Original Research Article

Antioxidant responses of three Pepper (*Capsicum annuum*) varieties against *Pepper Venal Mottle Virus*

ABSTRACT

Aims: This study aimed to investigate the changes in antioxidant activity and protein content between non-infected and infected leaves of three Capsicum annum varieties against Pepper Venal Mottle Virus.

Material and methods: PVMV isolated from infected pepper plants was inoculated to three healthy varieties of pepper (Pepper Narval, Yolo Wonder and Chili pepper) at an early stage of the growing period. Control peppers of each variety were treated in the same way with distilled water. The infection of inoculated plants was confirmed by the ELISA method. Control and infected leaves were collected three weeks after inoculation and used for biochemical analyses. Change in different biochemical parameters (catalase, superoxide dismutase, MDA and protein) in infected pepper plant was observed compared to control non-infected ones.

Results: CAT and SOD activities were increased in Pepper Narval and Pepper Yolo Wonder infected leaves compared to non-infected, while a significant decrease was observed in infected Chili pepper compared to control. Higher MDA content was found in Pepper Yolo Wonder and Chili pepper infected leaves ($P < 0.05$) than control while a non-significant difference was shown between the infected and non-infected of Pepper Narval variety ($P > 0.05$). Infected Chili pepper showed high protein content compared to control ($P < 0.05$). An opposite trend was observed in pepper Narval and Yolo Wonder varieties ($P < 0.05$).

Conclusion: The results of this study showed that PVMV infection induces changes in enzymes, malondialdehyde and protein levels. These biochemical components were greatly expressed differentially between PVMV infected and non-infected in Pepper Yolo Wonder variety. Further studies with more biochemical parameters may contribute to improve the pepper tolerance mechanism to PVMV in a breeding program.

Keywords: Pepper; PVMV; catalase; superoxide dismutase; malondialdehyde; protein.

1. INTRODUCTION

Pepper (*Capsicum* spp.), including sweet pepper and hot pepper, is an important spice and vegetable crops worldwide [1]. Peppers belong to the Solanaceae family, genus *Capsicum* (Greenleaf, 1986). Among the five domesticated species of the genus *Capsicum* (*C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum*, *C. pubescens*), *C. annuum* is the most widely grown in Africa (Grubben and El Tahir, 2004). In Burkina Faso, peppers are an important source of income for many small farmers. *C. annum* crop is grown in open fields

on an estimated area of 1639 hectares with a production of 8230 tons/year [4]. However, their average yields are severely affected by the presence of pests and diseases. Pepper crop is infected by several fungal, bacterial, and viral diseases.

Among viral diseases, *Pepper veinal mottle virus* is endemic and the most devastating pepper virus and other solanaceous crops in several West African countries [5]. The virus was first discovered in Ghana [6] and then in others West African countries [7,5], Ethiopia [8], and South Africa [9].

Pepper veinal mottle virus (PVMV) is a virus species in the genus Potyvirus of the family Potyviridae [6]. PVMV is transmitted by aphids in a non-persistent manner and is transmissible experimentally by mechanical inoculation. Symptoms expressed on the leaves plants infected with PVMV are characterized by chlorotic vein banding, mottling, mosaic, and distortion with puckering of leaves. Infected plants may show stunting with reduced and distorted fruit set [10]. PVMV causes significant losses for growers of solanaceous crops in several African countries [11, 12]. The incidence of the virus can reach 50 to 100%, leading to significant losses of production causing whole field to be abandoned before harvest and in some areas [13,14].

To control these pests, synthetic chemical pesticides are the most used solution. However, several studies conducted in Burkina Faso [15,16] have highlighted the existence of poor phytosanitary practices. The consequences are the intoxication of farmers and consumers, environmental pollution and the selection of strains resistant to pesticides. Considerable efforts have focused on the development of pepper varieties resistant to the virus. Early work resulted in materials that were tolerant or only partially resistant [17,18].

Another approach to select resistant plant by using biochemical parameters was developed [19,20].

Indeed, the contact of the plant with the pathogen induces biochemical reactions leading to the production of defense substances. The level of antioxidant activity and total phenolic content of peppers infected with the virus reflects the condition of resistance or susceptibility of pepper plants [21]. The identification of pepper biochemical products expressed under virus infection will be helpful to improve *Capsicum annum* tolerance mechanism to PVMV in a breeding program. This study aimed to investigate the changes in antioxidant activity and protein content of three peppers varieties against *Pepper Venal Mottle Virus* infection in order to understand the biochemical tolerance mechanism of *Capsicum annum*.

