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Full Length Research Paper

Water use efficiency in soybean crop after inoculation with *Azospirillum brasiliense* in the Cerrado of Tocantins State, Brazil

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Crops in the Cerrado experience short drought stress periods that affect cultivation development, especially the water use efficiency (WUE), which can be modified by the use of *Azospirillum brasiliense* bacteria. In this sense, this study evaluated soybean plants' response to *A. brasiliense* inoculation, in relation to WUE, in the Cerrado of Tocantins State. During the growing season of 2015/2016, three experiments were conducted in Palmas municipality, with analyses in the phenological stages: R3 for the first planting and R2 for the second and third plantings. The experimental design used in each experiment was randomized block with ten treatments and four replications. The treatments were disposed in 2x5 factorial scheme, represented by two cultivars (M-9144RR and TMG 1288RR) and five different doses of *A. brasiliense* (0, 100, 200, 300 and 400 ml of the commercial product per hectare). They were applied through the leaf at V2 and R1 stages of the crops. WUE determination was done through photosynthesis measurement (IRGA), through which the photosynthesis and transpiration values were obtained. There was no WUE difference between the cultivars. *A. brasiliense* doses had effect in relation to WUE, on stressed crops. *A. brasiliense* inoculation did not show any significant change in WUE in crops with favorable conditions.

Key words: *Glycine max*, diazotrophics, photosynthesis, transpiration.

INTRODUCTION

The State of Tocantins, with an area of 227.720,567 km^2, is located at a region designated as Legal Amazon. It involves both the Amazon and the Cerrado biomes, in which territorial space is located in the largest hydrographic basin fully located inside the Brazilian territory (Tocantins-Araguaia River Basin). In this region, cultivation land is equivalent to 50% of the state territory. This, combined with logistic facilities for connecting to all parts of the country, has attracted the attention of farmers, national companies and international trading, who aim the internal and external market (EMBRAPA, 2016).

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Table 1. Soil chemical analysis results from the experimental area in Palmas, growing season of 2015/2016.

pH	P	K	Al	H+Al	Ca+Mg	CTC	V	MO
(H_2O)	$mg.dm^{-3}$			$Cmol_c.\ dm^{-3}$			%	
5.65	17.28	40.0	0.0	1.5	3.3	5.5	61.82	1.8

PH: Hydrogen ionic potential; P: phosphorus; K: potassium; Al: aluminum; H + Al: potential acidity; Ca+Mg: calcium + magnesium; CTC: cationic exchange capacity; V: saturation by bases; MO: organic matter.

In the Cerrado, under low latitude conditions, due to drought periods and high temperatures, plants are subjected to stress periods, resulting in a reduction of the photosynthetic rate. This is caused by the decrease in plant metabolism that occurs due to increased diffusion resistance of atmospheric CO_2 to reach the carboxylation site caused by stomata closure (Cruz et al., 2016). Therefore, the utilization of parameters such as the water use efficiency (WUE) would allow effect fixing of carbon while transpiring (Ramos et al., 2014).

As the water use efficiency is related to the photosynthetic rate and transpiration of the plant, the application of this concept allows plants to adapt to water stress (dry), caused by the closure of the stomata, and makes plant to have osmotic adjustment, which is among other mechanisms used to adapt to the effective control of water use, even under low availability of water. It aims to stabilize production in the face of climatic changes. Thus, the adoption of cultivars with greater efficiency in the use of water, associated with the use of growth-stimulating microorganisms and increased photosynthetic rate, even under adverse conditions, could increase the tolerance of plants to water and thermal stress (Bulegon et al., 2016).

The use of plant growth promoter bacteria (PGPB) is a promising alternative to meet the growing food demand and also the necessity of natural resources conservation in the Cerrado, under favorable and unfavorable cultivating conditions (Cohen et al., 2015).

Revising the literature, there are several reports related to the beneficial effects of growth promoter bacteria (PGPB) under the following conditions: nitrogen biological fixation (Huergo et al., 2008), increased nitrate reductase activity when it develops as an endophytic in plants (Cassán et al., 2008), biological control of pathogens (Correa et al., 2008), enhanced plants' productivity (Rodrigues et al., 2014) and the production of hormones such as auxins, cytokinins, gibberellins, ethylene and a variety of other molecules (Bulegon et al., 2016).

Hungria (2011) indicated that corn and wheat showed high yield when they were inoculated with *Azospirillum brasiliense* as compared to the control plants. *A. brasiliense* supplied nitrogen to the inoculated plants and produced phytohormones that stimulated root growth, due to the liberation of indole acetic acid (IAA), gibberellins and cytokinins. In addition, green corn plants in Marilia-SP exhibited higher chlorophyll content, stomatal conductance, hydric potential, cell wall extensibility, biomass production and high plant height after being inoculated with *A. brasiliense* compared to the control counterpart plants (Neto et al., 2013).

