Journal of Biotechnology and Crop Science
Vol. 10, No. 15, January-December, 2022, 54-60

doi: 10.5958/2582-5089.2022.00007.X

Research Article

Molecular diagnosis of '*Candidatus* Phytoplasma australasia' related strain (16SrII-D) associated with flat shoot and shoot proliferation of radish in Uttar Pradesh, India

Noopur Singh, Durgesh Kumar Dubey, Sushil Kumar Singh and Govind P. Rao

ABSTRACT

Radish is an important vegetable crop used in our daily dishes as salad, vegetable and other common cuisines. In the present study, phytoplasma associated with the flat shoot and shoot proliferation son radish plants grown at Narendra Dev University of Agriculture & Technology, Ayodhya campus, India were attempted. The DNA was extracted from diseased and healthy shoots and subjected to nested PCR assays with P1/P7 and R16F2n/R16R2 phytoplasma specific primer pairs. Amplification of 1250 bp product was achieved in all the symptomatic radish samples. The amplified product was cloned and directly sequenced. Sequence analysis of *16Sr RNA* gene through BLASTn, phylogeny and virtual RFLP comparison confirmed radish isolate as *Ca.* P. Australasia related strain (16SrII-D sub group). To our best knowledge, this is the first report of the 16Sr II-D subgroup phytoplasma associated with radish in India.

Keywords: *Candidatus*, diagnosis, molecular, phytoplasma, proliferation, radish

INTRODUCTION

Phytoplasmas are cell-wall less bacteria with very small genome size and are amongst the smallest self-replicating living organisms (Bertaccini et al., 2022). They infect numerous vegetable crops including cucurbitaceous crops and cause significant crop losses, and are transmitted by sap-feeding insects (Kumari et al., 2019; Rao, 2021). *Raphanus sativus* L. (radish) belongs to family *Brassicaceae* and it is originated from the Central and Western China and India (Thamburaj & Singh, 2005). Apart from Uttar Pradesh Haryana, West Bengal, Punjab, Assam Chhattisgarh, Bihar, Madhya Pradesh are the major radish growing states. In year 2021-2022, the total production was 33.47 million tonnes in 209 million hectare area grown in India (National Horticulture Board and Ministry of Agricultural, 2022). Radish is used as vegetable and its younger tuberous roots and young leaves are consumed raw as salad but it can also be cooked or salted together with other vegetables (Nishio, 2017). *Raphanus sativus* is a good source of nutrients like proteins, glucosinolates, flavonoids, β-carotene, vitamin B6, vitamin C, folate, minerals like calcium, magnesium, copper, manganese potassium, and low in calories (Gamba et al., 2021; Gupta et al., 2003). It has refreshing and depurative and also

Noopur Singh[1], Durgesh Kumar Dubey[2], Sushil Kumar Singh[3] and Govind P. Rao[4](✉)

[1]Centre of Genomics and Bioinformatics, Institute of Agriculture and Natural Sciences, DDU Gorakhpur University, Gorakhpur-273009, Uttar Pradesh, India

[2]Department of Plant Pathology, Institute of Agriculture& Natural Sciences, DDU Gorakhpur University, Gorakhpur-273009, Uttar Pradesh, India

[3]Department of Plant Pathology, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya-224229, Uttar Pradesh, India

[4]Institute of Agriculture and Natural Sciences, DDU Gorakhpur University, Gorakhpur-273009, Uttar Pradesh, India
Email: gpraossrp@gmail.com

medicinal properties (Dongarwar et al., 2018). Radish roots are considered to be good for patients suffering from liver trouble, gallbladder ailments, hemorrhoids (piles), jaundice, and enlarged spleen (Swaamy, 2023). In traditional medicine, radishes are used to treat coughs, cancer, whooping cough, constipation, dyspepsia, arthritis, and kidney stones (Adams, 2008). Radish production is hampered by many pathogens including fungi, bacteria, virus and phytoplasma. A few of the important diseases of radish are Alternaria blight, clubroot, downy mildew, wilt, damping-off, white rust, black root, mosaic and phyllody (Selvakumar et al., 2019). Very limited information are available on phytoplasma disease association on radish from India.

