


Candidatus Phytoplasma trifolii (16SrVI) infection modifies the polyphenols concentration in pepper (*Capsicum annuum*) plant tissues

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Abstract

Of late, the presence of *Candidatus* Phytoplasma trifolii was reported as a serious threat to the pepper crop in Zacatecas, México; therefore, asymptomatic and symptomatic pepper plants were collected from a commercial field among three samplings after the fruit set stage was reached. Total DNA was extracted using the CTAB-based method and tested for phytoplasma using a nested PCR assay, followed by a BLAST, and restriction fragment length polymorphism (RFLP) analysis of 16S rDNA sequences, which confirmed the presence of phytoplasma group 16SrVI, "*Candidatus* Phytoplasma trifolii" in the symptomatic plants. As the metabolic pathways of pathogen-infected plants tend to change, resulting in a biochemical differentiation with the noninfected plants, the polyphenolic compound concentrations were quantified from the vegetative tissues (root, stem, leaves and developed fruit/big bud) and were analysed based on a principal component analysis (PCA). Results revealed that, in general, plants tend to a progressive increase in total phenols, flavonoids, condensed tannins and anthocyanins related to the plants exposure to "*Ca. P. trifolii*" infection, and PCA demonstrated that almost 90% of the observed variance was explained by the first two components. Hence, the phenolic content of the plants increases as a response of the defence mechanism, which reflects its condition and resistance.

KEYWORDS

pepper, phytoplasma, plant tissues, polyphenols

1 | INTRODUCTION

Pepper is a crop of major agricultural and economic importance, heavily consumed worldwide as a season-free vegetable, and it is known for its pungency, rich flavour, and nutritional value (Park et al., 2012 and Reddy et al., 2014). Pepper crops can be grown in fields and in greenhouses; however, their average yields are severely impacted by the presence of pests and diseases (Dikilitas, Guldur, Deryaoglu, & Ozcan, 2011). Besides well-known fungal and virus pathogens,

recently, phytoplasma has become increasingly important as a serious threat in many different crops around the world (Junqueira, Bedendo, & Pascholati, 2004; Margaria et al., 2014), and the pepper crop is not the exception. Phytoplasma associated with *C. annuum* diseases have been reported worldwide, including bell pepper leaf size reduction (16SrIII) in Bolivia, bell and chilli pepper big bud in Iran and the United States, respectively (16SrII and 16SrVI) and pepper witches' broom (16SrI) in China and México (Arocha et al., 2010; Faghihi, Taghavi, Safaei, Siampour, & Najafabadi, 2016; Randall, Bosland, &

Hanson, 2009; Santos-Cervantes, Chávez-Medina, Mendez-Lozano, & Leyva-Lopez, 2008; Zheng-Nan, Zhang, Song, & Wu, 2013). Of late, Mauricio-Castillo, Salas-Muñoz, Velásquez-Valle, Ambríz-Granados, and Reveles-Torres (2015) reported the presence of "*Candidatus Phytoplasma trifolii*" (16SrVI) in Zacatecas, México. A floral structure modification known as big bud characterized the disease; this pathogen was found in Mirasol type pepper plants, which remains as the main cultivar in the principal productive regions of Zacatecas.

Plants infected with phytoplasma exhibited a number of anatomical, physiological, biochemical and molecular changes. According to Prezelj et al. (2016), changes in photosynthate translocation along with other impaired physiological functions, including reduced photosynthesis, stomatal conductance, hydrogen peroxide accumulation, disturbed plant hormone balance and altered secondary metabolism, could account for symptoms exhibited by phytoplasma infection. In this regard, different studies have shown that phytoplasma infection can change the levels of some compounds in plant tissues, as plants have evolved multiple defence signalling pathways to cope with adverse pathogen attack (Jones & Dangl, 2006; Junqueira et al., 2004). In regard to the secondary metabolism, plants produce an immense number of secondary compounds to interact with harmful organisms, and these compounds mainly act as signal components and chemical defence (Leiss, Choi, Verpoorte, & Klinkhamer, 2011). Secondary metabolites are the end products of gene expression and are controlled in response to environmental cues. Therefore, the changes in their abundance may be regarded as an important feature of plant-phytoplasma interactions. Furthermore, the presence of phytoplasma in the host plant can cause an increase in metabolites related to the phenylpropanoid biosynthetic pathways and thus a higher accumulation of polyphenols (Kim & Hwang, 2014; Ying-Ping et al., 2014).

Although few studies have linked the incidence of phytoplasma infection to increase in the phenolic compounds in different crops, such as *Malus pumina* and *Prunus domestica* (Musetti, Favali, & Pressacco, 2000), *Zea mays* (Junqueira et al., 2004), *Vitis vinifera* (Margaria et al., 2014), *Theobroma cacao* (Ondobo et al., 2014) and *Vigna radiata* (Hameed et al., 2017), there is insufficient information regarding the effect of phytoplasma over the polyphenols content in the pepper plants. In a more specific manner, biochemical changes at the phenylpropanoid pathway level among different pepper plant tissues are less known. Therefore, the objective of this study was to evaluate the phenolic compound concentrations in different pepper plant tissues as a response to phytoplasma infection within different dates during the fructification phenological stage; this information will assist in revealing the complex interactive nature of the plants' metabolic network and the responses to phytoplasma infection.

