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REVIEW

Evaluation of germplasm: An avenue of identifying resistant donor in addition to characterization of emerging pathogen

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Germplasm with the ability to withstand biotic and abiotic pressures are the keys for sustainable agriculture under the contexts of exploisive population and changing climate driven demand from agriculture. Identification of resistant sources against biotic stresses is very much essential to support crop improvement programmes. Thus evaluation of germplasm resources to identify suitable germplasm with target trait is essential for their utilization. A host is considered resistant when it has the ability to exclude, hinder or overcome the effects of a given pathogen. A plant may be resistant to one pathogen but not to others. The success of the identification of resistant germplasm will depend upon the through proof methodology, which has been adopted to screen such trait specific germplasm. During the period of 2011 to 2014 lots of germplasm of different crops were evaluated at ICAR – National Bureau of Plant Genetic Resources, New Delhi following international standard screening methodologies. These gave us ample opportunity to identify resistant germplasm vis-á-vis revealation of some pathogens which are characteristically different from the earlier same pathogen open our eyes to revaluate the germplasm against new target. Yellow mosaic disease (YMD) of black gram is an important production constraint in India. Black gram germplasm, consisting of 344 accessions, originally collected from different geographic regions of India and conserved in National Gene Bank at National Bureau of Plant Genetic Resources, India, were evaluated for their response against YMD. None of the accessions tested was found to be immune, but four accessions IC144901, IC001572, IC011613 and IC485638 were identified as resistant against Mungbean yellow mosaic virus (MYMV) but the virus is detected as new recombinant type of MYMV, prevalent mostly in southern India and Mungbean yellow mosaic india virus (MYMIV), prevalent mostly in nrthern India. Similarly, during evaluation of Brassica, emergence of a weed-infecting begomovirus–betasatellite complex in rapeseed-mustard germplasm in India were detected and this raises the concern on utilization of such susceptible germplasm in crop improvement programmes.

Key words: Blackgram, begomovirus, biotic stresses, brassica, CYVMV, disease, evaluation, germplasm, MYMV

INTRODUCTION

The projected world population will pass the 9 billion mark by 2050, and by 21st century it will reach to 10 billion (UN, 2011). The global agricultural production required to be nearly double to match the demand (The Royal Society, 2009; Kearney, 2010). The International Food Policy Research Institute (IFPRI) estimates that a 70% increase in production of grains will be needed to feed the world by 2050, unless major changes in consumption patterns occur (Rosegrant *et al.* 2009). This is a challenge to the agriculturist to produce more. In country like India, where maximum land holding is under marginal, rainfed, saline and waterlogged conditions, the productivity have still remained low. Several factors are responsible for low productivity, of which biotic and abiotic stresses also have major role. Therefore more emphasis on research aiming at resistance to biotic and abiotic stresses is necessary. Plant genetic resources (PGR) are the backbone of agriculture which plays a positive role in the development of new cultivars, including improvements of the existing ones that are handicapped due to poor expression or lack of one or the other attributes. Plants, which could withstand better under biotic and abiotic pressures, are thus the keys for sustainability in agriculture. Obviously, it is a tough task and require all-round

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efforts of collecting and preserving germplasm, and also evaluating it under appropriate stress conditions, and then to identify the desired germplasm with appropriate genetic background (Bag and Dutta, 2014).

Identification of resistance donor to pathogen and utilization of that genetic factor to improve and protect crop production is one of the most important objectives of evaluation of germplasm. Germplasm are the main sources for incorporating the trait of target in the cultivated varieties. Genes of desirable traits are available in landraces and wild species. Screening of germplasm to identify resistant sources against biotic stresses is very much essential to support crop improvement programmes. A host is considered resistant when it has the ability to exclude, hinder or overcome the effects of a given pathogen or other damaging factor. A plant may be resistant to one pathogen or pest but not others. Often true resistance is not observed rather tolerance is more common phenomenon in host-pathogen interaction. The success of the identification of resistant/tolerant germplasm depends upon the methodology which has been adopted to screen trait specific germplasm.