2. MATERIAL AND METHODS

2.1 Plant material and experimental dispositive

Seeds of *C. annuum* were purchased from a commercial supplier in Ouagadougou, Burkina Faso. Three varieties of peppers, pepper Narval (Na), pepper Yolo Wonder (Y) and Chili pepper were sown in pots (25 cm diameter) containing sterilized sand and peat (1:1). For each variety, two seeds were sown in pots in three replications. A control group and infected group were defined for each variety. Plants were well watered and grown in a greenhouse under insect-proof conditions all the experiment.

2.2 Inoculation of plants with *Pepper Venal Mottle Virus*

Virus isolates were obtained from the naturally PVMV infected pepper plants grown in greenhouses and propagated in pepper plants. The isolated PVMV was confirmed serologically by DAS-ELISA. The inoculum was prepared according to the method described by Dikilitas et al. [22]. Each pepper variety was then inoculated with the supernatant containing PVMV by gently rubbing on the leaves of 2-week-old seedlings [23]. Control plants were treated in the same manner using distilled water. All tests were performed in triplicate. After three weeks of inoculation, ELISA and biochemical tests were carried out.

2.3 ELISA test

Pepper leaf samples were tested for the presence of PVMV in inoculated peppers by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Leaves were ground in phosphate buffered saline with Tween 20 using a pre-chilled mortar and pestle. The extract was tested using polyclonal antisera produced by SEDIAG. Samples were considered positive when absorbance values at 405 nm (A405) were at least three times greater than the mean absorbance value of five healthy control samples [24,11,25].

2.4 Determination of antioxidant enzymes activities

2.4.1 Extraction of antioxidant enzymes

500 mg of fresh leaves were homogenized with 50 mM sodium phosphate (pH 7.8) and centrifuged at 4000 rpm during 10 minutes. The supernatant was used to measure superoxide dismutase and catalase enzymes activities.

2.4.2 Superoxide Dismutase (SOD) enzyme activity

SOD activity was measured using the method described by Ranjitha and Vijiyalakshmi [26] at 420 nm. The enzyme activity was expressed in terms of µmol/g protein.

2.4.3 Catalase (CAT) enzyme activity

CAT activity was measured using the method described by Ranjitha and Vijiyalakshmi [26]. The absorbances were measured at 240 nm for each interval of 30 seconds during 3 minutes. The CAT activity was expressed in terms of µmol of H_2O_2 consumed/g protein.

2.5 Lipid peroxidation assay

The Malondialdehyde (MDA) content as the marker of lipid peroxidation was determined as described by Mahi et al. [27]. The MDA content of samples was expressed in micromole per milligram (µmol.mg-1) of leaves fresh weight.

2.6 Protein content

500 mg of leaves were homogenized in 5 ml of 0.1 M NaCl. The samples were centrifuged at 4400 rpm during 30 min, and the supernatant was used to determine the protein content. Protein concentration was determined by Bradford method as described by Mimouni et al. [28].

2.7 Statistical analysis

The results are presented as mean ± SD for triplicate analysis and were subjected to one-way analysis of ANOVA variation with Tukey's Significant Difference test and $p < 0.05$ was considered significant. The statistical analysis was performed using XLSTAT Version Pro-2017 and the graphs were drawn using Graph Pad Prism software version 5.0.

3. RESULTS

3.1 Pathogenicity test

The different varieties of pepper inoculated by PVMV showed more or less severe symptoms. Inoculated Chili pepper developed disease symptoms 2 weeks after inoculation and developed severe symptoms such as chlorotic vein banding, mottling, mosaic, and

distortion. However, the inoculated Pepper Na and Pepper Y varieties developed slight symptoms of chlorotic vein banding on some leaves three weeks after inoculation. These observations were confirmed by ELISA-positive result for PVMV. On the contrary, no symptoms were observed in control peppers plant and confirmed by ELISA-negative result for PVMV. The results of the pathogenicity test are presented in Fig. 1.