2 | MATERIALS AND METHODS

2.1 | Plant tissue sampling and preparation

An epidemic outbreak of phytoplasma infection in the pepper crop was observed in Zacatecas, México, in 2015; however, few plants

were detected with the "big bud" symptom in the sampled field. Therefore, three pepper plants (Mirasol type) showing big bud symptoms caused by phytoplasma infection (Figure 1a) were collected in a commercial field located in the municipality of Calera de V.R., Zacatecas (N: 23° 03.522'; W; 102° 39.732'; Altitude: 2,103 masl). In addition, three asymptomatic plants (Figure 1b) were collected to be used as the healthy control after confirming the absence of phytoplasma by polymerase chain reaction (PCR). Sampling of symptomatic and asymptomatic plants was carried out in three different dates after the fructification phenological stage was reached: 31st August, 08th September and 24th September 2015 (N = 18). As this is a preliminary study of metabolic alterations caused by phytoplasma infection, the analysis was carried out with the purpose of stratifying the source of variation. Therefore, all asymptomatic samples constituted a representative sample, except for phytoplasma isolation and identification, where asymptomatic samples were analysed individually.

Plants were processed within ~15 min after uprooting, by separating them into different tissues (root, stem and leaf). The fruit/big bud from the infected plants and developed fruit from apparently healthy plants were only collected on the last sampling date. Samples were transported to the laboratory in liquid nitrogen and were freeze-dried

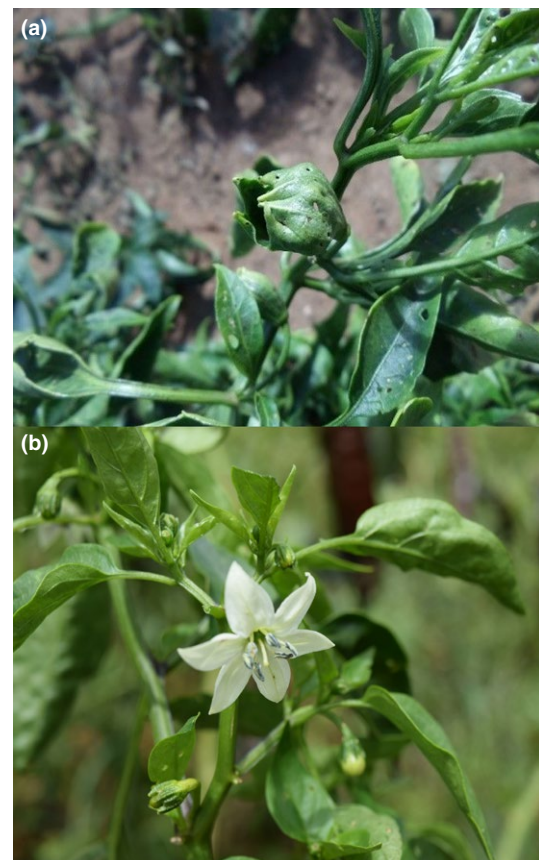


FIGURE 1 Mirasol type pepper plants. (a) Big bud symptomatology of a phytoplasma-infected plant develop instead of the floral structure. (b) Floral structure of a noninfected plant

(LABCONCO, Kansas City, USA), followed by milling in a domestic grinder (Krupps GX4100, Mexico) and stored at 4°C in sealed plastic bags until use.

2.2 | DNA extraction from chilli pepper

DNA was isolated from phytoplasma-infected and noninfected plants using the method described by Dellaporta, Wood, and Hicks (1983) with some modifications. Briefly, each sample was macerated in liquid nitrogen and placed in a 1.5-ml Eppendorf tube with 750 µl extraction buffer (2% CTAB, 1.4 mM NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1% β-mercaptoethanol). The DNA pellet was suspended in 50 µl of TE Buffer (Tris-EDTA 0.01 mM pH 8) and stored at -20°C.

2.3 | Nested PCR for phytoplasma

The phytoplasma was detected by amplifying the 16S rRNA gene. The oligonucleotides used for direct PCR were P1 5'-AAGAGTTTGTATCTGGCTCAGGATT-3' and Tint 5'-TCAGGCGTGTGCTCTAACCAGC-3' (Smart et al., 1996) and for nested PCR were R16F2n 5'-GAAACGACTGCTAAGACTGG-3' and R16R2 5'-TGACGGGCGGTGTGTACAAACCCCG-3' (Gundersen & Lee, 1996). The reagent mixture contained 2.5 µl PCR buffer (10×), 1.5 µl MgCl₂ (50 mM), 2.5 µl dNTPs (20 mM), 0.5 µl of each oligonucleotides (20 pM), 0.15 µl Taq polymerase (5 U/µl), 2.5 µl DNA extract (50 ng/µl) and 25 µl of H₂O milliQ. Regarding the reagent mixture for the nested PCR, 1 µl of the direct PCR was used. PCR products were performed in a programmable thermocycler (Applied Biosystems) with the following parameters: 95°C for 5 min, 35 cycles of 94°C for 1 min, 56°C for 2 min and 72°C for 2 min, and a final extension cycle of 72°C for 5 min for direct and nested PCR. PCR products were analysed by electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination. DNA samples extracted from pepper plant tissues were used as template for PCR. DNA extracted from asymptomatic peppers and sterile water was used as negative controls.