Important points to consider before screening

- Prioritization to be given on one stress at one time.
- Evaluation of germplasm against biotic stress means evaluation against the pathogen not against the disease. So, it is essential to identify the pathogen based on morphological or molecular tools. Then, mass culture of identified pathogen needs to be done. In case of viruses, infectious viral construct needs to be prepared for challenge inoculation purpose.

- Identification of the pathogen is also important to know the race/strain/isolate/biotype which exists at that particular location.

Screening of germplasm for resistance to pathogens causing diseases

Evaluation of germplasm of any crop against diseases is very critical because expression of the disease and their degree of variation not only depends upon the germplasm but also rely upon degree of virulence of the pathogen, environmental conditions as well as host – pathogen interaction. Time is also important factor because it allows a pathogen to infect a host plant. These four factors i.e. susceptible host, virulent pathogen, environment and time, which cause disease is called "disease quadrangle". In case of virus diseases often another factor, "vector", which transmits virus is important to establish a disease. Furthermore, different disease management strategies and other cultural practices may either prevent or predispose any hosts against diseases. So, non-occurrence of symptoms in any germplasm accession or reduced symptom production in any accession does not always imply resistance or tolerance of those germplasm, rather there may be chances of escape from the infection. Under these circumstances, repeated field screening with proper experimental design followed by stringent screening under artificial challenged inoculation condition under field and glasshouse are essential to establish a real resistance or tolerance response of germplasm. Interesting findings were come out during evaluation of black gram and brassica germplasm. These observations and outcomes are discussed under case study I (Black gram) and case study II (Brassica).

Methodology of screening

(i) *Field Screening*

(a) Preliminary field screening for one year with large number of accessions to narrow down the numbers to a manageable extent. Only percent disease incidence should be recorded and field tolerant accessions (<20% incidence) are selected.

(b) Advanced field screening of the selected field tolerant accessions should be done atleast two years. The advance screening plot and germplasm maintenance plot should be different. In advance screening plot no plant protection measures are adopted so that the germplasm should be exposed to the maximum load of inoculum pressure of pathogen or vectors of viruses. Experiments should be done in replicated trials with susceptible check as infector row, preferably one line of infector row after every three lines of germplasm and border lines of susceptible check. The data should be recorded at regular intervals.

In case of fungal and bacterial foliar diseases Standard evaluation system (SES) scale should be followed during data recording.

For soil borne pathogen the selected germplasm is grown in sick plot and maintained with optimum inoculum load. At least 80-100% infection in susceptible check confirms optimum inocula load.

In case of air-borne pathogen, spraying of spore suspension at regular intervals is must and 80-100% infection in the susceptible check should confirm the optimum inocula load.

In case of viral diseases, per cent disease incidence and average disease severity should be taken into account. A severity index grade is formulated for individual disease based on the progress of the disease and coefficient of infection (CI). CI=% disease incidence (PDI) X Response value (severity grade). As per the CI value, a scale is prepared and the response of the germplasm is categorized. The area under disease progress curve (AUDPC) value be calculated to understand the rate of spread of the disease. The germplasm with low CI and AUDPC value should be selected as promising accession.

(ii) *Screening through challenged inoculation under greenhouse condition*

For virus and other vector borne pathogens, advance screening under natural field condition are carried out for two years and the promising accessions are challenged under controlled glasshouse conditions with viruliferous insects (for insect transmited virus), sap from the infected plant (for mechanically transmitted virus) and grafting (for the viruses transmitted by vegetative propagules).

Artificial inoculation under controlled conditions should also be carried out for fungal and bacterial pathogens. For such experiments advanced facilities like temperature humidity controlled glasshouse, insect maintenance chamber, plant inoculation chamber etc are essentially required.

Finally, the identified accessions may be tested under multi-location evaluation at hot-spot to evaluate its performance against other race/strain/ isolate of the pathogens those exist in different locations.

