genus *Tobamovirus*; Blackeye mosaic virus (BICMV), genus *Potyvirus*; Cucumber mosaic virus (CMV), genus *Cucumovirus*; Cowpea mottle virus (CMeV), genus *Carmovirus*; Cowpea yellow mosaic virus (CPMV) genus, *Comovirus*; Cowpea mild mottle virus (CPMMV), genus *Carlavirus*. (Shoyinka, 1974; Hughes and Shoyinka, 2003; Taiwo, 2003). The qualitative and quantitative effects of these viruses have been reported (Taiwo and Akinjogunla, 2006). Mixed infections of these viruses lead to several symptoms and virus synergism cause the decline of cowpea plants and serious crop losses (Gillaspie et al., 1998). Owolabi et al. (1988) reported a 78 – 100% reduction in the pod number of cowpea (Ife brown and Nigeria B7) inoculated with Blackeye cowpea mosaic virus (BICMV) and Cowpea yellow mosaic virus (CYMV). The identification of these viruses is by symptomatology and serology (Taiwo, 2003). The viral infection of cowpea is transmissible through sap, seeds and insects like, *Mycus persicea*, *Meoythia quartena*, *Ootheca mutabilis, Paraluperodes quaternius*, *Aphis craccivora*, *Aphis gossypii* , and are readily transmissible in a non persistently manner (Bock, 1973; Bock and Conti, 1974; Lana and Adegbola, 1997; Shoyinka et al., 1978; Bock and Conti, 1994; Taiwo, 2003). Southern bean mosaic virus, cowpea mottle virus and cowpea aphid-borne mosaic virus have been reported to be fairly prevalent and of moderate incidence of cowpea cultivated in Nigeria (Taiwo and Akinjogunla, 2006).

In order to improve the quality and quantity of the germplasm and to significantly reduce the infection of virus to different cultivars, proper diagnosis and control is essential, and diagnosis of viruses equally helps in exporting planting materials to countries where-in strict quarantine conditions have been imposed. Management of the viral diseases is based primarily on the development of cowpea resistant varieties (Thottappilly and Rossel, 1992).

This overview examines the use of immunological and molecular diagnostic techniques with respect to Cowpea aphid-borne mosaic virus (CABMV), Cucumber mosaic virus (CMV), and Cowpea mottle virus (CMeV).

IMMUNOLOGICAL AND MOLECULAR DIAGNOSTIC METHODS

The method of diagnosis, detection and identification of viruses in plants play a vital role. Traditional diagnosis of plant viruses requires bioassay, an indicator plant, determination of host range, symptomatology, virus particle morphology (size and shape), and vector relations. A single diagnostic test or assay may provide adequate information on the identity of a virus but a combination of methods is generally needed which are specific, sensitive and inexpensive (Naidu and Hughes, 2003). However, progress in molecular biology, biochemistry and immunology has led to the development of many new, accurate, rapid and less labour-intensive methods of virus detection. Technologies for the molecular detection of plants pathogens have already undergone two major breakthroughs well over the past three decades. The first was the advent of antibody based detection, in particular monoclonal antibodies and enzyme-linked immunosorbent assay (Kohler and Milstein, 1975; Clark and Adams, 1977). There are various immuno-diagnostic and molecular-diagnostic techniques presently available in field of virology and these are divided into two: Protein based techniques which include precipitation/agglutination tests, enzymes linked immunosorbent assay (ELISA), Immunosorbent electron microscopy (ISEM), fluorescent antibody test, dot immunoblotting assay (DTBIA). Viral nucleic acid based techniques are dot blot hybridization/slot blot hybridization, polymerase chain reaction (PCR), nucleic acid hybridization with radio labelled and nonradio-labelled probes, DNA/RNA probes. (Hampton et al., 1992). Lawson (1981) has mentioned that appropriate screening procedures have been conducted in order to certify any plant free of certain pathogen using ELISA, PCR, DNA probes.

Occurrence of CABMV in cowpea in several African, Asian and Europeans countries has been reported. (Mali et al., 1988; Hampton, 1992; Patel and Kuwute, 1992). CABMV was first reported in Italy (Lovisolo and Conti, 1966) and also reported in Nigeria in Mid-70's (Ladipo, 1976). CABMV can be found in virtually all the ecological zones of Nigeria, and has flexuous rod shaped particle of about 750 nm Length. CABMV belongs to family *Potyviridae* and genus *Potyvirus.* CMeV was first isolated from Bambarra groundnut (Thottappilly and Rossel,