Several authors reported the use of *Bradyrhizobium* bacteria associated with *A. brasiliense* in different regions, covering different types of soils and climate. However, there are no reports of work in the state of Tocantins on the inoculation of this bacterium in association with *A. brasilienses* that influences the water use efficiency in soybean crop.

In this context, it is known that in legumes and/or grasses, the concomitant use of these bacteria has economic and environmental benefits. Due to the scarcity of information on the behavior of soybean plants inoculated with *A. brasilienses* regarding water efficiency use (WUE) in the Cerrado biome, the present study was carried out to evaluate the behavior of two soybean cultivars when inoculated with different doses of *A. brasiliense* in relation to WUE.

MATERIALS AND METHODS

In 2015-2016 growing season, three experiments were developed in the experimental area of the Tocantins Federal University (TFU) in the municipality of Palmas-TO (latitude 10°45' S, longitude 47°14' W, 220 m of altitude above sea level). Plantations were carried out in November 18th, December 2nd and December 17th 2015. The soil of this region is classified as Dystrophic Yellow Red Latosol (EMBRAPA, 2013); the region has plain relief, Aw climate, and is tropical with a dry season. Soil sample collection was performed at 0 to 20 and 20 to 40 cm depth layers according to the EMBRAPA methodology (2011). The soil chemical analysis was performed by a private soil analysis laboratory that meets the requirements of the Program of Quality of Analyzes of Laboratories of Fertility-PAQLF (EMBRAPA, 2011). The results are presented in Table 1.

The experiments were laid out in a randomized block design with ten treatments and four replications. The treatments were organized in factorial scheme 2x5, represented by two cultivars (M-9144RR and TMG 1288RR) and five doses of *A. brasiliense* (0, 100, 200, 300 and 400 ml of the commercial product per hectare). They were applied on the leaf surface: half of each dose was applied at V2 stage and the other half at R1 stage of the crops. The *A. brasiliense* strains used were AbV5 and AbV6, with the commercial concentration of 2×10^8 $Ufc.ml^{-1}$.

Meteorologic data (Figure 1) were obtained through the INMET, 2016 web site, based on the meteorologic station installed in the same municipality where the experiments were conducted.