CASE STUDY I

Black gram (Vigna mungo (L.) Hepper), the third important pulse crop of India, contributes 70% of world's total black gram production but very low productivity (0.41- 0.53 t/ha) during last two decades (http://www.aicrpmullarp.res.in). One of the most important factors is yellow mosaic disease (YMD) and mostly account for the low harvest index of the present day black gram cultivars (Tuba Anjum *et al.* 2010). Vector control through application of synthetic and nonsynthetic insecticides is neither sustainable nor economically viable. Hence, use of resistant varieties, is the only feasible, economic, environment friendly and sustainable approach to alleviate occurrence of YMD in areas where it is a major constraint to grain legume cultivation.

In search of resistant black gram germplasm, preliminary screening was started with 344 accessions. On preliminary field screening, 32 accessions of blackgram were selected as resistant to yellow mosaic disease based on incidence and severity of the symptoms under natural disease pressure. These 32 accessions were free from other diseases like leaf curl, powdery mildew etc. (based on visual observations only). The response to yellow mosaic disease was found to differ greatly among the accessions. However, resistance of a host can also been influenced by several other factors, like presence or absence of virulent strains of the viruses, climatic conditions and inoculum pressure. All of these factors can have significant influence on the measure of the disease in individual plant (severity) as well as in population (incidence) (Matthews, 1991). Hence, for estimating the response of germplasm against a virus, PDI or PDS alone cannot provide conclusive evidence. CI is a routine parameter to screen germplasm (Singh and Singh, 2000). Therefore, we used a combination of these two parameters in the form of CI to determine the response of germplasm accessions under natural disease pressure in field. We had also estimated the cumulative progress of disease using AUDPC, which varied greatly between susceptible and resistant groups. In general, accessions within the resistant group showed a lower AUDPC value than the susceptible group, indicating a small amount of cumulative disease progress in resistant accessions. These 32 accessions were undergone advance field screening and 8 accessions were selected as resistant to yellow mosaic disease. The 8 accessions, that showed consistent resistance in the field, were further tested by inoculation with whiteflies. Of the 8 field-resistant accessions, 2

accessions IC144901 and IC001572 were grouped into the HR category as <10% plants exhibited minute yellow specks or spots after 17-22 days post infection (dpi). Two other accessions viz. IC011613 and IC485638 showed <20% incidence with bright yellow specks or spots (some coalesced) after 15-20 dpi and were grouped into the R category (Bag *et al.* 2014). The artificial screening of germplasm needs to be performed to confirm the resistance in the field; however, it should only be a supplement to the field screening. Disease screening using the vector transmission alone may produce misleading results as vector resistance can be interpreted as resistance against virus (Akhtar *et al.* 2010). Hence, the resistance showed by the 4 accessions may be against either whitefly or virus. Agroinoculation of infectious virus constructs was employed to further evaluate the resistance of the accessions. The resistance (HR or R) of 4 accessions, viz. IC144901, IC001572, IC011613 and IC485638, was further evaluated by agroinoculation with the infectious cloned DNA-A and DNA-B components of a New Delhi isolate of MYMV. Agroinoculation is a proven technique to introduce geminiviruses into host (Bi *et al.* 2010). Southern blot hybridization analysis, a standard technique for detection and relative quantification of begomovirus (Singh *et al.* 2012), indicated relative accumulation of viral DNA was consistently lower in the four resistant accessions, particularly in IC144901 and IC001572, compared with the susceptible check (Bag *et al.* 2014). This suggests that the replication and accumulation of virus is restricted in the resistant accessions. Low accumulation of begomoviral DNA in resistant plants may be due to degradation of begomoviral RNA, as such a phenomenon has been observed in case of MYMIV infection in resistant soybean accessions (Yadav *et al.* 2009).