1988b) and was first reported from Nigeria (Robertson, 1966). CMeV has isometric particle and contain 20% RNA and the natural occurrence of CMeV in several legumes has been reported (Kaisser et al., 1968). Cucumber mosaic virus (CMV) is the type of member of the genus *Cucumovirus*, family *Bromoviridae*. This virus has icosahedra particle of diameter of 28 nm and has a segmented genome of three single stranded RNAs (Palukaitis et al., 1992) and was first found in Cucumber in the USA (Price, 1934) and first reported in cowpea by Robertson (1966). CMV has been confirmed to be very ubiquitous plant virus and is the most commonly found in the riverine area of the middle belt of Nigeria. (Shoyinka et al., 1997). CABMV, CMV and CMeV cause mosaic, leaf distortion, stunting, mottling and death. Viruses have been purified from the host by different workers. ELISA and other modified forms e.g. direct antigen coating enzymes linked immunosorbent assay (DAC-ELISA), double antibody sandwich ELISA (DAS-ELISA), antigen-coated plate (ACP-ELISA), plate trapped antigen (PTA-ELISA), triple antibody sandwich (TAS-ELISA) have been extensively used for the detection of CABMV, CMV, CMeV from different parts of the cowpea with a wide range of sensitivity. (Clark and Adams, 1977; Hobbs et al., 1987; Bashir and Hampton, 1996). ELISA proved sensitive and reliable for the detection of Cucumber mosaic virus (CMV) in different tissues (Abdullahi et al., 2001).

Evaluating ELISA for CMV detection revealed that virus concentration was highest in flowers and lowest in primary leaves (Abdullahi et al., 2001). Many factors can therefore influence the sensitivity and reliability of ELISA assay, among these are quality of antibodies, preparation and storage of reagents, incubation time and temperature, selection of appropriate parts of sample and the use of suitable extraction buffer (McLaughlim et al., 1981; Hewings and D'Aray, 1984). ELISA is an excellent technique for detection of seed borne viruses (Bashir and Hampton, 1997). It is critical that positive and negative controls are included in each assay to define a threshold for differentiating between infected and non inflected cowpea cultivars. Generally a sample is regarded as positive if the absorbance value exceeds the mean value of a negative control by 2 - 3 standard deviation (Naidu and Hughes, 2003). A biotin/streptavin ELISA was found to be more sensitive than a standard ELISA protocol for detecting CABMV infection in cowpea seeds and also indicated that ELISA technique is reliable for selecting CABMV – free stock of cowpea seeds. (Kunate and Neya, 1996).

In a three year survey for the incidence and distribution of cowpea viruses in Nigeria, (Shoyinka et al., 1997) detected viruses in 390 out of 649 cowpea collected from all agro ecological zones in Nigeria using ELISA, and CABMV had the highest incidence and was the most prevalent of all the viruses detected. A set of 2930 cowpea germplasm accessions, mostly from Botswana and Senegal were examined under field conditions for detection and identification of seed-borne viruses, only CABMV was detected using DAC-ELISA and DAS-ELISA (Bashir and Hampton, 1996). The evaluation of 158 *V. unquiculata* assessions provided evidence that at least CABMV and CMV occurred in cowpea germplasm seed stocks maintained in United State (Bashir and Hampton, 1996). The surveys conducted in Nigeria using ELISA between 1991 and 1993 showed incidence rate of 9.8, 0 and 7.9% and prevalence rate of 29.2, 0 and 40.9% for CMeV for the three years respectively (Shoyinka et al., 1997). The types of antibodies used in ELISA affect the detection of CABMV, CMeV and CMV. ELISA with antise-ra against cowpea isolates could detect CABMV, in lea-ves flowers etc. The polyclonal antibodies showed cros-sed reactivity. Thus, the use of monoclonal antibodies was preferred. CMV was detected by DAS-ELISA in the flowers, primary and trifoliate leaves and stem of cowpea plants. When trifoliated leaf samples of cowpea plants were subjected to DAS-ELISA much higher CMV infec-tion rates were recorded compared to the least amount of detectable CMV in stem and primary leaves (Abdullahi et al., 2001). The usefulness of DAS-ELISA and tissue printing ELISA for CMV detection in cowpea plants and for indexing of seed has been reported (Abdullahi et al., 2001). ISEM, a technique introduced by Derrick (1973) can be used to estimate the degree of serological rela-tionship among CMV, CABMV and CMeV. ISEM combi-nes the specificity of serological assays with the visuali-zation capabilities of the electron microscope.