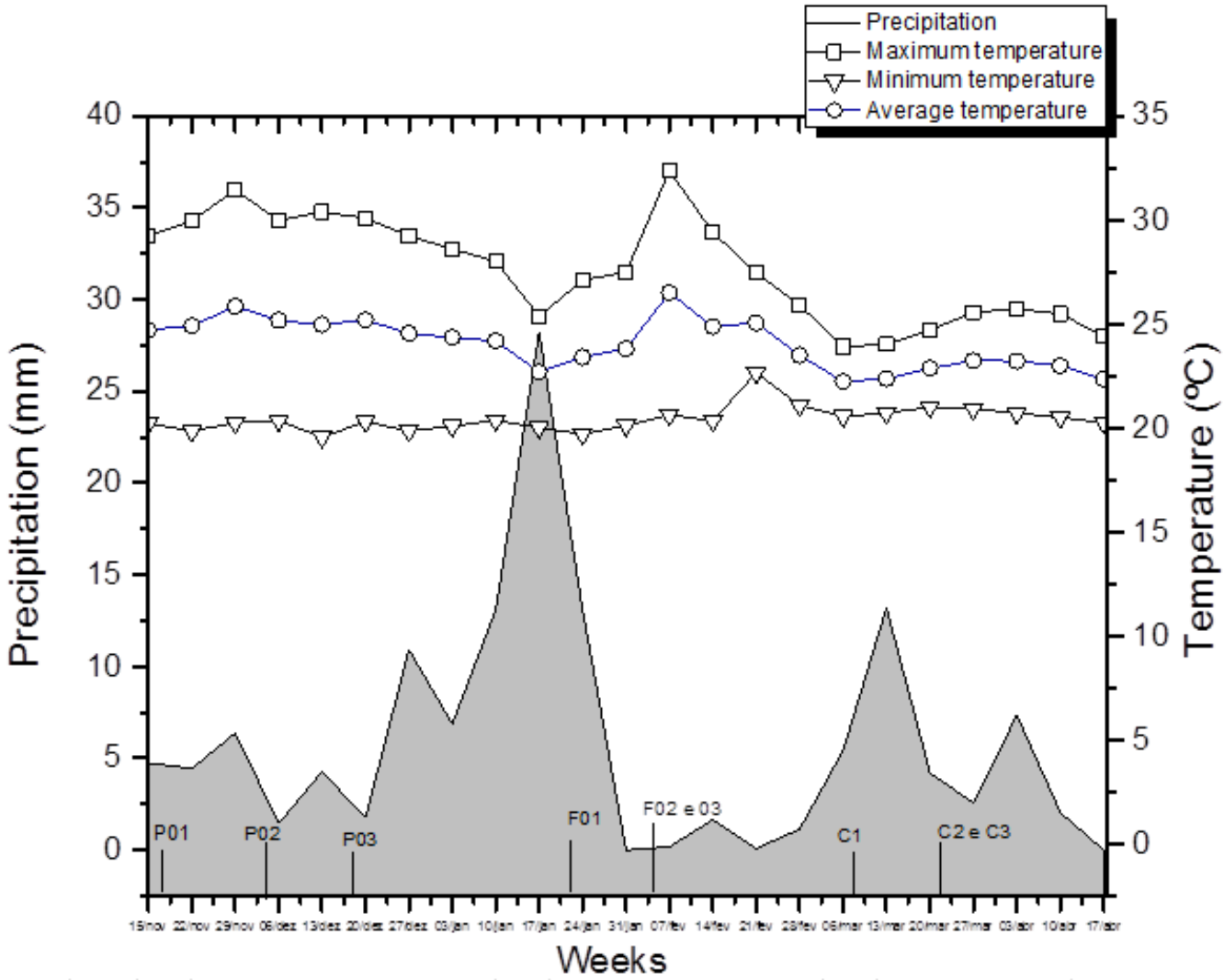

Figure 1. Weedkly climatic data from Palmas between Novemeber 15th, 2015 and April 17th, 2016.

Both cultivars M9144RR and TMG 1288RR are traditionally planted in the region, presenting determined growth habit and high fertility requirement. Based on maturity groups, cultivar M9144RR belongs to group 9.1 and TMG 1288RR to group 8.8.

Before planting, the soil was plowed and harrowed. The experimental plots had four rows of 5.0 m length, spaced by 0.5 m, totaling a plot area of 10 m^2 with 300 plants. When the inoculant was applied via the leaf, the sprayer jets were directed to the plants of the two central rows of the plot so that the lateral rows functioned as a border for avoiding the contamination of the other plots. 400 kg ha^{-1} of simple superphosphate corresponding to approximately 80 kg of P_2O_5 ha^{-1} was applied. Planting density was carried out in order to obtain 15 plants per linear meter, and 10 days after emergence, thinning was done when necessary. Final plant population was about 300.000 plants/ha^{-1}. The seeds were treated with fungicide (Carboxin + Thiram 200 SC -> 2.5 mL + 2.5 mL of water kg^{-1}). During planting, they were inoculated with *Bradyrhizobium japonicum* strains, using the product Biomax® Premium Peat- Soybean (Strains SEMIA 5079 + SEMIA 5080), at 60 g/50 kg dose of seed. At harvesting stage, only plants from the net plots (two center rows in each plot) were used. During the crop cycle, no irrigation was used, as water was available to the plant through precipitation.

The pests were controlled by applying the active principle Bifenthrin + Imidacloprid (50 + 100 gL^{-1}). Azoxystrobin (0.5 L ha^{-1}) was applied to control diseases and weeds where Glyphosate (360 gL^{-1}) was needed. Fertilization with potassium chloride at 166 kg ha^{-1} (approximately 100 kg ha^{-1} K_2O) was performed; half of the dose was applied at V2 stage and the other half at V4 stage.

Photosynthesis (A) and transpiration (E) were measured at R3 stage for the first planting time, and at R2 for the second and third planting time, using a portable photosynthesis measurement system (IRGA Infrared Gas Analyzer) model Li- 6400 (Li-Cor, Biosciences Inc., Nebraska, EUA). Measurements were performed at 9:00 am, when leaves were totally expanded and mature at the two central rows of each plot.

Table 2. Summary of the variance analysis of water use efficiency (WUE) due to planting time, cultivars and leaf *A. brasiliense* inoculation doses in soybean plants cultivated in Palmas-TO, during 2015/2016 growing season.

VS	DF	MS
Planting times	2	2.1501*
Cultivars	1	0.0804*
Leaf inoculation doses	4	0.0823*
Planting time x cultivars	2	0.1024*
Planting time x leaf inoculation doses	8	0.0642*
Cultivars x leaf inoculation doses	4	0.0637ns
Planting time x cultivars x leaf inoculation doses	8	0.0403*
Block/planting time	6	0.0060^{ns}
Error	54	0.0177
Total	89	
CV (%)	6.05	

*Significant p<0.05 and non-significant p<0.05, by the F test.