CASE STUDY II

Rapeseed-mustard (*Brassica* spp.) are significant crops grown throughout the world mainly for oilseed and condiments, as well as vegetable and fodder crops also. India is the third largest rapeseed-mustard producer in the world. Mustard is the second important edible oilseed after groundnut in India's oilseed economy (Chand *et al.* 2004). Like other crops brassica also faced lot of diseases like *Alternaria* blight, white rust, powdery mildew etc. but rarely by viral diseases. During evaluation of brassica germplasm, peculiar leaf curling and stunting like symptom were observed and it aroused inquisitiveness in our mind to find out the reason behind it. These kinds of symptoms resembled with some viral diseases and curling & stunting are common symptoms in case of geminivirus infection. No leaf curl disease or any geminivirus have previously been reported to infect rapeseed-mustard crops under natural conditions. However, in recent years, the majority of the begomoviruses reported from the Old World were found to be monopartite, which lack the DNA-B component. Most of these monopartite begomoviruses are associated with betasatellites, a recently identified group of symptom-modulating, single-stranded DNA satellites that occur only in the Old World (Briddon *et al.* 2008). In tropical and subtropical regions of the world, begomoviruses are emerging as a major threat to a number of economically important crops (Varma *et al.* 2011). Furthermore, evolution of newer begomoviruses and the occurrence of co-infection with heterologous begomoviruses and betasatellite molecules have aggravated the disease spectrum and thus posed an additional threat to the cultivation of crops (Varma and Malathi ,2003). The persistence of whitefly populations throughout the year is influenced by the changes in climate and this allow adaptation of begomoviruses in newer hosts, which were earlier not reported to be natural hosts. Thus we shifted our focus to identify the causal organism and its characterization instead of screening. We describe here the first natural occurrence and spread of a leaf curl disease in the rapeseed-mustard germplasm in India and report the detection and characterization of a weed-infecting begomovirus-betasatellite complex associated with such disease following standard methodologies.

Disease occurrence and response of germplasm

Only16 plants of 7 germplasm accessions showed symptoms of upward leaf curl, leaf rolling, thickening of veins and stunted growth during 2011–2012. Next year similar symptoms were observed in 201 plants of another 62 accessions. Severe rolling of leaves and stunted growth were observed in *B. rapa* cv. Yellow Sarson and *B. rapa* cv. Brown Sarson while mild upward leaf curl symptoms were observed in *B. nigra*. Although considerable populations of whitefly were observed in the field, uniform spread of the

disease, which was otherwise expected in cultivars, was not observed in germplasm accessions of all the species, indicating a differential response of these accessions to the disease. Among the symptomatic germplasm accessions, the majority were *B. rapa* cv. Yellow Sarson (including 3 check cultivars) while none of the accessions belonging to *B. rapa* cv. toria, *B. juncea, B. napus* and *B. carinata* showed any leaf curl disease for consecutive two years (Roy *et al.* 2013). A leaf curl disease found in germplasm of rapeseed mustard was not known previously in this group of crops. Germplasm accessions of *B. rapa*, more precisely those belonging to cvs Yellow Sarson and Brown Sarson, were found to be more sensitive to the disease, whereas in some other species, there was apparent field resistance which will need to be confirmed. Based on symptom expression and presence of whiteflies on the plants, begomovirus infection was suspected.

Whitefly transmission

As second step to confirm the disease caused by begomovirus, white fly transmission experiment was conducted. After 21–28 days of whitefly transmission, typical symptoms appeared on the healthy plants of *B. rapa* cv. Brown Sarson with 40–60% transmission efficiency under glasshouse conditions.