Till now there are few incidences known on phytoplasma disease associated with radish from India and abroad. In radish, 16SrII group phytoplasma was identified associated with leaf yellowing, mosaic, crinkling, and small leaves symptoms from Cuba and Spain (Arocha et al., 2008; Alfaro-Fernández et al., 2011). Due to phytoplasma disease, 5-20% yield losses were also recorded in radish in India (Selvakumar et al., 2019). Recently, '*Candidatus* Phytoplasma Australasia' related strain (16SrII-D subgroup) was identified in radish from Andhra Pradesh in South India with symptoms of little leaf and proliferation of axillary buds (Dungavath et al., 2021). Many phytoplasma strains have been reported on vegetable crops, viz. cucurbits, tomato, leafy vegetables, chilli, brinjal, cowpea etc. from Uttar Pradesh which is reported to cause severe losses (Rao, 2021). But, there is no report available of any phytoplasma strain in radish from Uttar Pradesh. In 2020, radish plants showing flat shoot and shoot proliferation symptoms were observed in agricultural fields of ND University of Agriculture & Technology campus, Ayodhya, Uttar Pradesh, India. Hence an investigation was done to detect the phytoplasma strain associated with flat stem and shoot malformation disease of radish from Uttar Pradesh.

MATERIALS AND METHODS

Shoot samples from symptomatic and non-symptomatic radish plants were collected from NDUAT, Ayodhya campus, Uttar Pradesh. The total DNA was extracted from symptomatic (3 samples) and healthy shoot tissues by using modified CTAB method (Ahrens & Seemuller, 1992). The extracted DNA was subjected to nested PCR assay for amplification of 16Sr RNA gene of phytoplasmas. The DNA used as a template in first round PCR for amplification with P1/P7 primers (Deng & Hiruki, 1991; Smart et al., 1996) followed by nested PCR using 2 µl of diluted standard PCR product with phytoplasma specific primers R16F2n/R16R2 (Gundersen and Lee.1996). The nested PCR products were analysed in electrophoresis using 1.0% (w/v) agarose gel, and DNA fragments were documented using gel documentation system (Alpha Innotech, USA). The PCR amplified 1250bp DNA fragment from gel slices was extracted using the QIA quick columns (Qiagen, Germany) kit as per the manufacturer's protocol. Cloning and sequencing analyses of 1.25 kb R16f2n/R2 primed PCR products of the radish phytoplasma strain was done and used for further analyses.

The extracted 1250 bp product was sequenced at automated DNA sequencing facility (Eurofin Genomics India Pvt. Ltd., Bangalore). Sequence assembling, nucleotide alignments and percent identity matrix were done with BioEdit version 7.0 software (Hall, 1999). The sequences were compared with 16S rDNA sequences of phytoplasmas in GenBank using Blast from the NCBI database (http://www.ncbi.nlm.nih.gov).

Sequence phylograms were constructed from aligned sequences using neighbor-joining method and boot strap option using Mega 7.0 software (Tamura et al., 2007). The other references downloaded from NCBI GenBank database (http://www.ncbi.org) was used for sequence analysis of the radish phytoplasma strain in the present

study. The 16S rDNA virtual RFLP patterns of radish phytoplasma isolates were analyzed and compared to that of other phytoplasmas using *i*Phy Classifier (Zhao et al., 2009).

RESULTS AND DISCUSSION

The phytoplasma typical disease symptoms of flat shoot and shoot proliferation were observed on radish plants (Figure 1) cultivated in the campus of Narendra Dev University of Agriculture & Technology, Ayodhya, and Uttar Pradesh. Amplification of 1800 bp and 1250 bp products were obtained in all the three symptomatic radish samples but not in the healthy collected samples in nested PCR assays with P1/P7 and R16F2n/R16R2 primers (Figure 2).

The 1250 bp amplicons were eluted from 1% agarose gel and sequenced directly. The partial 16Sr RNA gene sequences of radish isolate was aligned with other phytoplasma species (downloaded from NCBI GenBank) using BioEdit software to confirm the identity of phytoplasma species associated with radish flat shoot and shoot proliferation. The NCBI nucleotide blast analysis revealed that 16Sr RNA gene sequences of present radish isolates shared 99.84% similarity with '*Candidatus* Phytoplasma Australasia' related strains. All the three radish isolate sequences were found 100%

