International Journal of Agriculture Sciences
ISSN: 0975-3710&E-ISSN: 0975-9107, Volume 9, Issue 21, 2017, pp.-4232-4234.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

Research Article

MOLECULAR IDENTIFICATION OF PHYTOPLASMA ASSOCIATED WITH SOYBEAN WITCHES'-BROOM IN VIDARBHA REGION, MAHARASHTRA

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Received: March 19, 2017; Revised: April 15, 2017; Accepted: April 16, 2017; Published: May 06, 2017

Abstract- Soybean (*Glycine max* L.) plants showing witches'-broom symptoms typical of phytoplamas were observed from different locations of Vidarbha region, Maharashtra. Symptoms observed under field condition include phyllody, stunting growth, proliferation of auxiliary shoots resulting in a witches-broom appearance and deformed pods if any. PCR detection of soybean phytoplasma by using phytoplasma-specific universal primer pairs (P1/P7) produced an amplified product of 1.8kb lengths when DNA extract from symptomatic samples was used as template. Asymptomatic plant samples yielded no amplification. On mechanical inoculation none of the test plants inoculated showed any visible symptoms even after 120 days after inoculation.

Keywords- *Glycine max*, Mechanical inoculation, P1/P7, PCR, Phyllody, Symptomatic and asymptomatic plant, Test plant and virescence.

Citation: Hajong M., et al., (2017) Molecular Identification of Phytoplasma Associated with Soybean Witches'-Broom in Vidarbha region, Maharashtra. International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 9, Issue 21, pp.-4232-4234.

Introduction

Soybean (*Glycine max* L.) is one of the few most important crops in the world and serves as a principal dietary food and oil source. It contains almost 38-45% protein and approximately 20% oil. It is the major cash crop of central zone of India. Recently, symptoms of virescence (i.e. the development of green flowers and the loss of normal flower pigments), phyllody (i.e. the development of floral parts into leafy structures), sterility of flowers and proliferation of auxiliary shoots in soybean have been observed in several locations of Vidarbha region, Maharashtra. The development of such type of symptoms are usually attributed to phytoplasma which is cell wall-less plant pathogenic prokaryotes found in phloem of many plant species [1].Other symptoms like generalized stunting (i.e. small flowers and leaves and shortened internodes), discolorations of leaves or shoots, leaf curling or cupping and bunchy appearance of growth at the ends of the stems [2]. Phytoplasmas can only transmit by graft, insects and dodder but cannot transmit mechanically by inoculation with phytoplasmas containing sap [3].

Diagnosis of phytoplasma-associated diseases is one of the most difficult aspects of research on plant diseases due to the inability to culture the pathogens *in-vitro* [4]. Henceforth, PCR has become a powerful, extremely sensitive, fairly inexpensive and simple tool in molecular biology and diagnosis [5]. Detection of phytoplasmas by PCR is at least 100 to 1,000,000 times as sensitive as that by dot-blot hybridization methods [6]. PCR assays using universal primer pairs (P1/P7) designed on the basis of 16S and 23SrDNA sequence have been employed effectively to detect and identify a broad array of known and unknown phytoplasmas from various host plants and insect vector. The objective of this study was to determine the possible association of phytoplasma with witches'-broom in soybean and to identify the associated phytoplasma.

Materials and Methods

Soybean phytoplasma sample collection

The soybean plants showing symptoms of witches'-broom (deformed pod formation), bushy appearance and dark green with thicker stem were collected from the fields of Departments of Plant Pathology and Entomology, Dr. PDKV, Akola. Samples were also collected from different locations of Vidarbha region (i.e., Buldhana, Nagpur, Amravati and Akola), Maharashtra. The samples were kept at -80°C in plastic bags in deep freeze with labels indicating date of collection and the location from where it was collected.