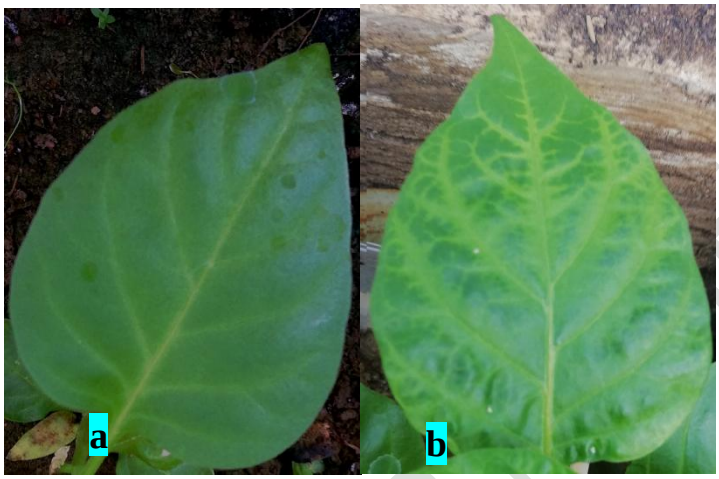
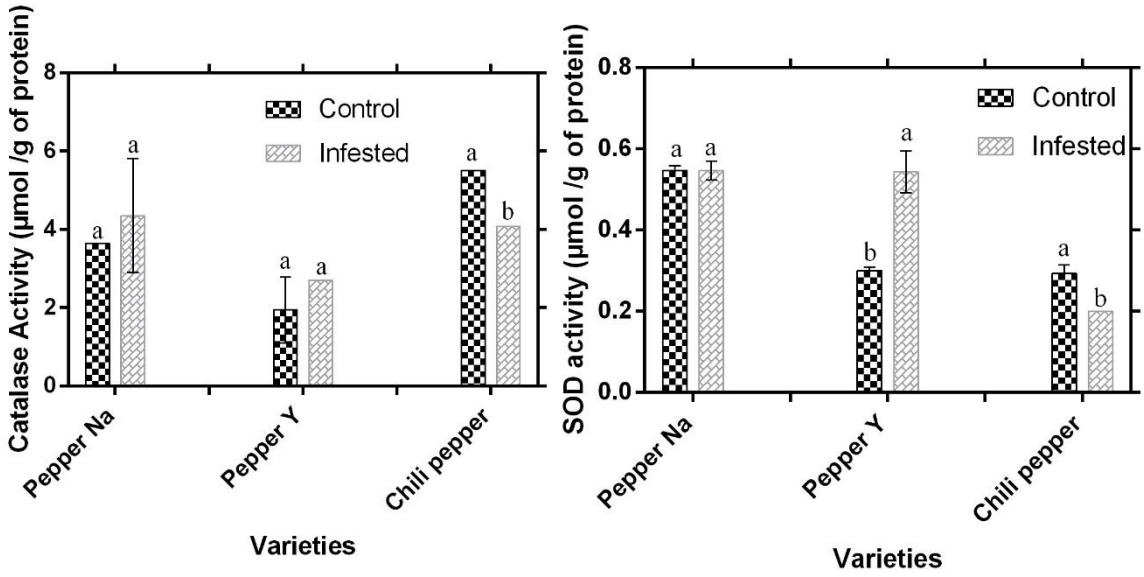


Fig. 1. Pepper leaf structures (a) healthy, (b) infected

3.2 Enzymes antioxidant activities of non-infected and infected pepper varieties

Catalase (CAT) and superoxide dismutase (SOD) activities of control and PVMV-infected peppers are shown in Fig. 2. The activities of these enzymes were increased non-significantly in infected pepper Na variety compared to control ones. The activity of catalase enzyme increased non-significantly while the SOD enzyme activity increased significantly ($P < 0.05$) in infected pepper Y as compared to non-infected. A significant decrease ($P < 0.05$) in catalase and superoxide activities was observed in Chili pepper variety.



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130 **Fig. 2. Enzymes antioxidant activities of non-infected and PVMV-infected varieties of**
131 **pepper**

132 **3.3 Lipid peroxidation of non-infected and infected pepper varieties**

133 The MalonDiAldehyde (MDA) content of control and PVMV-infected peppers is shown in Fig.
134 3. The results showed non-significant decrease of MDA content only in infected Pepper Na
135 variety compared with control ones. On the contrary, the MDA content of Pepper Y and Chili
136 pepper varieties infected with PVMV significantly increased ($P < 0.05$) when compared to
137 control ones.