Detection of a begomovirus and betasatellite

DNA was isolated from 22 field samples and 4 glasshouse samples showing symptoms along with one asymptomatic sample from the field and one healthy sample. All the 22 symptomatic samples obtained from field-grown *B. nigra* (2), *B. rapa* cv. Brown Sarson (6), *B. rapa* cv. Yellow Sarson (9), *B. rapa* subsp. chinensis (3), *B. juncea* subsp. *rugosa* (2) yielded expected 1.2 kb and 1.3 kb amplicons with DNA-A- and betasatellite-specific primers respectively; however, amplification with DNA-B primers failed even with repeated attempts, indicating presence of a monopartite begomovirus and a betasatellite with symptomatic samples. Glasshouse-inoculated plants also showed the similar results. Neither the asymptomatic samples (AS), obtained from the field, nor the healthy samples (H), grown under glasshouse, have produce any amplicon (Roy *et al.* 2013).

Isolation of complete genome of begomovirus

The targeted viruses were amplified through RCA method and were digested with five different restriction enzymes, among which one resulted 2.7 kb fragments, which is the unit length of begomovirus. The complete begomovirus genome was cloned and sequenced from these symptomatic *Brassica* species to know whether similar begomovirus is present in all these samples (Roy *et al.* 2013).

Genome organization of begomovirus and betasatellite

Sequences of four clones of begomovirus and betasatellites showed 99.8–100% and 99.6–100% sequence identity, respectively. These results clearly showed an uniform association of a begomovirus and betasatellite species in all the four genotypes showing leaf curl symptoms. Hence, one complete sequence each for begomovirus and betasatellite isolated from *B. rapa* cv. Brown Sarson was deposited in the nucleotide database under accession numbers, JX270684 and JX270685, respectively (Roy *et al.* 2013).

Sequence identities with other begomoviruses and betasatellites

On comparison of JX270684 with begomoviruses sequences showed highest (98.1%) identity with *Croton yellow vein mosaic virus* (CYVMV, FN645901) isolated from *Acalypha* sp. in Haryana, India and clustered with CYVMV isolates reported from *Acalypha* sp. in India. The identities were 96–98% with other CYVMV isolates reported on Acalypha sp. (FN645926, FN645898, FN645902), radish (*Raphanus sativus*) (FJ593629) and cluster bean (*Cyamposis tetragonoloba*) (FN645915) from India. It showed 92–96% sequence identities with CYVMV isolates reported on *Croton bonplandianum* (JN817516, AJ507777, JN817517, JN831446) and *Jatropha gossypifolia* (EU727086) from India. One isolate of CYVMV reported on *Alcea rosea* (FN678906) and another isolate of a new begomovirus, *Croton yellow vein virus* (CYVV), reported on *C. bonplandianum* (FN543112) from Pakistan, showed 90.2 and 89.5% sequence identities, respectively, with the present isolate. ORF wise sequence identities both at nucleotide and protein level showed highest sequence identities with different isolates of CYVMV. The entire sequence of JX270685 showed 96 and 84% nucleotide sequence identities, respectively, with *Croton yellow vein mosaic betasatellite* (Cro-YVMB), isolated from papaya in India and

clustered with them. Based on the species demarcation criterion for the begomoviruses and betasatellites (Briddon *et al.* 2008; Fauquet *et al.* 2008), the present begomovirus isolate and betasatellite are an isolate of CYVMV and its associated betasatellite, respectively, and hence, the following descriptors are proposed: Croton yellow vein mosaic virus-(India:Delhi:Brassica:2011) (for begomovirus) and Croton yellow vein mosaic betasatellite-(India: Delhi:Brassica:2011) (for betasatellite).

Recombination analysis

The Neighbor-Net analysis of our begomovirus (JX270684) and betasatellite (JX270685) sequences indicates clear evidence of phylogenetic conflicts within the analysed sequences that are caused by recombination as cycles within unrooted bifurcating trees. Notably, every sequence represented within these trees was implicated as a potential recipient of horizontally acquired sequences at some time in its evolutionary past. Recombination analysis with respect to both begomovirus and betasatellite indicated the occurrence of multiple overlapping inter- and intraspecific recombination events with different parental combinations.