Extraction of nucleic acid and PCR

Soybean phytoplasma DNA was isolated by DNeasy Plant Mini Kit (QIAGEN) following the instruction provided with the kit. The extracted DNA was run with PCR using phytoplasma specific universal primer set (P1/P7). P1 (16S)-Forward primer 5'-AAGAGTTTG ATC CTG GCT CAG GAT T-3' and P7 (23S)-Reverse primer 5'-CGTCCTTCATCGGCTCTT-3'. PCR master mix was prepared with total of 49 µl reaction. PCR Reaction Buffer, 10X without $MgCl_2$ 5µl; $MgCl_2$ 25mM, 2.5 µl; dNTPs 10mM each, 4µl; Primer-forward and -reverse 10mM each, 1.0µl each; Double distilled sterile water, 35.25µl and Tag DNA polymerase, 5 Unit μl^{-1}. PCR programmes for amplification of DNA is 94°C for 4min for initial denaturation, 45 sec for denaturation; 58°C for 1 min for annealing; 72°C for 2 min for extension and 10 min for final extension for total of 30 cycles. Amplified products were separated by 1% agarose gel alongside 1kb plus DNA ladder as molecular weight marker. Electrophoresis was done at 70V for initial 10 min and then at 70V for 2 hr. The buffer used was 1xTAE (pH 8.0). The DNA bands in the gel were visualized on a UV transilluminator and documented using a gel documentation system.

Mechanical transmission

The standard extract was prepared by macerating the infected plant material in a pre chilled pestle and mortar by adding 0.1M phosphate buffer (pH 7.0), containing 1% of 2-mercaptoethanol. The sap was then clarified by straining two fold muslin cloth and inoculated with cotton swab dipped in extract inoculums to the first true leaf of test hosts (*Glycine max*, *Solanum lycopersicum*, *Vignaunguiculata*, *Nicotiana tabaccum*, *Parthenium hysterophorus* and *Datura stramonium*) by previously dusted carborandum 600 mesh as an abrassive. Immediately after inoculation, the leaves were washed thoroughly with tap water to remove excess of inoculum and abrassive.

For each test plant, healthy seedlings were also maintain to compare the symptom i.e. control. All the inoculated plants were maintained in an insect proof cage house with proper labelling till the development of symptoms. Inoculated plants were confirmed by PCR.

Results and Discussion

Survey of disease and symptomological study

Soybean is not only attacked by phytoplasmas but also by different viruses and leads to severe yield losses [Fig-1]. Field survey for soybean phytoplasma from different locations of Vidarbha region during the year 2012-2014, the observed symptoms under field conditions were phyllody, stunting growth, witches;-broom and leaf like structure of flower parts with deformed or no pod formation at all [Fig-2a]. The characteristic symptom of soybean phyllody is the transformation of the floral calyx and corolla into leaf-like structures. Phytoplasmas occurring in severe form during the *kharif* season and causing economic loss to the growers of this region. Similar symptoms as above were observed and reported from different region of Maharashtra [7]. Plant height of symptomatic plant is shorter than the asymptomatic plants. Similar symptoms of phyllody, stunting growth and witches'-broom on Indian fig, soybean, mungbean and fababean were found by various workers [8-11].

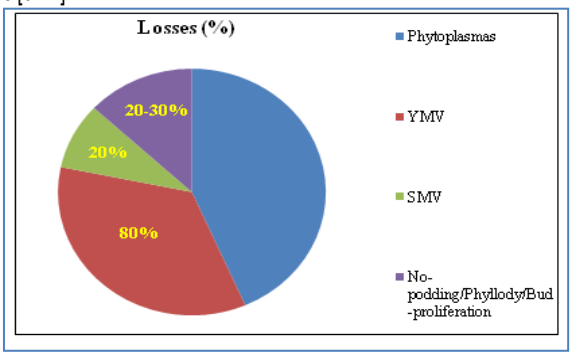
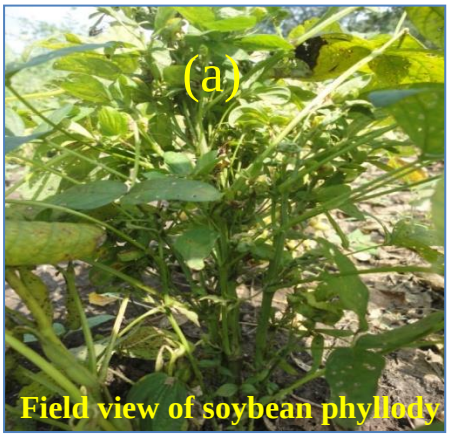