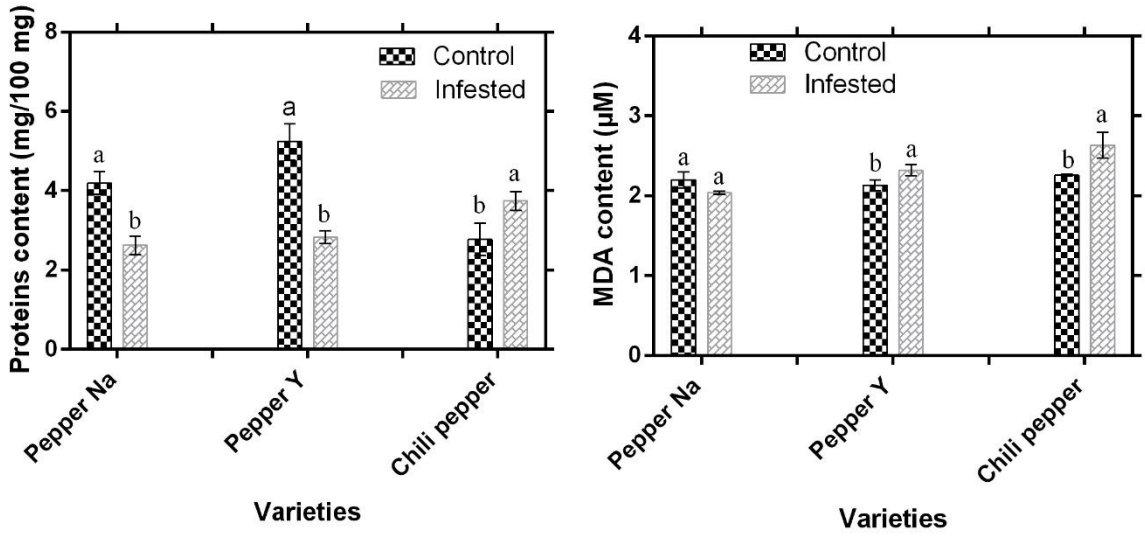

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Fig. 3. Protein and MDA contents of non-infected and PVMV-infected varieties of pepper

3.4 Proteins content of non-infected and infected pepper varieties

The protein content of control and PVMV-infected peppers is shown in Fig. 3. The protein content differed significantly ($P < 0.05$) among control and infected of the three varieties of pepper. The results revealed that the protein content decreased in infected pepper Na and Y. However, Chili pepper showed significantly increased in protein content due to PVMV infection compared with healthy ones.

3.5 Comparative analysis of the protein content and antioxidant response of pepper varieties to PVMV infection

The principal component analysis was performed on the basis of variations in enzyme antioxidant, MDA and proteins contents of different pepper varieties three (3) weeks after inoculation. Fig. 4 presents the repartition of different parameters evaluated in the biplot axis.

The first two principal components explained 82.96 % of the total variance. The first principal component (F1) and the second principal component (F2) account, respectively for 42.07 % and 40.88 % of the total variation. The first principal component (F1) separated Chili pepper control from Chili pepper infected in one hand and Pepper Y infected, Pepper Na infected from Pepper Y control and Pepper Na control in other hand. The F1 axis divided the control non-infected group from infected group of the different varieties of pepper. The second principal component (F2) separated Chili pepper (control and infected) from Pepper Y and Pepper Na (control and infected). The F2 axis divided the pepper in different part according to the type of pepper variety. Analysis of the correlations between the evaluated variables and factors showed a strong contribution of Pepper Y infected, Pepper Na infected and Pepper Na control to SOD while Chili pepper infected contributes strongly to MDA. Pepper Y control contributes strongly to proteins, while Chili pepper control contributes strongly to catalase. After the construction of dendrogram of the different treatments, the treatments were grouped into three main classes, I, II and III (Fig. 5). Class I comprised Pepper Na control, Pepper Na infected and Pepper Y infested. Class II comprised Chili pepper control and Chili pepper infected. Class III is constituted only of Pepper Y control.