Figure 1: Radish plants flat shoot and shoot mal formation ssymp to msat Ayodhya

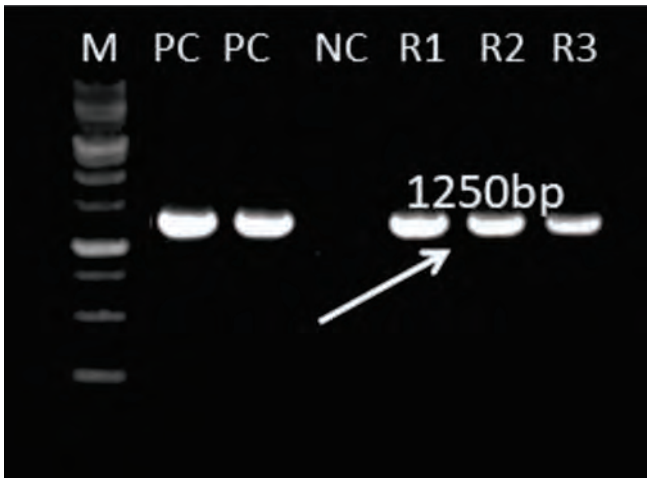

Figure 2: Electrophoresis of nested PCR products in 1.0% agarose gel showing ~1.2 kb bands in symptomatic radish samples (lanes R1, R2, R3) as compared to positive control, C*atharanthus roseus* little leaf strain (lane PC) and a negative control, healthy asymptomatic radish plant (lane NC). M= Lambda DNA digested with *Eco*RI and *Hind* III (Genei Pvt Ltd, Bangalore, India) DNA marker (lane M).

identical among themselves, and hence one representative sequence was submitted to NCBI database using online submission portal with accession number OQ78079. The phylogenetic analysis also confirmed its close relationship of radish phytoplasma strain with peanut witches' broom (16SrII-D) related strains (Figure 3). Virtual RFLP analysis results derived from in silico digestions of the R16F2n/R2 sequence of *16S rRNA* gene indicated that radish phytoplasma strain produced similar virtual RFLP profiles identical to '*Ca*. P. Australasia', representative of 16SrII-D subgroup (Y10097-papaya yellow crinkle disease). This confirms the identity of radish shoot proliferation phytoplasma strain as member strain of peanut witches' broom group (16SrII-D subgroup) (Figure 4).

Phytoplasma diseases are emerging threat to vegetables crops in India and abroad and induce serious yield losses in cases of early infection (Kumari et al., 2019; Rao, 2021). Phytoplasma belonging to the 16SrII-D subgroup was found as the most widely distributed phytoplasma strain on other vegetables and legume crops in India