2.4 | Cloning of PCR products and sequencing of DNA

Amplified products of 1.25 kb were ligated in pGEM-T Easy vector (Promega, Madison, WI, USA), as indicated by the supplier. Transformation of *Escherichia coli* Top-10 calcium competent cells was performed by the heat shock method. Different clones obtained from DNA samples of chilli pepper were completely sequenced at the National Laboratory of Agricultural, Medical and Environmental Biotechnology (LANBAMA) in San Luis Potosí, Mexico.

2.5 | Virtual RFLP analysis

Computer-simulated RFLP analysis of 16S rRNA gene was performed on sequences of phytoplasma isolates from chilli pepper.

The aligned sequences were exported to the virtual RFLP analysis programs of iPhyClassifier website (<https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) for computer-simulated restriction digestion and virtual gel plotting (Zhao et al., 2009). Each aligned DNA fragment was digested in silico with 17 distinct restriction enzymes that have been routinely used for phytoplasma 16S rRNA gene RFLP analysis (Lee, Gundersen-Rindal, Davis, & Bartoszyk, 1998). These enzymes were *AluI*, *BamHI*, *Bfal*, *BstUI* (*Thal*), *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI* (*Mbol*), *MseI*, *RsaI*, *SspI* and *TaqI*. After in silico restriction digestion, a virtual 3% agarose gel electrophoresis image was plotted and captured as a device-independent PDF file. The RFLP analysis sequences of phytoplasma were automatically compared to each 16Sr groups and subgroups reference strains of phytoplasma for group and subgroup recognition.

2.6 | Polyphenols extraction

For polyphenols extraction and quantification, each sample, including the asymptomatic representative sample for root, stem, leaves and developed fruit, and all 36 individual samples (nine for each tissue) corresponding to the infected plants collected among the three different dates, was extracted with three replicas.

The total phenols, flavonoids and condensed tannins extraction were performed as described by Xu and Chang (2007). Briefly, 1 g of dry sample was mixed with 10 ml of acetone/water/acetic acid (70:29.5:0.5, v/v/v) and shaken in amber tubes for 24 h at room temperature, then centrifuged at 4,000 g for 10 min. Extraction of total anthocyanins was carried out as described by Abdel-Aal and Hucl (1999) with slight modifications. An acidified ethanol solution (85:15 v/v) was prepared with 95% ethanol and HCl 1N; 4 ml of this solution was added to 0.5 g of the sample and shaken for 2 min at room temperature, and then, the pH was adjusted to 1 with concentrated HCl. In addition, a 30-min shake of samples was performed before centrifugation at 10,000 g for 20 min. Supernatants from both extractions were recovered and kept at 4°C until analyses.

2.7 | Total phenolic quantification

The total phenolic compounds were quantified by the Folin-Ciocalteu assay described by Singleton, Orthofer, and Lamuela-Raventós (1999). An aliquot of 40 µl of the phenolic extract was vortexed with 460 µl of distilled water, and then, 250 µl of the Folin-Ciocalteu reagent 1N (Sigma-Aldrich, Saint Louis, MO, USA) was added, and samples were incubated for 5 min at room temperature. A dose of 1,250 µl of 20% Na₂CO₃ was incorporated, and the absorbance of samples was read at 765 nm in a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) against a reagent blank. Gallic acid was used as the standard. The total phenols were expressed as milligrams of gallic acid equivalents per gram of dry sample (mg GAE/g) through a calibration curve of the standard, with a linearity range of 0–0.032 mg/ml.

2.8 | Total flavonoid quantification

Total flavonoid concentration was determined as described by Liu et al. (2002). In brief, 0.25 ml of the acetonic extract was diluted with 1.25 ml of distilled water and 75 μ l of a 5% NaNO₂ solution; after 6 min, 150 μ l of a 10% AlCl₃·6H₂O solution was vortexed with the sample and the mixture was maintained at room temperature for 5 min. Finally, 0.5 ml of NaOH 1M was added and the volume was made up to 2.5 ml with distilled water. The absorbance of the solution was measured at 510 nm. The linearity range of the calibration curve was 0–0.074 mg/ml using (+)-catechin as the standard. Results were expressed as milligrams of (+)-catechin equivalents per gram of dry sample (mg CAE/g).

2.9 | Total anthocyanin quantification

The extract for anthocyanins quantification was made up to 10 ml with acidified ethanol, and the absorbance was measured at 535 nm. The anthocyanin content was expressed as milligrams of cyanidin 3-glucoside equivalents per gram of dry sample (mg EC3G/g) and calculated using the equation obtained by Abdel-Aal and Hucl (1999):

$$C = [(A/\epsilon) \times (\text{vol}/1,000) \times \text{MW} \times (1/\text{sample weight}) \times 10^6] / 1,000$$

where C is concentration of total anthocyanin (mg/g), A is the absorbance of the sample, ϵ is the molar absorptivity of cyanidin 3-glucoside (25,965/cm/M), vol represents the total volume of the anthocyanin extract, and MW is the molar weight of cyanidin 3-glucoside = 449.

