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EVALUATION OF MORPHOLOGICAL AND PHYSIO-CHEMICAL CHANGES IN PHYTOPLASMA INFECTED *BRASSICA NAPUS*

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ABSTRACT

Phytoplasmas are bacterial plant pathogens associated with various diseases causing yield losses in agricultural production worldwide. Symptoms of phytoplasma include small leaf size, stunted growth and malformation of floral leaves. Infected plants show a number of biochemical changes and activation of antioxidant enzymes to defend themselves from the plant pathogen. The objective of current experiment was to investigate the morphological and Physio-chemical changes induced in phytoplasma infected leaves of *Brassica napus* plants. Healthy *Brassica napus* plants were exposed to infection through the insect vector *Orosious orientalis* after which leaves from a set of healthy and inoculated plants were employed to analyze and compare their antioxidative enzyme activity and other defense related compounds. The data revealed that phytoplasma attack resulted in small leaf size and stunted growth along with malformation of flowers. Such plants showed lower chlorophyll a, b and total chlorophyll but higher level of phenolics, amino acids, free proteins and H_2O_2 contents as compared to the healthy plants.

Key words: Phytoplasma, *Orosious orientalis*, *Brassica napus*, superoxide dismutase, phenolics, flower morphology

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INTRODUCTION

Oil seed rape (*Brassica napus L*) is grown worldwide for food, feed and edible oil purposes (Ahuja *et al*., 2011). Phytoplasma are wall less obligate plant pathogens transmitted by phloem feeding insects belonging to order hemiptera (Ahmad *et al*., 2017; Weintraub and Beanland 2006). Phytoplasmas causes significant agricultural losses in more than 1000 plant species worldwide (Streten and Gibbs, 2006; Strauss, 2009). A number of plant diseases such as dwarf yellow and witches' broom were associated with phytoplasma presence. Phytoplasmas induce physiological and defense related disorders in cruciferous and other agronomic plants by causing 70-100% yield losses (Bhowmik 2003; Bertaccini, 2007; Ahmad *et al*., 2013, 2014). Infected plants show various symptoms at different stages of plant growth. At vegetative stage, plants show abnormal leaf color and stunted growth along with extra proliferation. However, at the floral stage, phyllody, virescence and abnormal floral development can be seen in infected plants.

Phytoplasma associated diseases are described as auxinic diseases, as they cause hormonal imbalance and modification in the growth of diseased plants (Bertamini *et al*., 2002b; Chang, 1998; Curkovic-Perica *et al*., 2007; Lepka *et al*., 1999; Pertot *et al*., 1998). Previously it was found that phytoplasma infection impaired photosynthesis and instigated increase of sugar and starch concentration in leaves, and decrease of soluble carbohydrates and starch in roots (Leon *et al*., 1996; Lepka *et al*., 1999; Tan and Whitlow, 2001; Bertamini *et al*., 2002a, b; Maust *et al*., 2003; Giorno *et al*., 2013; Buoso *et al*., 2019; Liu *et al*., 2016). However, only a few reports have been published on morphological and physiochemical alterations in plants after infection of microorganisms. Plants produce several shielding compounds to resist disease or pathogen attack through induced defensive responses. Oxidative stress in plants is managed through a highly efficient antioxidant system (Adwas *et al*., 2019) which maintains the threshold level of ROS (reactive oxygen species) inside the cell (Torres *et al*., 2006; Ali, *et al*., 2018). Several antioxidant enzymes such as catalase, superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase, monodehydroascorbate reductase and molecules like flavonoids, ascorbate, glutathione, proline, phenolics, tocopherols and carotenoids neutralize the ROS production during pathogen attack (He *et al*., 2017).

Phyllody disease of phytoplasma was first reported in Pakistan in 1987 and later symptoms of these diseases have been observed on different agricultural crops like chickpea, sesame, *Brassica napus*, parthenium,

tomato, and onion (Akhtar *et al*., 2008,2009a, b; Ahmad *et al*., 2015a, b, c; Ahmad *et al*., 2019a; Sharif *et al*., 2019) and other related molecular work of DNA barcoding and pest management have also been recently performed (Ahmad *et al.,* 2019b, 2020a, b; Malik *et al.,* 2020; Manzoor *et al.,* 2020). Brassica plants were found to be associated with symptoms of floral organ malformation, small leaves, and stunted growth. Since very little work on the effect of phytoplasma on plant physiology has been carried out, the present analyses were performed to identify and investigate the alterations in biochemical parameters viz., hydrogen peroxide, total phenols, antioxidants, total protein and amino acids in phytoplasma infected *Brassica napus* plants.