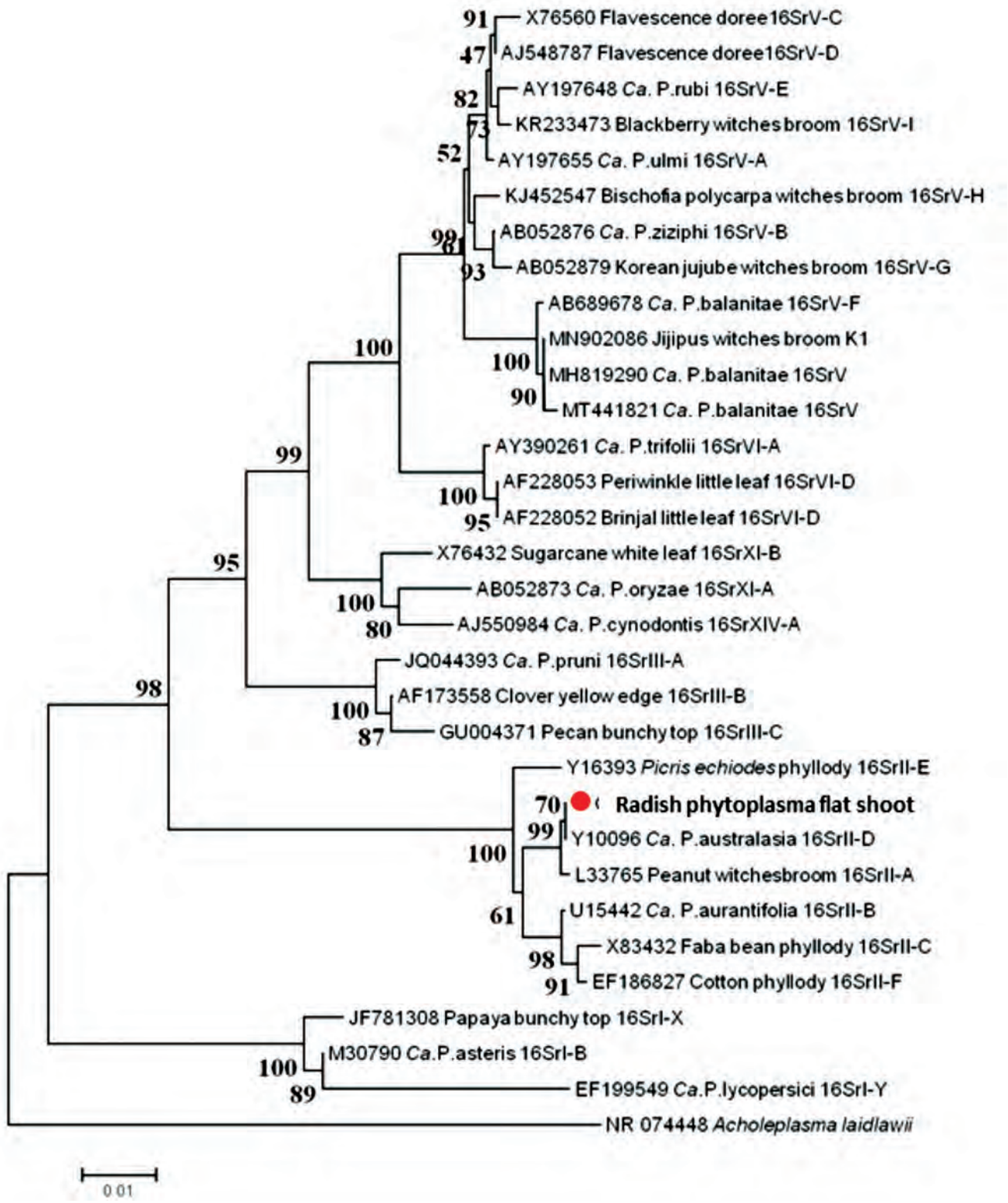

Figure 3: Phylogenetic tree constructed by neighbor-joining method showing the relationships between the radish flat shoot and shoot proliferation isolate (red circle) and other phytoplasmas strains on the of 16S rRNA gene sequence. *Acholeplasma laidlawii* was used as out group.

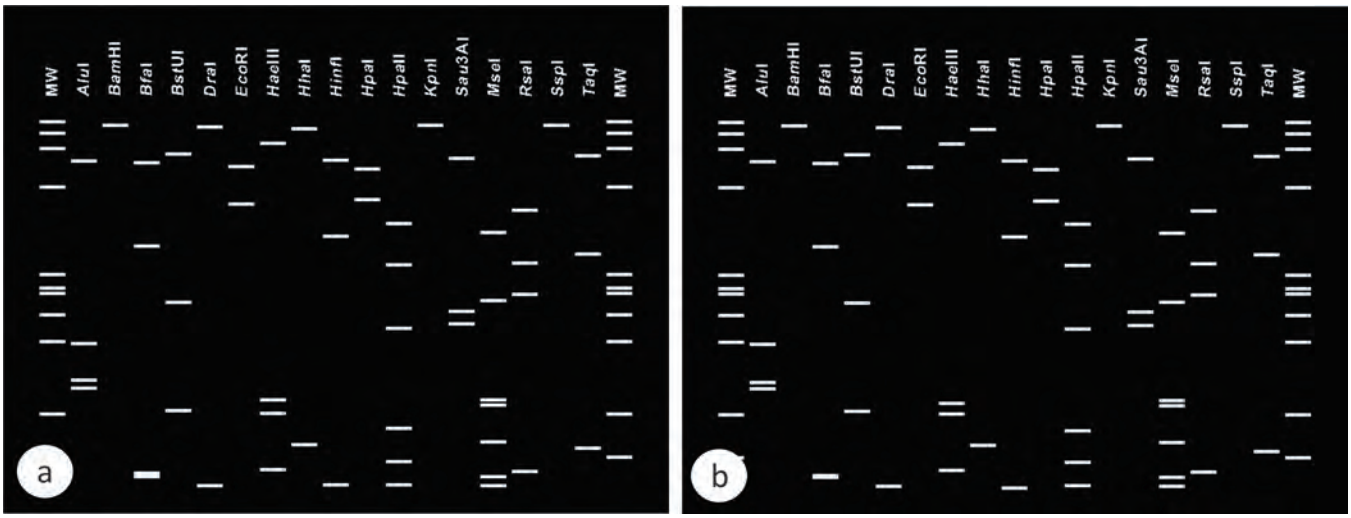

Figure 4: Comparison of virtual RFLP pattern derived from *in silico* digestion of 16SrRNA sequences of radish phytoplasma strain with 17 different restriction endonucleases using iphyClassifier programme a) 16SrII-D reference strain (Acc. No. Y10096), b) Radish flat shoot and shoot proliferation isolateusing the *i*PhyClassifier