2.10 | Condensed tannins quantification

From the acetonic extract, an aliquot of 100 μ l was used to perform the vanillin assay for condensed tannins quantification (Deshpande

& Cheryan, 1985). A volume of 500 μ l of vanillin 1%: HCl 8% (both prepared in methanol) (1:1 v/v) was vortexed with the acetonic extract; absorbance of samples was read at 492 nm against a reagent blank. Condensed tannins calculated on the basis of a linear curve (0–0.8 mg/ml of CAE) were expressed as milligrams of (+)-catechin equivalents per gram of dry sample.

2.11 | Statistical analyses

Results were expressed as mean values \pm standard error (SE). The statistical analyses were completed by the analysis of variance (ANOVA). Differences were considered to be significant whether the probability value was <0.05 ($p < 0.05$) with the Tukey test, and principal component analysis (PCA) was assessed to identify the polyphenols that characterize each tissue among pepper plant conditions using the statistical software JMP 5.0.1 (SAS Institute, Cary, NC, USA).

3 | RESULTS

3.1 | Molecular identification of phytoplasma

The nested PCR amplicons (1.2 kb) amplified from all symptomatic plants were cloned separately and directly sequenced. No PCR products were obtained from the symptomless plants (Figure 2). BLAST analysis of the 16S rDNA sequences revealed that they shared 99.9% sequence identity to each other and 99.0% sequence identity with those of the 16SrVI group, “*Candidatus* Phytoplasma trifolii” strains. Computer-simulated RFLP analysis of the chilli pepper phytoplasma sequence of the representative clone, Mch2-22 (GenBank Accession No. MG674595), was performed using the iPhyClassifier (<http://plantpathology>.

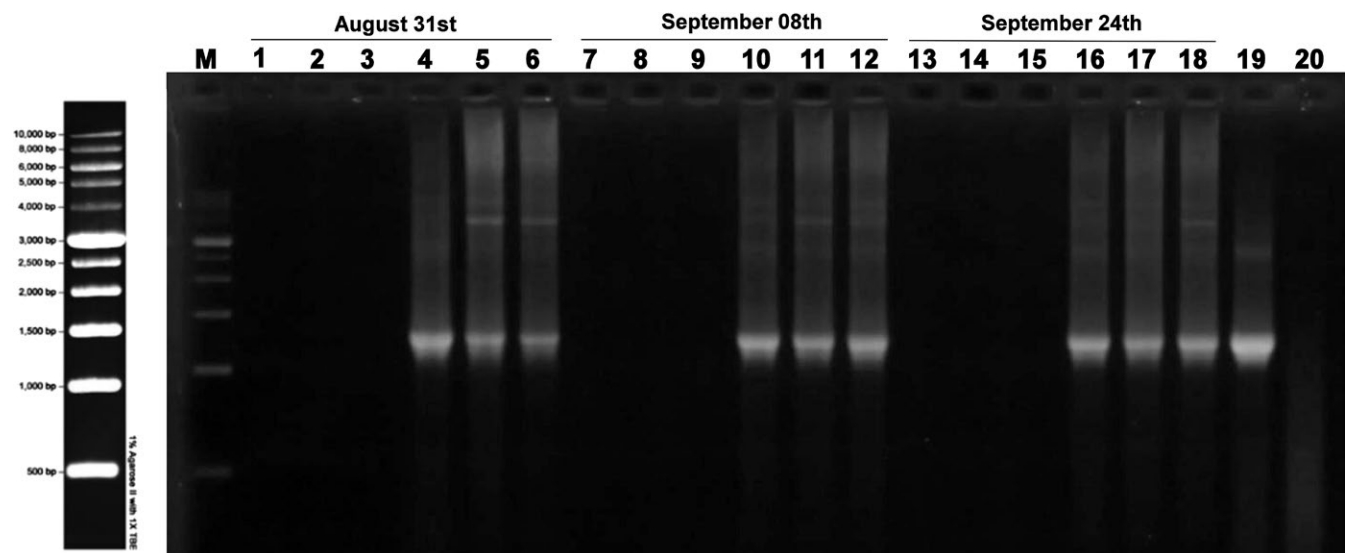


FIGURE 2 Nested PCR for the detection of phytoplasmas in chilli pepper plants. 1 kb DNA MW Marker (Amresco) (lane M), asymptomatic (lane 1–3) and symptomatic (lane 4–6) samples collected in 31 August 31, asymptomatic (lane 7–9) and symptomatic (lane 10–12) samples collected in 08 September, asymptomatic (lane 13–15) and symptomatic (lane 16–18) samples collected in 24 September, positive control (lane 19) and negative control (H₂O miliQ) (lane 20)

ba.ars.usda.gov/cgibin/resource/iphyclassifier.cgi) data base and RFLP profiles (Zhao et al. 2013), confirming that the analysed sequence shared 99.0% identity with the reference strain (GenBank Accession No. AY390261). Finally, the pattern of the virtual RFLPs showed a high similarity with the members of the group 16SrVI, subgroup B, with a coefficient of similarity of 0.97 (Figure 3).