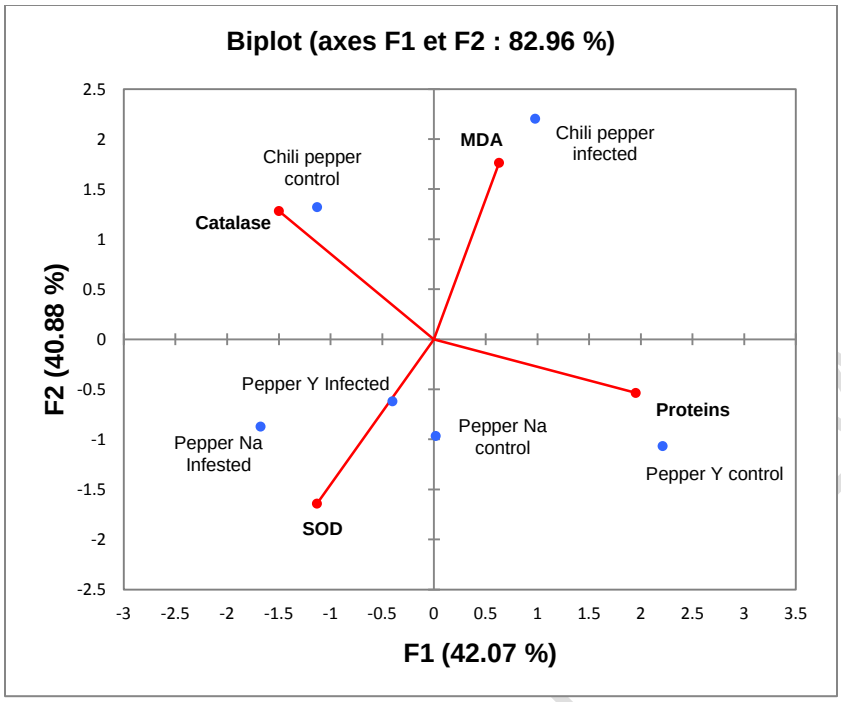

Fig. 4. Principal component analysis of the different pepper varieties responses on the protein content and antioxidant activities to PVMV infection

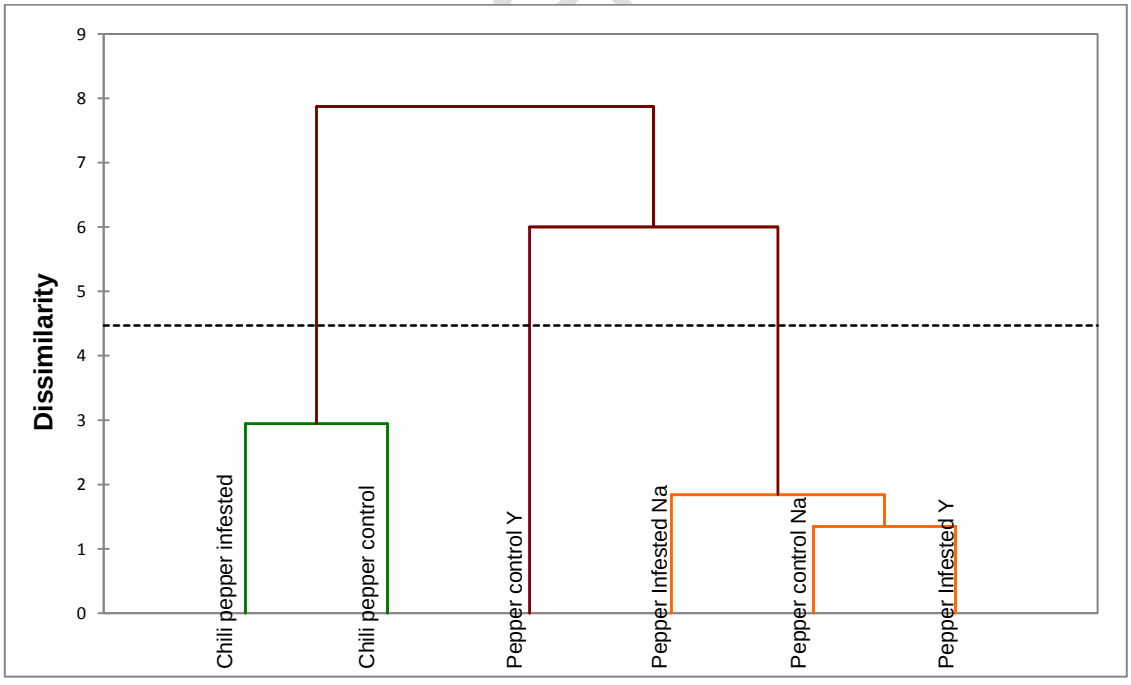

Fig. 5. Dendrogram of the different pepper varieties response based on the protein content and antioxidant activities to PVMV infection.