MORE CURRENT DIAGNOSTIC TECHNIQUES

Recently a novel real-time quantitative PCR assay was developed for the detection and quantification of plant viruses (Detzgen et al., 1999). Polymerase chain reaction (PCR) is a molecular biology method for enzymatically copying target nucleic acid sequence without using a living organism, in which repeated replication of a given sequence forms millions of copies within a few hours. PCR technique is a DNA based technology that permits a small sample of target nucleic acid to be copied multiple times for analysis (Mullis and Faloona, 1987). The method is a highly specific and versatile method of DNA amplification using thermostable DNA polymerase from *Thermus aquaticus* or *Pyrococus furiosus*. The PCR process consists of a series of twenty or thirty cycles. Each cycle consists of three steps:

(i) The double stranded DNA is heated to 94° – 96° to separate the strands.
(ii) Lowering of the temperature 45° - 60° so that the primer can attach themselves to the single DNA strand.
(iii) Extension of each primer, usually at 72° using a thermostable DNA.

The Reverse Transciptase PCR (RT-PCR) method has been found to be 10^5 times more sensitive than direct antigen coating enzymes-linked immunosorbent assay

(DAC- ELISA) in detecting cowpea mottle virus (CMV). The RT-PCR method gives no false positive reaction as is sometimes seen with ELISA. The comparison of DAC-ELISA and RT-PCR detection of CMeV in cowpea indicated that PCR is much more sensitive. CABMV, CMV and CMeV could be detected by ELISA, DTBIA, RT-PCR and by Nucleic acid hybridization. Availability of these diagnostic methods provides greater flexibility, increased sensitivity and specificity for rapid diagnosis of virus diseases. Nucleic acid hybridization has been extensively and successfully used for characterization of cucumber mosaic, cucumovirus seed borne in cowpea (Glimpse et al., 1999). Nucleic acid hybridization test for detecting specific DNA or RNA sequence have gained whole acceptability in recent years. The term hybridization was originnally proposed by Spiegelman (1964) to describe DNA-RNA hybrids; today the term hybridization includes the formation of DNA-DNA, DNA-RNA or RNA-RNA complexes.

Non-radioactive methods for detection of cowpea viruses now exist, but they are labour-intensive because of the steps required for antibody conjugate attachment and substrate reaction and also requires bulky hardware such as micro array reader to detect fluorescent probes. Dilution end point for CMV can be determined using slot blot hybridization (Choi et al., 1995). Dot immunoblotting assay (DTBIA) can detect CMV, CABMV and CMeV in cowpea plants. An optimized DTBIA is as sensitive as ELISA, simple, relatively inexpensive and the DTBIA result can be scored visually, but differs from ELISA as the plant extracts are spotted on to a membrane rather than using a microlitre plate as the solid support matrix. Abdullahi et al. (2001) evaluated the detection capacity of ELISA to prove its reliability using a reverse transcriptase PCR assay, thus, PCR confirmed ELISA. Differentiation of CMV isolates using the polymerase chain reaction has been reported (Rizos et al., 1992).

Reverse transcriptase PCR (RT-PCR) can be used for viral disease diagnosis because it is a quick and more reliable method in comparison to ELISA, and PCR can be used for further characterization of cowpea plant viruses. Combining PCR with molecular hybridization can detect even pictogram qualities of virus, and this combination is 4 - 5 orders magnitudes superior to direct molecular hybridization (Vunsh et al., 1990). Diagnostic approaches based on nucleic acid hybridization are not only highly specific but also applicable for routine testing of large number of samples.

CONCLUSION

Accurate identification and early detection of the viral diseases is the cornerstones of the management of cowpea cultivar. Cowpea viruses are difficult to identify using morphological criteria, which can be time consuming and challenging and requires extensive knowledge in taxonomy. Molecular and immunological detection such as ELISA and other modified forms, precipitation/agglutination, fluorescent antibody, DTBIA, PCR, nucleic acid hybridization are best suitable techniques to detect the various viruses viz., CABMV, CMV, and CMeV infecting cowpea. Until now ELISA and other modified forms have been extensively used, because these are quick. However PCR has been widely used with the varying degree of modification for detection of viral genomes in infected plant in the last two decades.

The disadvantage of PCR is that it requires sophisticated equipment like thermocycler which is expensive, where as ELISA/DAC-ELISA can be used for diagnoses even in field conditions and are very cost effective. Viruses and virus strains cannot be distinguished on the basic of common sources of resistance. Since unrelated viruses have been known to share sources of resistance, for instance, some cowpea lines found to be resistance to Nigeria isolate of CABMV were known to possess resistance to isolate SBMV and CYMV (Ladipo and Allen, 1979). Screening cowpea lines for resistance to viral infection is a useful approach to virus control and information obtained from such screening experience could be used in cowpea improvement programme. These various immunological and molecular diagnostic tests with symptoms and history are of immense value to diagnose cowpea viruses, thus, these diagnostic techniques can become a routine in plant pathology research.

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