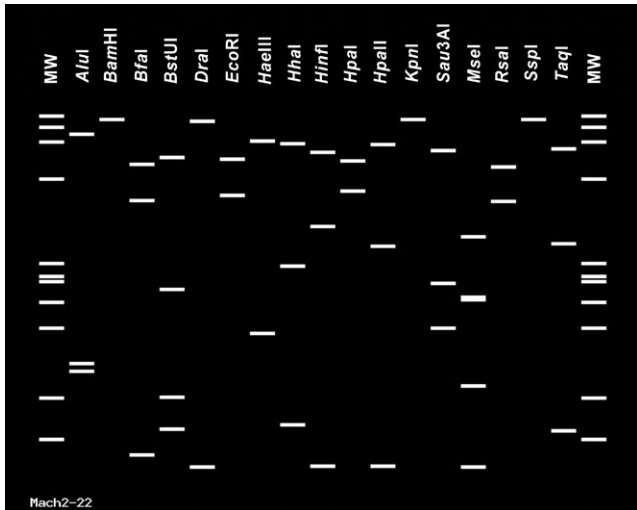


FIGURE 3 Analysis of the virtual RFLPs of the sequence Mch2-22 by the computer program iPhyClassifier, with the 17 enzymes *AluI*, *BamHI*, *BfaI*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI*, *MseI*, *RsaI*, *SspI* y *TaqI*

3.2 | Effect of phytoplasma infection on phenolic compounds synthesis

The effect of phytoplasma infection of pepper plants cv. Mirasol over the synthesis of phenolic compounds was assessed. Table 1 shows the phenolic compound concentration in different plant tissues among three samplings after the fructification stage was reached. Overall, statistical differences ($p < 0.05$) were observed in the polyphenol quantification of root, stem and leaf sampled from infected and noninfected plants. Regarding the root tissue, total phenols and flavonoids tend to a progressive increase related to the plants exposure to “*Ca. P. trifolii*” infection, with an augmentation of 280% and 112%, respectively, for the latest sampling which led to the maximum concentration of both compounds in comparison with the noninfected tissue. However, condensed tannins were highly accumulated in the roots from plants collected in the second sampling, which exhibited 103% more tannins comparing to the same control; meanwhile, the highest upsurge of total anthocyanins was observed in roots of plants subjected to a minor phytoplasma exposure, with a concentration five times greater than the noninfected plants.

The levels of polyphenols in the stems revealed that the main effect of “*Ca. P. trifolii*” in the pepper plants was observed by those collected from the second sampling, as these plants lean towards the greatest concentration of total phenols, condensed tannins and anthocyanins (40%, 16% and 440%, respectively) than the noninfected plants. Contrary to the above, only total flavonoid concentration was higher in stems corresponding to plants from the third sampling.

It is interesting that a nonconsistent level of the integral polyphenols profile in the foliar tissue was observed due to the presence

TABLE 1 Phenolic compounds content in different pepper plant tissues infected by phytoplasma

Compound/tissue	Noninfected	Infected		
		1st sampling	2nd sampling	3rd sampling
Root				
Total phenols ¹	1.08 ± 0.14 c	3.28 ± 0.07 b	3.79 ± 0.15 ab	4.11 ± 0.15 a
Total flavonoids ²	5.93 ± 0.35 b	6.44 ± 0.35 b	11.85 ± 2.78 ab	12.61 ± 0.08 a
Condensed tannins ²	11.80 ± 4.10 c	13.37 ± 0.08 b	23.94 ± 0.84 a	21.61 ± 0.8 a
Total anthocyanins ³	5.18 ± 0.00 c	32.33 ± 1.22 a	16.60 ± 0.48 b	29.91 ± 3.66 a
Stem				
Total phenols ¹	5.08 ± 0.39 b	5.41 ± 0.61 b	7.10 ± 0.00 a	5.98 ± 0.06 ab
Total flavonoids ²	14.90 ± 3.50 b	17.45 ± 2.96 b	30.74 ± 3.41a	32.33 ± 2.96 a
Condensed tannins ²	34.95 ± 3.03 ab	32.05 ± 2.05 b	40.52 ± 1.39 a	38.25 ± 0.49 ab
Total anthocyanins ³	25.93 ± 2.44 d	120.87 ± 1.71 b	140.93 ± 2.69 a	42.71 ± 0.73 c
Leaf				
Total phenols ¹	12.69 ± 0.68 d	40.96 ± 2.30 a	23.76 ± 0.80 c	30.37 ± 0.38 b
Total flavonoids ²	70.30 ± 0.53 b	56.69 ± 3.41 c	42.21 ± 2.06 c	91.54 ± 3.05 a
Condensed tannins ²	86.28 ± 0.16 a	53.86 ± 7.30 b	39.76 ± 0.98 b	71.67 ± 0.00 a
Total anthocyanins ³	43.57 ± 1.95 c	88.71 ± 11.89 b	121.04 ± 0.27 a	107.73 ± 3.17 ab