MATERIALS AND METHODS

The current experiment was carried out in order to assess the physiochemical alterations in phytoplasma infected brassica plants. Brassica seeds were allowed to grow in the greenhouse under defined conditions (27˚C day, 20˚C night). At 3 leaf seedling stage, five plants of equal length were maintained in each pot after thinning. Hoagland's solution (Full strength) (Hoagland and Arnon, 1950) was added weekly to each pot until the termination of the experiment to ensure proper nutrition of the plants.

The identified 16SrIX-H ribosomal subgroup of phytoplasma maintained in periwinkle plants was used as a source of inoculum. Accretion and transmission were carried out with the insect vector (*Orosious orientalis*). For two weeks, about fifty instars of *Orosious orientalis* were caged on infected periwinkle plants. After termination of the quiescent period, six leafhoppers were transmitted to one set of *Brassica napus* plants at flowering stage. The other set of plants was kept healthy in a separate insect-proof cage. Symptoms were recorded three times a week up to 21 days. After 3 weeks of phytoplasma inoculation, when phytoplasma disease symptoms of deformation and yellowing of leaves began to appear, plants were treated with insecticide and leaves of equivalent size and position were harvested from healthy as well as infected brassica plants. Data for physiochemical attributes were recorded in leaves of both healthy and symptomatic plants by the following methods:

Determination of photosynthetic pigments: Photosynthetic pigments of both healthy and phytoplasma infected leaves were determined following the protocol followed by Arnon *et al*. (1949). Leaf samples were triturated in 80 % acetone. The optical density of each content (Chl a and b) was determined at 663 and 645 nm through spectrophotometer respectively.

Determination of total phenolics: The phenolic contents in phytoplasma infected as well as healthy plant leaves were estimated by the protocol followed by Julkunen-Titto (1985). The fresh leaf sample of about 0.1 g was triturated in 5ml of 80 % acetone. After centrifugation 100 µL of aliquot was separated to make reaction mixture with 2 mL dH_2O_2 along with Folin-Ciocalteau's phenol reagent. Each mixture was homogenized thoroughly and raised the volume up to 10ml by adding 5 mL of 20% (w/v) Na_2CO_3 and dH_2O_2. The absorbance of each sample was read at 750 nm against 80% acetone. The standard solution of tannic acid (100 ug/ml stock) was used to prepare a standard curve.

Determination of hydrogen peroxide contents: The tri-chloroacetic acid (TCA) assay followed by Velikova *et al*. (2000) was followed for the estimation of hydrogen peroxide contents in brassica leaves. Pre-chilled pestle and mortar were used to extract fresh leaf samples 0.1% (v/v) TCA. After centrifugation, to the 0.5 mL of supernatant, 1 mL potassium iodide followed by 0.5 mL of potassium phosphate buffer was added. Then absorbance values were measured by using the pico drop at 390 nm wavelength. The concentration of H_2O_2 in each sample was collected from the values of absorbance by using the standard values.

Determination of free amino acid: The total free amino acid in fresh leaves was determined according to the method of Hamilton and Van-Slyke (1943). About 1 ml of each 10 % pyridine and 2 % ninhydrin were added to 1 ml of sample extract. After boiling in a water bath, the volume of each mixture was made up to 50 ml by adding dH_2O water and absorbance was noted at 570 nm.

Estimation of total soluble protein: Total soluble protein contents from fully emerged leaves were determined by following the dye-binding assay described by Bradford (1976). For this purpose 0.5g of fresh leaf sample was triturated in 5mM chilled phosphate buffer (pH 7.8). After centrifugation of reaction mixture, to 100 µl of supernatant was incubated at 37^0 C after the addition of 1 ml of Bradford reagent. Then absorbance was noted at 595 nm.

Antioxidant enzyme extraction: Pre-chilled pestle and mortar were used to triturate fresh leaf samples of about 0.5 g in 10ml of chilled phosphate buffer (pH 7.8). After the centrifugation of homogenate at 40 ºC, the supernatant was removed and used for further evaluation of enzymatic activities.

Superoxide dismutase (SOD): Giannopolitis and Ries (1977) method was proceeded to determine the SOD activity by measuring its ability to cause the photoreduction of 50% nitro blue tetrazolium (NBT). According to this method the reaction mixture was prepared in cuvettes by adding phosphate buffer (250 µL), 13 mM L-methionine (100 µL), 0.1% triton-X (100 µL), 1.3 µM (50 µL) riboflavin, 50 µL NBT, distilled

H_2O (400 µL) and 50 µL of enzyme extract. The absorbance of the irradiated reaction mixture was noted at a wavelength of 560 nm.

Determination of Peroxidase (POD) activity: Peroxidase activity was determined using the method of Chance and Maehly (1955) which describes the change of 1.0 A470 unit per min to be equal to 1 unit of enzyme activity. For the determination of POD activity, 100 µL of crude enzyme extract was poured into a cuvette. To it was added, the reaction mixture containing 900 µL 40 mM H_2O_2, 1 mL 20 mM guaiacol and 1 mL 50 mM phosphate buffer. The absorbance was noted after every 20s at a wavelength of 470 nm.