4. DISCUSSION

The results of the pathogenicity test showed an early development of diseases symptoms and severity in the Chili pepper variety due to PVMV infection compared to the Pepper Na and Pepper Y varieties. Besides, the ELISA tests were positive for all infested peppers. The Chili pepper showed to be more sensible to PVMV infection compared to the others varieties of pepper. The susceptibility of Chili pepper is confirmed by the decrease of catalase and superoxide dismutase activities after PVMV infection when compared to non-infected group. Only pepper Y variety showed a significant increase of SOD activity in infected group compared of non- infected group. The pepper Yolo Wonder variety is tolerant to PVMV by increasing the activity of superoxide dismutase, enzyme involved against the biotic or abiotic stress of plant. Appiah et al. [29] showed that pepper plants respond differently to a viral infection based on their susceptibility or resistance to viruses. Antioxidant enzymes (CAT, SOD) are produced by host plant to promote cells protection of oxidative damage from pathogens [30]. They induce resistance against pathogen [31]. Similar results have been reported by Siddique et al. [32]. They showed an increase of the CAT and SOD activities in the leaves of resistant varieties of cotton and a decrease in the susceptible varieties after infection with *Cotton Leaf Curl Burewala Virus*.

Generally, infected plants show a high content of protein, which could be due to both of the activation of the host defense mechanism and the pathogen attack mechanism [33]. In this study, protein content significantly decreased in infected Pepper Na and Pepper Y compared to control non-infected. An opposite trend was observed in Chili pepper plants. The increase in protein content in Chili pepper after infection may be due to viral replication which could explain it high susceptibility to PVMV. Indeed, Zinga et al. [34] showed that protein content is higher in cassava leaves infected by *African Mosaic Virus* than in healthy ones. However, other investigators have shown an increase in protein content in resistant infected varieties [32,35].

MDA is a general indicator of lipid peroxidation [36]. MDA produced during lipid peroxidation is an indicator of cellular membrane damage to the cell membrane caused by pathogenic infection [27]. Infection of Pepper Y and Chili pepper with PVMV resulted of an increase of the MDA content compared to control non-infected. Previous studies have shown that MDA content tends to increase in susceptible varieties due to infection. Lanubile et al. [37] obtained the same result with maize leaves corn infested by *Aspergillus niger*. Analysis of the principal components revealed a negative correlation between MDA and SOD. Chili pepper infected contributes strongly to MDA while infected Pepper Na and infected pepper Y contribute strongly to SOD. MDA increasing translates cellular degradation while SOD enhancement induces cellular defense mechanism [27,31]. The Pepper Na and Y varieties produce chemicals inducing resistance to PVMV than Chili pepper. Sama et al. [35] showed that the leaves of susceptible varieties of Jatropha strongly contribute to the MDA content after infection with *Lasiodiplodia theobramae*.

Combination in classes of three varieties of uninfected and infected peppers revealed a relationship between control and infected Chili pepper in class II. This closeness might be due to a weak response of the measured parameters (protein and MDA content, antioxidant enzymes activities) of this variety to the viral infection. Chili pepper presented a susceptible reaction against the PVMV. Pepper Y control and Pepper Y infected are in different classes. Likewise, Pepper Na control and infected are in the same class but in different subclasses. This may explain by the important biochemical response of Pepper Na and Y varieties due to viral infection.

In view of parameters of the oxidative enzymes (CAT, SOD) and MDA of the infected pepper varieties then the grouping into classes of the different uninfected and infected varieties, we

can conclude that Pepper Y induce resistance against PVMV infection than Pepper Na and Chili pepper.

4. CONCLUSION

This study found variations in the activity of oxidative enzymes, MDA and protein contents between the three infested and non-infested pepper varieties. SOD is involved in the biochemical defense mechanisms controlling the development of PVMV in Pepper Yolo Wonder variety. Under stressful conditions such as viral infection, stimulation of biochemical parameters plays a vital role in the defense mechanism. The results of this study suggest that the Pepper Yolo Wonder variety is more tolerant to *Pepper Venal Mottle Virus* than the Pepper Narval and Chili pepper varieties. Further studies with more biochemical parameters related to pathogenicity may contribute to improve the pepper tolerance mechanism to PVMV in a breeding program.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Lin S, Chou Y, Shieh H, Ebert WA, Kumar S, Mavlyanova R, et al. Pepper (*Capsicum* spp.) germplasm dissemination by AVRDC-The World Vegetable Center : an overview and introspection. Chronica Horticulturae. 2013;53(3):21-27.