Different letters in same row indicate significant differences ($p < 0.05$) by Tukey's test. Values are expressed as ¹mg equivalents of gallic acid/g, ²mg equivalents of +catechin/g, ³mg equivalents of cyaniding-3-glucoside/g.

of the pathogen in the host plants, leaves from each sampling date showed higher concentration of a different response variable. For instance, total phenols were higher in the foliar tissue of plants from the first sampling, while the highest content of total anthocyanins and flavonoids corresponded to the second and third samplings, respectively. In contrast, noninfected plants had significantly greater foliar levels of condensed tannins.

3.3 | PCA for the pepper plant tissues based on phenolics content

To provide a better visualization of the complete data set in a single dimension plot, PCA was performed. PCA demonstrates that almost 90% of the observed variance could be explained by the first two components. PC1 accounted for 71.8% of total variability from the analysis of polyphenolic compounds, although PC2 represents only the 17.9% (Figure 4). Positive values of PC1 indicate a higher concentration of total phenols, total flavonoids and condensed tannins. This group revealed a clear separation of the foliar tissue from the rest of the samples, indicating that although “*Ca. P. trifolii*” infection leads to greater synthesis of polyphenols on the host plant, in general, the leaves might represent the part of the plant with the highest concentration of these metabolites, even if the plant is free of the phytoplasma infection.

It is interesting that the total anthocyanins represented in the positive values of the PC2 appear in greater concentration in the stem collected from the second sampling, which suggest that although many compounds of the stem were imported from leaves, anthocyanins remain as the principal metabolites for this tissue. Negative values of the PC1 and PC2 did not represent a main effect of the infection over the response variables evaluated. By chance, the difference between infected and healthy roots was relatively small compared to the

difference between the infected and noninfected stems and leaves. This finding indicates that “*Ca. P. trifolii*” infection resulted in minor changes in the metabolite concentration in the root tissue.

Figure 5 depicts the concentration of polyphenols in the developed fruit and big bud collected from the healthy and infected plants of the later sampling date. It is important to mention that the collected big bud had an undeveloped fruit in the centre of the malformed structure. All response variables evaluated in this study were higher in the big bud compared to the healthy developed fruit; data indicated that the infected plant increased the accumulation of polyphenols in the big bud tissue with an upsurge of 11%, 171%, 285% and 122% for total phenols, flavonoids, condensed tannins and total anthocyanins, respectively.

4 | DISCUSSION

The efforts to control the dissemination of “*Candidatus Phytoplasma trifolii*” to new economically important crops has been limited to identifying its presence by molecular methods; nevertheless, we have to go beyond the diagnosis of the phytoplasmas and focus on the biochemical changes that occur as a result of the infection and thus understand the metabolic pathways that are inhibited or activated when the plant is infected. During this work, the presence of “*Ca. Phytoplasma trifolii*” associated with symptomatic chilli pepper plants confirms that it is the most widely distributed phytoplasma in the state of Zacatecas, Mexico, where it has been reported affecting chilli pepper (Mauricio-Castillo et al., 2015) and tomato (Salas-Muñoz, Velásquez-Valle, Teveles-Torres, Creamer, & Mauricio-Castillo, 2016). The high similarity at DNA sequence level among the phytoplasmas isolated from the symptomatic chilli plants allowed the selection of a clone (Mch2-22) to do the sequence analysis.