Whitefly transmission followed by PCR amplification confirmed the presence of a monopartite begomovirus and a betasatellite with the disease. Further characterization revealed that an isolate of a weed-infecting begomovirus species, CYVMV and its associated betasatellite were consistently associated with different species of rapeseed- mustard germplasm showing leaf curl symptoms in India. The occurrence of croton yellow vein mosaic disease was reported from India by Varma in 1963. The virus associated with the disease was described as *Croton yellow vein mosaic virus*, which was efficiently transmitted by whitefly in a persistent manner (Mandal and Muniyappa 1991a). Infectivity analysis was carried out to confirm the aetiology of the disease (Pramesh *et al.* 2013). The disease was also reported from Pakistan, but a new species of begomovirus, CYVV, was found to be associated with it (Hussain *et al.* 2011). Although CYVV was reported as an isolate of a distinct begomovirus based on 89% cut-off value (Hussain *et al.* 2011), but comparison with the present isolate indicated that a revision is required for their taxonomic identities. Different methods used for recombination breakpoint analysis using RDP software also provided strong evidence for the presence of past recombination events in most of the sequences analysed. The role of such overlapping recombination between different isolates or different species in adaptation to rapeseed-mustard may be an interesting aspect that needs to be resolved. Such inter- and intraspecific recombination is a predominant feature of begomovirus evolution (Lefeuvre *et al.* 2007) and has been implicated in the emergence of new begomovirus species and adaptation in new hosts in agricultural system (Garcia-Andres *et al.* 2007).

CONCLUSION

In case study I, strategic screening methodologies combining repeated field screening, whitefly transmission and agroinoculation have been adopted to screen a part of national collection of black gram germplasm against Mungbean yellow mosaic virus. The study allowed us in selecting four germplasm accessions of black gram, which showed consistent resistant reaction and exhibited at per or higher agronomic performance than the checks. These four accessions viz., IC144901, IC001572, IC011613 and IC485638 could be used in YMD resistant breeding programme or could also be released directly for cultivation after verifying their adaptation to various regions and other acceptable quality traits. We are fortunate to identify germplasm of some important crops following this stringent screening procedure like IC309064, IC393365, IC306465 and IC283866 accessions of pearl millet identified as resistant to *Sclerospora graminicola,* downy mildew causing pathogen and *Magnaporthe grisea,* causing blast disease (Kumari *et al.* 2016); accession IC410617 of cucumber identified as resistant to downy mildew pathogen (*Pseudoperonospora cubensis*) and Cucumber mosaic virus (Pragya *et al.* 2015); few wild okra accession viz., EC306731-P and EC305725 of *Abelmoschus caillei*; accessions IC117175, IC344598, IC433667 and IC331214 of *A. manihot*; IC140986 of *A. moschatus* resistant to *Bhindi yellow vein mosaic virus* (Gangopadhyay *et al.* 2017). Lot of wheat accessions were identified resistant against rust pathogens and spot blotch pathogens during mega screening of wheat germplasm involving multi-institutional and multi-disciplinary approach (Kumar *et al.* 2016)

In case study II, we are not claiming that this begomovirus complex is the cause of such leaf curl disease in rapeseed-mustard. Here, we tried to generate infectious clones to establish that the disease symptoms observed in infected plants is attributable to the begomovirus and betasatellite. Here, we have only demonstrated the association of this begomovirus complex with the symptomatic rapeseed-mustard germplasm (Roy *et al.* 2013). The role of such overlapping recombination between different isolates or different species in adaptation to rapeseed-mustard may be an interesting aspect that needs to be resolved. Such inter- and intra-specific recombination is a predominant feature of begomovirus evolution (Lefeuvre *et al.* 2007) and has been implicated in the emergence of new begomovirus species and adaptation in new hosts in agricultural system (Garcia-Andres *et al.* 2007). Later natural infection of *Croton yellow vein mosaic virus* (CYVMV) and its cognate betasatellite in germplasm of different Crambe spp is reported in India during screening of Crambe sp., an industrial crop belong to wild relatives of Brassica (Kumar *et al.* 2017).

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