2. Greenleaf WH. Pepper breeding. In Breeding Vegetable Crops. Westport: AVI Publishing; 1986.

3. Grubben GJH, El Tahir IM. Ressources végétales de l'Afrique Tropicale 2. Wageningen: Bakhuys Publishers; 2004.

4. FAOSTAT Database. Food and Agriculture Organization. Available: www.fao.org; 2017.

5. Konaté G, Traoré O. Caractérisation et distribution du virus de la panachure du poivron en Afrique de l'Ouest. Cah. Agric. 1999;8:132-4.

6. Brunt AA, Kenten RH. Pepper veinal mottle virus, a new member of the potato virus Y group from peppers (*Capsicum annuum* L. and *C. frutescens* L.) in Ghana. Ann. Appl. Biol. 1971;69:235-243.

7. Huguenot C, Furneaux MT, Clare J, Hamilton RI. Serodiagnosis of Pepper Veinal Mottle Virus in West Africa using specific monoclonal antibodies in DAS-ELISA. J Phytopathol. 1996;144:29-32.

8. Agranovsky AA. Virus diseases of pepper (*Capsicum annuum* L.) in Ethiopia. 1993; 138:89-97.

9. Brunt AA, Kenten RH, Phillips S. Symptomatologically distinct strains of pepper veinal mottle virus from four West African solanaceous crops. Ann App Biol. 1978; 88:115-119.

10. Green SK, Kim JS. Characteristics and control of viruses infecting peppers: a literature review. Asian Veg. Res. Dev. Cent. AVRDC Tech. Bull. 1991;18.

11. Moury B, Palloix A, Caranta C, Gognalons P, Souche S, Selassie KG, et al. Serological , molecular , and pathotype diversity of Pepper veinal mottle virus and Chili veinal mottle virus. Phytopathology. 2005;95(3):227-232.

12. Arogundade O, Balogun OS, Kareem KT. Occurrence and distribution of pepper veinal mottle virus and cucumber mosaic virus in pepper in Ibadan, Nigeria. Virol. J. 2012;9 (79):1-4.

13. Alegbejo MD, Uvah II. Effect of intercropping pepper with tall companion crops on the incidence of Pepper veinal mottle virus on pepper, Niger. J. Entomol. 1987;7:82-87.

14. Bolou Bi BA, Moury B, Abo K, Sorho F, Cherif M, G. Girardot et al. Survey of viruses infecting open-field pepper crops in Côte d'Ivoire and diversity of Pepper veinal mottle virus and Cucumber mosaic virus. Plant Pathol. 2018;67(6):1416-1425.

15. Toé AM. Étude pilote des intoxications dues aux pesticides agricoles au Burkina Faso. Secrétariat de la Convention de Rotterdam. Disponible sur : www.pic.int. 2010.

16. Naré RWA, Savadogo PW, Gnankambary Z, Nacro HB, Sedogo MP. Analyzing risks related to the use of pesticides in vegetable gardens in Burkina Faso. Agric. For. Fish. 2015;4(4):165-172.

17. Alegbejo MD, Abo ME. Ecology, Epidemiology and control of Pepper Veinal Mottle Virus (PVMV), genus Potyvirus, in West Africa. J. Sustain. Agric. 2002; 20:5-16.

18. Janzac B, Fabre F, Palloix A, Moury B. Phenotype and spectrum of action of the Pvr4 resistance in pepper against potyviruses, and selection for virulent variants. Mol. Plant Pathol. 2009;10(5)443-449.

19. Rai VP, Jaiswal N, Kumar S, Singh S, Kumar R, Rai AB. Response of total phenols and peroxidase activity in chilli exposed to pepper leaf curl virus disease. Vegetral Sci. 2010;37(1):78-80.

20. Vagiri M, Eva J, Kimmo R. Phenolic compounds in black currant leaves -an interaction between the plant and foliar diseases. J. Plant Interact. 2017;12 (1):193-199.

21. Petrova D, Marinova G, Chaneva G, Kapchina-Toteva V, Stoimenova E. Local and systemic responses of antioxidants to *Cucumber Mosaic Virus* infection in pepper plants local and systemic responses of antioxidants to cucumber. Biotechnol. Biotechnol. Equip. 2009;23(1):516-518.

22. Dikilitas M, Guldur ME, Deryaoglu A, Erel O. Antioxidant and oxidant levels of pepper (*Capsicum annuum* cv . Charlee) infected with Pepper Mild Mottle Virus. Not Bot Horti Agrobo. 2011a;39(2):58-63.