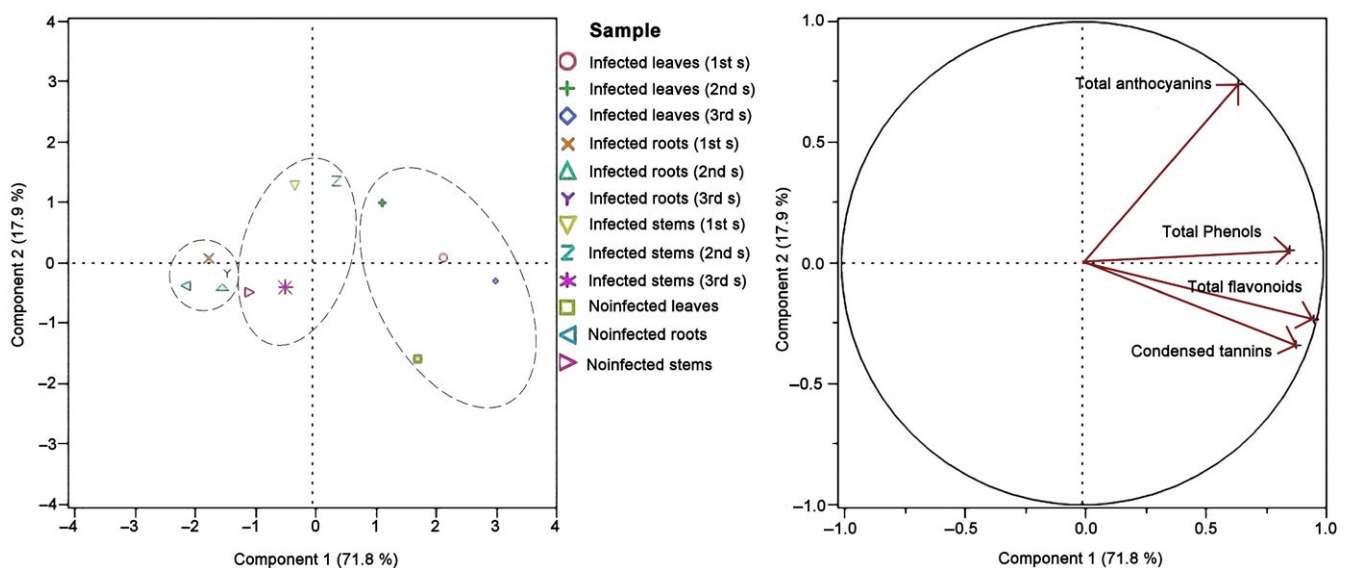


FIGURE 4 Principal components analysis for the root, stem and leaf tissue based on phenolics content: total phenols, flavonoids, anthocyanins and condensed tannins. s, sampling. Results are grouped into different plant tissues

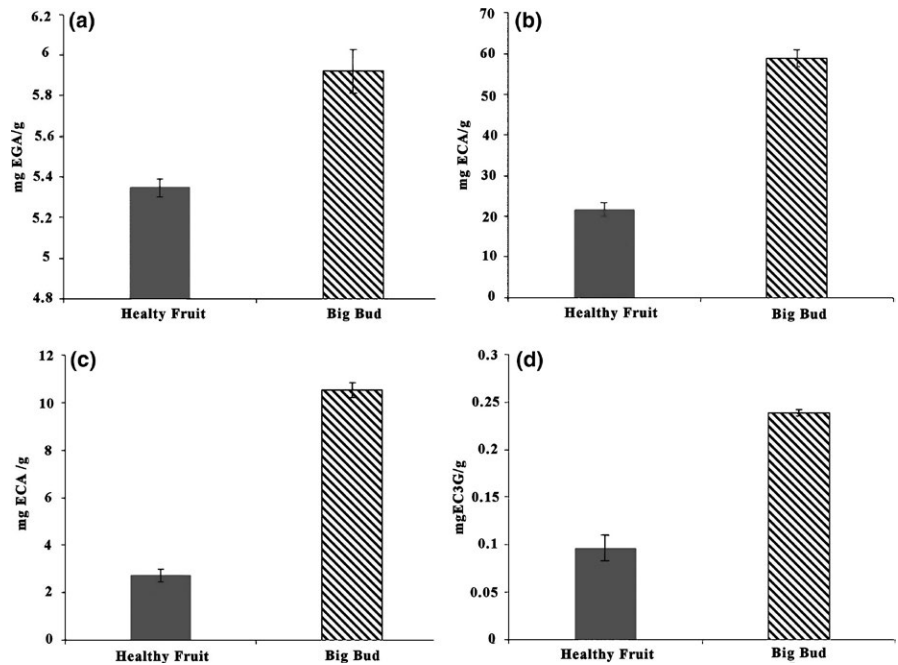


FIGURE 5 Phenolic compound concentration in the healthy fruit and the big bud of infected plants. (a) total phenolics. (b) Total flavonoids. (c) Condensed tannins. (d) Total anthocyanins

When faced with pathogen infection, the host plant activates a multicomponent physical and biochemical response involving radical changes in the expression patterns of genes, proteins and metabolites (Ying-Ping et al., 2014). Some studies have revealed that phytoplasma infection can modify the levels of different components in plant tissues through modifications in the cell wall and local accumulation of secondary metabolites such as polyphenols (Himeno et al., 2014; Musetti et al., 2000). The current results reveal that phytoplasma infection changes the level of polyphenols in the root tissue; it has been well established that phytoplasma severely affects phloem function in susceptible plants, impairing the transport of soluble organic material, particularly to the roots (Weintraub & Jones, 2010). However, this study shows that “*Ca. P. trifolii*” presence in the pepper plants was not followed by a decrease in phenolics in the infected root tissue. On the other hand, our data showed that the polyphenolic compounds in the infected roots were lower than those quantified in the remaining tissues, and these findings suggest that phytoplasma-infected plants tend to accumulate less polyphenols in the sink organs such as roots, in comparison with the source leaves and stems. In other words, “induced” responses among plant tissues may be active shifts in resource allocation, arising because the biotic elicits responses caused by phytoplasma, affecting source and sink units unequally. In addition, the higher accumulation of different phenolic compounds was time sampling dependent; these results suggest that phenylpropanoids including flavonoids and phenolic compounds, that play important roles in the ability of plants to fight against attacking pathogens (Dixon et al., 2002), may take part in multiple branches of the phenylpropanoids pathway differently, according to the severity of the infection.