Determination of Catalase (CAT) activity: According to Chance and Maehly (1955) catalase activity was determined by mixing crude enzyme extract in 50 mM buffer and 5.9 mM H_2O_2. The increase in absorbance was noted at 240 nm. One unit (U) of catalase activity was equal to the amount of enzyme that caused 0.001 per min change in absorbance under assay conditions.

Statistical analysis: All analyses were established based on a completely randomized design. The data were examined by Student's t-test (Student, 1929) using the COSTAT software. Results were considered to be significant at $P<0.05$.

RESULTS

The symptoms and morphological and physiochemical changes observed in the phytoplasma infected *Brassica napus*: In the present study, the effect of the phytoplasma infection, through insect vector *Orosious orientalis* on morphological and physiochemical alterations in *Brassica napus* plants was studied. The first symptom detected in the phytoplasma infected *Brassica napus* was the reduced size of leaves. Later on symptoms like severe virescence, the transformation of the entire inflorescence to bushy appearance and green leafy structure is seen. Furthermore, various symptoms appeared on phytoplasma infected plants including irregular green flowers looking like green shoots, plants that produced yellow-colored flowers with petals, sepals, asparagus, and carp. The results of our study showed that at maturity, healthy plants usually produced yellow flower buds contrary to infected brassica plants that produced green flowers.

Figure 4.1. Flower buds of healthy and phytoplasma inoculated *Brassica napus; (*A) Healthy flower buds, (B) Healthy flower, (C-D) Abnormal flowers and buds, (phyllody and virescence symptoms), (E) Abnormal pod formation, big bud like pod formation, (F) Healthy flowers, (G), Abnormal floral development.

Various physiochemical fluctuations in *Brassica napus* leaves were also found in phytoplasma infected plants. The mean data showed that phytoplasma reduced the chlorophyll a and total chlorophyll contents of infected plants. The chlorophyll b contents were reduced significantly (Table 4.1). Phytoplasma attack also induced alterations in amino acid content, proteins, total phenolics and activities of SOD, POD, and catalase enzymes as presented in table 4.1.

Amino acid, protein, total phenolics and H_2O_2 contents of brassica plants increased considerably in phytoplasma inoculated as compared to the healthy plants. Moreover, our results demonstrated that amino acid and hydrogen peroxide contents, proteins, and total phenolics significantly increased upon phytoplasma infection.

Moreover, our results indicate that antioxidant enzyme activity was also enhanced in phytoplasma infected leaves. Among these antioxidants, SOD activity was significantly higher than the other two enzymes. In the infected leaves, the percent increase in the activities of SOD, POD, and catalase were ca. 70, 44 and 37 % of the control value, respectively.

Table 1. Differences in various physio-chemical contents in phytoplasma-infected and control/healthy *Brassica napus* leaves.

Parameters	Control	phytoplasma Infected	
Chl a (mg/g f.w)	0.21 ± 0.006	0.11 ± 0.004	(-48)
Chl b (mg/g f.w)	0.522 ± 0.048	0.046 ± 0.024	(-91)
Total chl (mg/g f.w)	0.044 ± 0.002	0.022 ± 0.006	(-49)
Total Phenolics (micro g/gallic acid)	27.6 ± 1.25	61.6 ± 5.94	(+45)
H_2O_2 (g/mol)	3.46 ± 0.31	9.36 ± 0.44	(+37)
Soluble proteins (mg/g)	0.30 ± 0.033	0.73 ± 0.153	(+41)
Total amino acids (mg/g)	5.36 ± 0.21	6.26 ± 0.34	(+86)
SOD (mg/protein)	19.56 ± 1.92	27.94 ± 3.17	(+70)
POD µmol/mint/g f.wt	0.16 ± 0.014	0.36 ± 0.132	(+44)
Catalase µmol/mint/g f.wt	0.252 ± 0.044	0.68 ± 0.030	(+37)

The above values correspond to Means ± SE of each attribute
Percent reduction values are written in parentheses with reference to controls.