23. Dikilitas M, Guldur ME, Deryaoglu A, Erel O. A novel method of measuring oxidative stress of pepper (*Capsicum annuum* var . Charlee) infected with tobacco mosaic virus. J Biosci. 2011b;37:2425-2433.

24. Clark MF, Adams AN. Characteristics of the microplate method of Enzyme-Linked Immunosorbent Assay for the detection of plant viruses. J. Gen. Virol. 1977;34:475-

483.

25. Janzac B, Fabre MF, Palloix A, Moury B. Characterization of a new potyvirus infecting pepper crops in Ecuador. Arch. Virol. 2008;153:1543-1548.

26. Ranjitha JS, Vijiyalakshmi MA. Biological assay of In vitro antioxidant and antibacterial activity of the whole plant material *Cleome gynandra* Linn. Res. J. Pharm. Biol. Chem. Sci. 2013;4:97-102.

27. Mahi Z, Dedaldechamp F, Maurousse L, Lemoine R, Belkhodja M. Etude de la peroxydation lipidique (MDA) et l'activité antioxydative (POD) chez deux halophytes : *Atriplex halimus* L. et *Atriplex canescens* (Pursh) Nutt sous l' éffet du sel Int. J. Innov. Appl. Stud. 2015;10(1):450-458.

28. Mimouni H, Wasti S, Manaa A, Gharbi E, Chalh A, Vandoorne B et al. Does Salicylic Acid (SA) improve tolerance to salt stress in plants? A study of SA effects on tomato plant growth, water dynamics, photosynthesis, and biochemical parameters. OMICS A J. Integr. Biol. 2016;20(3):1-11.

29. Appiah AS, Quartey EK, Amoatey HM, Nunekpeku W, Owusu-Ansah M, Ofori S. Response of nine cultivars of pepper (*Capsicum* spp) to infection by four viruses under natural field conditions in the coastal savanna zone of Ghana Biotechnology Center , Nuclear Agriculture Center , Radiation Entomology and Pest Management Center. Res. J. Appl. Sci. Eng. Technol. 2014;7(5):903-907.

30. Brunner K, Zeilinger S, Ciliento R, Woo SL, Lorito M, Kubicek CP et al. Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both antagonism and induction of plant systemic disease resistance. Appl. Environ. Microbiol. 2005;71(7):3959-3965.

31. Rao SG, Nageswara NRR, Surekha C. Induction of plant systemic resistance in legumes cajanus cajan , *Vigna radiata* , *Vigna mungo* against plant pathogens fusarium oxysporum and alternaria alternata-a trichoderma viride mediated reprogramming of plant defense mechanism. Int. J. Recent Sci. Res. 2015;6:4270-4280.

32. Siddique Z, Akhtar KP, Hameed A, Sarwar N, Imran-Ul-Haq, Khan SA. Biochemical alterations in leaves of resistant and susceptible cotton genotypes infected systemically by cotton leaf curl Burewala virus. J. Plant Interact. 2014;9(1):702-711.

33. Agrios G.N., Plant Pathology, 4th ed. Academic Press, San Diego, CA., USA., ISBN-13: 9780120445646; 1997.

34. Zinga I, Longue RD, Komba EK, Beaumont C, Semballa S. Evaluation de la teneur en protéines et en chlorophylle dans des feuilles de cinq variétés locales du manioc infectées par la mosaïque en République Centrafricaine. Tropicultura. 2016;34 (1):3-9.

35. Sama H, Sombié PAED, Hilou A, Bonzi S, Somda I. Biochemical resistance mechanism study of *Jatropha curcas* (*Euphorbiaceae*) against *Lasiodiplodia theobramae*, a leaf blight and necrosis agent. J. Agric. Crop. 2018; 4(12):176-185.

36. Louerrad Y, Haddi R, Kaid Harche M. Etude de la peroxydation lipidique chez une plante médicinale Haloxylon scoparium POMEL. J. Bioresour. Valorization. 2016;1(1):28-33.

353 37. Lanubile A, Maschietto V, De LS, Battilani P, Paciolla C, Marocco A. Defense
354 responses to mycotoxin-producing fungi *Fusarium proliferatum* , *F . subglutinans* ,
355 and *Aspergillus flavus* in kernels of susceptible and resistant maize genotypes. Mol.
356 Plant-Microbe Interact. 2015;28(5):546-557.

358 357