(Rao, 2021). Besides, the 16SrII-D subgroup phytoplasma has also been reported widely in several weed species in India and several leafhoppers are reported as natural vectors for transmission of '*Ca.* P. Australasia' related strains in India (Reddy et al., 2021; Rao, 2021). Earlier, association of '*Ca*. Phytoplasma Australasia' and '*Ca*.P. asteris' and '*Ca*. P. solani' related strains were reported on radish crops from Cuba, Spain and south India (Arocha et al., 2008; Alfaro-Fernández et al., 2011; Dungavath et al., 2021). In the present study, the presence of 16SrII-D subgroup was identified based on sequence comparison, phylogeny and RFLP analysis of 16S rDNA sequences. This is the first report of the association of 16SrII-D phytoplasma subgroup with flat shoot and shoot proliferation disease in radish from Uttar Pradesh. Earlier also pedant witches broom phytoplasma group has been reported on several crops from Ayodhya, Uttar Pradesh (Rao et al., 2017). This indicates that 16SrII-D subgroup phytoplasma is wide spread and prevalent to diverse crop species in Ayodhya. This increasing wide spread incidence of peanut witches' broom phytoplasma strain strongly suggests to study the sources and other epidemiological factors involved in natural spread of the disease for a suitable management approach. It needs immediate attention towards planning further studies on epidemiology, and management of the disease.

REFERENCES

Adam, M. 2008. Radish (online) available http://www. Heading food reference .com/com/radish.html.

Ahrens, U., & Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology*, *82*, 828-832.

Alfaro-Fernández, A., Del Carmen Cebrián, M., Villaescusa, F.J., & Font-San-Ambrosio, M. (2011). Detection and identification of aster yellows and "stolbur" phytoplasmas in various crops in Spain. *Bulletin of Insectology*, *64*(Supplement), S63-S64.

Arocha, Y., Pinol, B., Almeida, R., Acosta, K., Quinones, M., Zayas, T., Varela, M., Marrero, Y., Boa, E., & Lucas, J.A. (2008). First report of phytoplasmas affecting organoponic crops in central and eastern Cuba. *New Disease Reports*, *18*, 24.

Bertaccini, A., Arocha-Rosete, Y., Contaldo, N., Duduk, B., Fiore, N., Montano, H.G., Kube, M., Kuo, C.H., Martini, M., Oshima, K., Quaglino, F., Schneider, B., Wei, W., & Zamorano, A. (2022). Revision of the

'*Candidatus* Phytoplasma' species description guidelines. *International Journal of Systematic and Evolutionary Microbiology, 72*(4).

Deng, S., & Hiruki, C. (1991). Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *Journal of Microbiological Methods, 14*, 53-61.

Dongarwar, L.N., Kashiwar, S.R., Ghawade, S.M., & Dongarwar, U.R. (2018). Varietal Performance of Radish (*Raphanus sativus* L.) Varieties in Black Soils of Vidharbha-Maharashtra, India. *International Journal of Current Microbiology and Applied Sciences, 7*(1), 491-501.

Dungavath, V.K.N., Bhaskara Reddy, B.V., Lakkireddy, P., & Gurivi Reddy, M. (2021). Molecular identification of a '*Candidatus* Phytoplasma Australasia' strain in radish showing a little leaf disease in Andhra Pradesh, India. *Phytopathogenic Mollicutes, 11*(2), 147-150.

Flores, D., Amaral Mello, A.P.O., Massola Junior, N.S., & Bedendo, I.P. (2013). First report of a group 16SrVII-C phytoplasma associated with shoot proliferation of sunn hemp (*Crotalaria juncea*) in Brazil. *Plant Disease Notes, 97*, 12.