Fig-1 Yield losses (%) caused by different viruses including phytoplasma on soybean (7, 3, 19, 20)

The leaves of symptomatic plants were deformed, dark green in colour and reduced in size ('little leaf') [Fig-2b]. Both bud proliferation and reduced pod set minimizes yield on symptomatic plants. The symptomatic plant produced thicker stem and shorter internodes as compared to the asymptomatic plants [Fig-2c].



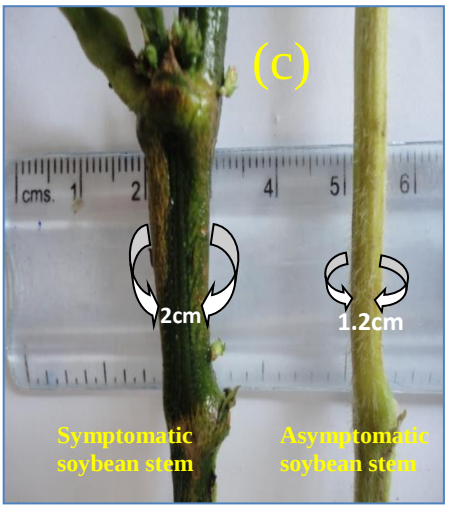


Fig-2 Phytoplasma infection on soybean (a) Field view of soybean witches'-broom or phyllody; b) Bushy infected and healthy soybean plants; (c) Dark green and thicker stem of symptomatic plant as compared with asymptomatic plant

Mechanical transmission

The result showed that the phytoplasma was not sap transmissible. None of the test plants inoculated by infected sap developed any symptoms even up to 120 days after inoculation. PCR detection also showed negative result.

The present results were in agreement with the results obtained by Lee et al. [13] and Kumar [10], where they did not found symptoms even after 120 days after inoculation. Coffee crispiness disease caused by phytoplasma failed to develop symptoms after mechanical inoculation of young plants with extracts derived from diseased plant tissues [12].

Detection of phytoplasma by PCR assays

PCR was found to be the most sensitive method for detection of phytoplasmas and viruses of soybean. Soybean phytoplasma detected by universal primer sets (P1/P7) showed positive detection with PCR amplification product of 1.8kb size [Fig-3].

Earlier reported similar result by using phytoplasma specific primers (P1/P7) and produce a PCR product of 1.8kb on aster yellows, *Catharanthus*, *Callistephus chinensis*, *Calosia argentea* and Periwinkle [13-17, 3, 11].

Conclusion

Phytoplasma associated with soybean phyllody was detected and confirmed by using phytoplasma 16S rDNA primer pairs. Phytoplasma was not sap transmissible.

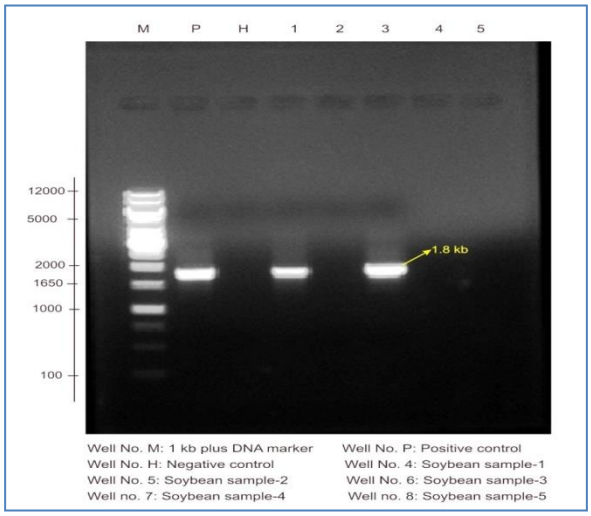


Fig-3 Soybean phytoplasma PCR amplification product of 1.8kb length by using primer pair P1/P7

Acknowledgement

Authors thank Dr. S. S. Mane, Head, Dept. of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra for providing necessary facilities and supporting this research throughout the course of investigation.