There is insufficient information about how “*Ca. P. trifolii*” infection may affect metabolism in pepper stems. The current study shows

that, in general, delayed sampling and thereby a prolonged exposure to phytoplasma infection were not followed by proportional increase in these compounds in the stem tissue; however, it is clear that accumulation of phenolic compounds is higher in the infected plants than in the healthy ones. In accordance, Wallis et al. (2015) evaluated the effect of “*Candidatus Liberibacter solanacearum*” in potato plants and found that this infection caused a high accumulation of phenolics in the stems, particularly hydrolysable tannins. In this context, they mention that depending on the tissue, phenolics involved in cell wall strengthening, as a response to bacterial infection, might be targeted around making thicker cell walls to limit pathogen movement and access to resources. Likewise, because the “*Ca. L. solanacearum*” bacterial infection process involved psyllid feeding, the thicker cell walls also could be a hypothesized host defence response against the vectors. In the current study, direct wall measurements were not evaluated; therefore, studies regarding this response variable should be assessed and correlated with pathogens titres.

Interestingly, anthocyanin concentration in the second-sampled stem tissue was the only manifestation of a higher accumulation of phenolics in the stem compared to the foliar tissue; an anthocyanin that is highly synthesized and accumulated among different chilli pepper organs (such as leaves, stems, flowers and fruits) is delphinidin-3-trans-p-coumaroyl-rutinoside-5-glucoside (Aguilar-Barragán & Ochoa-Alejo, 2014). In this sense, an individual anthocyanins profile is suggested, to verify whether this compound is over synthesized by infected plants and translocated to the stems.

As previously described, the foliar tissue exhibited the highest concentration of the phenolic compounds evaluated. In this regard, Foyer and Noctor (2003) mention that the function of phenolic compounds is more important for leaves compared to other organs of plants during pathogen infection, as chloroplasts, where the formation of H_2O_2 and other reactive oxygen species (ROS) occur, are

located in the leaves. When plants are attacked by pathogens, they respond by activating a variety of defence mechanisms, including the rapid production and accumulation of ROS; hence, the phenolic content of the plants increases (Dikilitas et al., 2011). In agreement to the current results, Huseynova et al. (2017) reported the overexpression of some metabolites in pepper plant leaves due to the effect of "*Candidatus Phytoplasma solani*," an amount four times higher of total phenols was detected in the infected foliar tissue in comparison with the healthy control. They attributed their finding to the high activity that polyphenolics have due to the aromatic rings and free hydroxyl groups in their composition, which easily react with free radicals and scavenge ROS formed during stress. In regard to proanthocyanin synthesis, although condensed tannins biosynthesis has been well characterized as wound-induced defences and it is known to be elicited by herbivore wounding (Arnold et al., 2004), these proanthocyanin type phenolics were found to also be affected by the presence of "*Ca. P. trifolii*" in the root and stem of pepper plants, but not in the leaves. Therefore, there is an unresolved issue regarding the metabolic mechanism at the phenylpropanoids level at which the plant controls the allocation of resources between source and sink organs at the whole-plant level.

Concerning the polyphenolics concentration of the developed and undeveloped (big bud) fruit, the outstanding result was the dramatic accumulation of anthocyanins in the big bud; this structure had the highest concentration of total anthocyanins among the different tissues collected from the whole-infected plants. Margaria et al. (2014) suggest that an explanation for the activation of anthocyanins biosynthesis may reside in the sugar concentration increase in the infected leaves, as accumulation of soluble carbohydrates has been reported in phytoplasma-infected leaves of several host cultivars, and they induce the anthocyanin synthesis pathway. Anthocyanins are particularly important for protecting photosystems from ROS generated by photon-saturated photosystem in leaf cells, therefore could have an antioxidant role, limiting further oxidative damage (Hoch, Singsaas, & McCown, 2003; Margaria et al., 2014). Furthermore, the observed results may indicate that when phytoplasma infection persists in the host plant as the fruit set progresses, anthocyanins are strongly reallocated to the modified flower structure or undeveloped fruit forming the structure known as big bud.

In conclusion, the observed results, besides providing a biochemical description of the phenylpropanoids synthesis in response to "*Ca. P. trifolii*" infection in pepper plants cv. Mirasol, raise interesting data concerning the allocation of polyphenols among different tissues, which indicates that phytoplasma infection affects the transport of resources between sink and source organs. Furthermore, this allocation is also affected by the exposure time to the pathogen after the fructification stage, thus the severity of the disease. In addition, it is worth mentioning that the first report of the "*Ca. P. trifolii*" in the state of Zacatecas, México, was made in 2015; therefore, the infection symptomatology was not clear enough at the sampling dates, and hence, phenotypic detection in the pepper samples used in the current

study relied on the big bud symptom. To our knowledge, this is the first paper that reports the effect of phytoplasma infection over secondary metabolites synthesis in four different vegetative tissues of the pepper plant; however, further studies are required to elucidate metabolic changes in a much larger group of metabolites. Moreover, to understand the interactive effects that may occur with other factors such as environmental conditions (by increasing sampling sites), and different pepper types and phenological stages, additional investigation is highly recommended.

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CONFLICT OF INTEREST

There is no conflict of interest to declare.

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