DISCUSSION

The *B. napus* plants suffered serious damage from the phytoplasma indicating a weak resistance to phytoplasma infection. Future threats to *Brassica napus* can be alleviated by tailoring phytoplasma-resistant varieties. But, this requires a lot of more work in order to understand the adaptive mechanisms and response of brassica plants toward phytoplasma infection that may help plants for their survival in the world. Knowledge about the biochemistry, molecular biology and physiology of phytoplasma is very limited. Furthermore, no previous studies were reported regarding biochemical and physiological responses against phytoplasma infection in *Brassica napus* plants. Thus, the present study was conducted to evaluate the contribution of the antioxidant defense system and primary and secondary metabolites in reducing the drastic effects of phytoplasma in *Brassica napus* plants. Unhealthy plants show several symptoms including witches' broom, stunting, yellowing, proliferation, and reduced growth. In our study reduction in chl. a and b and their total contents in phytoplasma infected plants can be attributed to damage in antenna complex. The decrease in chlorophyll synthesis enhances the leaf senescence (Zafari *et al.,* 2012). Plants accumulate glycinebetaine to protect themselves against various abiotic stress by maintaining osmotic homeostasis (Annunziata *et al*., 2019; Xu *et al*., 2018). Different enzymes such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and polyphenol oxidase (PPO) expressed in plants to mitigate the effect of oxidative damage by removing catalytically free radicals and reactive oxygen species (Karthishwaran *et al*., 2017; Jaleel *et al.,* 2008; Saffar *et al*., 2009). In the current study, SOD activity was higher in phytoplasma attacked plants as compared to healthy ones. Ray *et al*. (1998) found that antioxidants play an important role against microorganisms. Our results correspond with earlier report by Zafari *et al*. (2012) which explain that more antioxidants like SOD are produced to mitigate the detrimental effects of ROS produced upon phytoplasma infection. In the current analysis, significant differences in POD and CAT production were observed in both healthy and phytoplasma infected brassica plants. Similar observations were reported by Wallis *et al*., (2012) who verified that some enzymes including POD can be produced in plants upon pathogen attack. Significant raise in total soluble protein was detected in phytoplasma infected leaves of *Brassica napus* plants as compared to healthy plants. Our results were much similar to previous studies where maize and *C. coronarium* plants infected by bushy stunt phytoplasma showed more accumulation of protein contents (Junqueira *et al*., 2004; Zhong and Shen, 2004). In contrast to these results, decreased level of soluble protein contents was observed in different phytoplasma infected plants like apple proliferation infected apple trees (Bertamini *et al*., 2002a). H_2O_2 contents were high in phytoplasma inoculated *B. napus* plants as compared to healthy ones. Our results correspond to previous studies reported by Rojo *et al.* (2010) who explained that pathogen attack can activate the overabundance of ROS in cells, which can cause

oxidative stress and severe physiological damage. The same results were reported in apple, apricot, and grapevine by Musetti *et al.,* (2005, 2007). Phytoplasma infection impaired the amino acid translocation in host plants (Carginale *et al*., 2004). In our results, level of total free amino acid contents were high in phytoplasma affected plants as compared to healthy plants. High amino acid concentration were also observed in source and sink leaves of *Catharanthus roseus* (periwinkle) plant infected by ash yellows (ASHY) and in source leaves of tobacco plants infected by apple proliferation (AP) (Lepka *et al*., 1999). The possible reason is that phytoplasma attack affects the amino acid accumulation due to limited phloem transport which imparts a negative impact on growth and hence the size of the plant. Plant phenolics or polyphenol are aromatic compounds that play key role in plant growth, development, and reproduction. They protect the plant against abiotic stresses, such as high light, low temperatures, UV-B radiations, heavy metals and nutrient deficiency (Naikoo *et al*., 2019). In the present analysis, phenolic contents increased significantly in *B. napus* infected leaves which is in accordance with the finding of Musetti *et al*., (2000) who revealed that total phenolic contents were increased up to three-fold in phytoplasma infected plums and apples. The same results were reported in *Zea mays* by Junqueira *et al.* (2004). Protein contents in plants are involved in disease resistance (Tornero *et al*., 2002; Carvalho *et al*., 2006). Plants became resistant against pathogen invasion upon the stimulation of defense-related proteins (Van *et al.,* 1997). Agrios *et al.,* (1997) studied the activation of host defense and pathogen attack mechanism in phytoplasma infected plants which result in more production of protein contents. STOL infected tomato plants (Favali *et al*., 2001) and different Mollicutes infected maize plants. The difference in these results might be due to the use of more susceptible plants in later analyses. However, contrary to these results reduction in protein contents was also reported in phytoplasma infected tomato and corn plants (Favali *et al*. 2001). This reduction in protein contents might indirectly correlate with chloroplast damage in phytoplasma infected leaves which results in reduced activity of the major soluble protein in leaves that is RuBPC and hence $14CO_2$ fixation (Bertamini *et al*., 2002b).

Conclusion: From the current analysis, it can be concluded that the concentration of different metabolic contents increased in *Brassica napus* leaves upon the phytoplasma attack. These changes in levels of metabolic contents might cause disruption in many physiological a well as biochemical processes which result in the appearance of various severe symptoms in phytoplasma infected *Brassica napus* plants. The current work reveals the role of antioxidative enzymes upon phytoplasma infection in the brassica plant to enhance its resistance against the phytoplasma attack. Furthermore there is need to develop phytoplasma resistant cultivars.

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