Author Contributions: All author equally contributed for this present investigation to identify and characterize phytoplasma associated with soybean witches' broom.

Abbreviations:

DNA: Deoxyribonucleic acid
dNTPs: Deoxynucleotide Triphosphates
EDTA: Ethylenediaminetetraacetic acid
kb: Kilobase
M: Molar
$MgCl_2$: Magnesium chloride
mM: Millimolar
PCR: Polymerase Chain Reaction
TAE buffer: Tris base acetic acid and EDTA
µl: Microlitre
%: Percentage

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest: None declared

References

[1] Doi Y.M., Teranaka M., Yora K. and Asuyama H. (1967) *Ann. Phytopathology Soc. Jpn.,* 33, 259-266.
[2] Marcone C. (2010) Movement of phytoplasmas and the development of disease in the plant. In: Jones P, Weintraub P. (Eds.). Phytoplasmas: Genomes, Plant Hosts and Vectors .Wallingford: CABI Publishing; p. 114-131.
[3] Kumar S. (2010) Studies on phytoplasma disease of periwinkle [*Catharanthusroseus*(L.) G. Don.], M.Sc. Thesis, Uni. Agril. Sci., Dharwad, India.
[4] McCoy R.E., Caudwell A., Chang C.J., Chen T.A. and Chiykowski L.N. (1989) Plant diseases associated with mycoplasma like organisms. In: Whitcomb RF, Tully JG. (Eds.). The Mycoplasmas New York, Academic Press; p. 545-60.
[5] Henson J.M. and French R.C. (1993) *Annu. Rev. Phytopathol.,* 31, 81-109.
[6] Deng S. and Hiruki C. (1991) *Proceedings of the Japan Academy Series B,* 66, 140-144.
[7] Jadhav P.V., Mane S.S., Nandanwar R.S., Kale P.B., Dudhare M.S., Moharil M.P. and Dani R.G. (2013) *Egypt. J. Biol.,* 15, 59-65.
[8] Hernandez-Perez R., Noa-Carrazana J.C., Gaspar R., Mata P. and Flores-Estevez N. (2009)*J. Biol Sci.,* 9(3), 62-66.
[9] Kumar S., Sharma P., Sharma S. and Rao G.P. (2014)*J. Phytopathol.,* 163(5), 395-406.
[10] Ragimekula N., Chittem K., Nagabudi V.N. and del Rio Mendoza L.E. (2014) *Plant Dis.,* 98, 1424.2-1424.2.
[11] Al-Saleh M.A. andAmer M.A. (2014) *The J. Anim. & Plant Sci.,* 24(1), 221-228.
[12] Lee I.M., Gundersen-Rindal D.E., Davis R.E., Bottner K.D., Marcone C. andSeemuller E. (2004) *Int. J. Syst. Evol. Microbiol.,* 54, 1037-1048.
[13] Galvis C.A., Leguizamon J.E., Gaitan A.L., Mejia J.F., Alvarez E. and Arroyave J. (2007) *Plant Dis.,* 91, 248-252.
[14] Torres L., Galdeano E., Docampo D. and Conci L. (2004)*J. Plant Pathol.,* 86(3), 209-214.
[15] Navalinskiene M., Samuitiene M. and Jomantiene R. (2005) *Phytopathol.,* 35, 109-112.
[16] Samuitiene M. and Navalinskiene M. (2006) *Agron Res.,* 4, 345-348.
[17] Sertkaya G., Martini M., Musetti R. and Osler R. 2007) *Bull Insectol.,* 60(2), 141-142.
[18] Chen W.Y., Huang Y.C., Tsai M.L. and Lin C.P. (2011) *Australas. Plant Pathol.,* doi:10.1007/s13313-011-0062-x.
[19] Nene Y.L. (1972) *India J. Re. Bull.,* 4,191.
[20] Ross J.P. (1977) *Crop Sci.,* 17, 869-872.