Gamba, M., Asllanaj, E., Raguindin, P.F., Glisic, M., Oscar, H., Franco, Minder, B., Bussler, W., Metzger, B., Kern, H., & Muka, T. (2021). Nutritional and phytochemical characterization of radish (*Raphanus sativus*): A systematic review, *Trends in Food Science & Technology, 113*, 205-218.

Gundersen, D.E., & Lee, M.I. (1996). Ultrasensitive detection of phytoplasmas by nested PCR assays using two universal primer pairs. *Phytopathologia Mediaterranea, 35*, 144-151.

Gupta, K., Talwar, G., Jain, V., Dhawan K., & Jain, S. (2003). Salad Crops Root, Bulb, and Tuber Crops. In: Encyclopedia of Food Sciences and Nutrition (Second Edition).

Hall, T.H. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposia Series, 41*, 95-98.

Jyothi, G., Chandrakala, A., Chetan, S., Janawad Rangaswamy, K.T., Shruthi, T.B., & Ravikumara, B.M. (2012). Detection of phytoplasma associated with *Crotalaria* phyllody disease. *Global Journal of Biology, Agriculture and Health Sciences, 1*(2), 28-32.

Kumari, S., Nagendran, K., Rai, A.B., Singh, B., Rao, G.P., & Bertaccini, A. (2019). Global status of phytoplasma diseases in vegetable crops. *Frontiers in Microbiology, 10*, 1349.

Mitra, S., Kumar, M., Vemana, K., Saratbabu, K., Johnson, A.M., Mishra, S., Marcone, C., & Rao, G.P. (2021). Multilocus sequence analysis of a '*Candidatus* Phytoplasma Australasia'-related strain associated with peanut little leaf disease in India. *Journal of Plant Pathology, 103*(1), 311-316.

Murray, M.G., & Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research 8*, 4321-4326.

Nishio, T. (2017). Economic and academic importance of radish. In: H.K. Takeshi Nishio (Ed.), The radish genome (pp. 1–10). Springer.

Rao, G.P. (2021). Our understanding about phytoplasma research scenario in India. *Indian Phytopathology, 74*, 371–401.

Rao, G.P., & Aiyanathan, K. (2015). '*Candidatus* Phytoplasma asteris'-and '*Ca*. P. aurantifolia'-related strains associated with phyllody and witches' broom disease of sunn hemp (*Crotalaria juncea* L.) in India. *Phytopathogenic Mollicutes, 5*(2), 117-119.

Rao, G.P., Thorat, V., Manimekalai, R., Tiwari, A.K., & Yadav, A. (2017). A century progress of research on phytoplasma diseases in India. *Phytopathogenic Mollicutes, 7*(1), pp.1-38.

Reddy, M.G., Baranwal, V.K., Sagar, D., & Rao, G.P. (2021a). Molecular characterization of chickpea chlorotic dwarf virus and peanut witches' broom phytoplasma associated with chickpea stunt disease and identification of new host crops and leafhopper vectors in India. *3 Biotech, 11*(3), 1-23.

Selvakumar, R., Singh, D.B., Nabi, S.U., Malik, G., Sharma, A., & Gangadhara, K. (2019). Scientific Management of Pests and Diseases in Radish Cultivation. *Indian Farmer, 6*(3), 198-202.

Smart, C.D., Schneider, B., Blomquist, C.L., Guerra, L.J., Harrison, N.A., Ahrens, U., Lorenz, K.H., Seemuller, E., & Kirkpatrick, B.C. (1996). Phytoplasma specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Applied and Environmental Microbiology, 62*(8), 2988-2993.

Swaamy, K.R.M. (2023). Origin distribution genetic diversity and breeding of Rasdish (*Raphanus sativus* L.). *International Journal of Development Research, 13*(2), 61657-61673.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24*, 1596-1599.

Thamburaj, S., & Singh, N. (2005). Vegetables Tuber Crops and Spices. New Delhi: Indian council of Agriculture Research. pp 40.

Win, N.K.K., Jung, H.Y., & Ohnga, S. (2011). Characterization of sunn hemp witches' broom phytoplasma in Myanmar. *Journal-Faculty of Agriculture Kyushu University, 56*(2), 217-221.

Zhao, Y., Wei, W., Lee, I-M., Shao, J., Suo, X., & Davis, R.E. (2009). Construction of an interactive online phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *International Journal of Systematic and Evolutionary Microbiology, 59*, 2582-2593.