During measurements, photosynthetic active radiation (PAR), atmospheric CO_2 inside the leaf chamber and the chambers block temperature were kept constant, with values of 2000 µmol photons m^2 s^{-1}, 380 to 400 µmol CO_2 m^2 s^{-1} and 26 to 27°C, respectively. The reference air was collected and homogenized in a 5 L gallon before reaching the assimilation chamber. The net photosynthetic rate per unit leaf area (A) (µmol CO_2 $m^2.s^{-1}$) and the transpiration (E) (mol H_2O $m^2.s^{-1}$) were calculated using the variations of CO_2 and steam values inside the chamber, measured by the infrared gas analyzer of the portable photosynthesis system (Von Caemmerer and Farquhar, 1981). With the results of this system, WUE was calculated through the ratio between A (net photosynthetic rate) and E (transpiration).

The WUE data obtained in each planting time were subjected to analysis of individual variance and, in sequence, to an analysis of combined variance in which the smallest mean residual square did not differ more than seven times from the highest (Cruz and Regazzi, 2004). In the following step, the cultivars rates and planting time were compared by the Scott-Knott test, at 5% significance. For each *A. brasiliense* dose, in each cultivar and planting time, a polynomial regression model was adjusted. In the absence of functional relationship between doses and cultivars, the Scott-Knott test was applied, at 5% significance.

The statistical program used to analyze the data obtained was SISVAR 5.0 (Ferreira, 2011) and for the construction of the graphics, the software Origin Pro 8.0 was used.

RESULTS AND DISCUSSION

The analysis of variance for WUE (Table 2) revealed significant effect for all factors, except for the interaction of cultivars x leaf inoculant doses of *A. brasiliense.* The significance of the triple interaction indicates the cultivars' differential effect due to different doses and planting times. The latter is represented by variation in precipitation and temperature during the experimental period (Graphic 1). The coefficient of variation obtained was of 6.05%, indicating a good accuracy in conducting the experiments (Scapim et al., 1995). The statistical average for WUE, net values of photosynthesis rate (A) and transpiration (E), resulting from the triple interaction planting time x cultivar x *A. brasiliense* doses are presented in Table 3. As there was no functional relationship between *A. brasililiense* doses and the cultivars or/and planting times, a mean grouping test was used in the comparative study between the doses of A. *brasiliensis*. The WUE values obtained in this study were superior to the ones obtained by Nonato (2016), in soybean plantations subjected to different doses of *A. brasiliense* and *B. japonicum* inoculation.

In relation to the cultivars, for each dose and planting time, no significant differences were detected in the WUE, probably due to cultivars that had similarities in the number of days to complete their cycles, as they belonged to groups having very close maturation to each other. Procópio et al. (2004) studied WUE in soybean crops, beans and weed but did not find differences in WUE, as compared to plants with similar cycle. In addition, they observed that plants with long cycle tend to be more efficient in using water in water-limited environments. In this way, when selecting cultivars for regions with periods of drought, we must take into account not only the WUE, but also the cycle of cultivars to be used in order to maintain production. When *A. brasiliense* doses were compared in each cultivar and within the same planting time, no significant effects were detected in relation to WUE between the inoculant doses in the first planting time (Table 3). On the other hand, for the following planting times, there were significant differences between *A. brasiliense* doses to soybean cultivars. This might be related to the response of different cultivars to *A. brasiliense* doses used in this study.

At the first planting time ($11/18^{th}$), there were more favorable conditions of precipitation and temperature (Figure 1) for the plant development in relation to the second ($12/02^{nd}$) and third ($12/17^{th}$), when some hydric

Table 3. Deployment averages between planting time x cultivar x dose to water use efficiency in soybean crops, in Palmas – TO, during the growing season of 2015/2016.

Planting times	Dose 0		Dose 100		Dose 200		Dose 300		Dose 400	
	9144	1288	9144	1288	9144	1288	9144	1288	9144	1288
18/11	2.50^{Aa1}	2.46^{Aa1}	2.61^{Aa1}	2.44^{Aa1}	2.71^{Aa1}	2.48^{Aa1}	2.44^{Aa1}	2.36^{Aa1}	2.48^{Aa1}	2.32^{Aa1}
02/12	2.21^{Ba1}	2.07^{Ba2}	2.08^{Ba2}	2.19^{Ba1}	2.55^{Aa1}	2.39^{Aa1}	2.13^{Ba1}	2.04^{Ba2}	2.27^{Aa1}	2.10^{Aa1}
17/12	2.02^{Ba1}	2.06^{Ba1}	1.81^{Cb2}	1.98^{Ba1}	1.78^{Ba2}	1.97^{Ba1}	1.90^{Ca2}	2.02^{Ba1}	1.99^{Ba1}	1.70^{Ba2}
A/E (1º)	19.6/9.3	18.35/7.4	21.6/8.7	17.8/7.1	16.1/6.8	17.0/7.4	20.2/6.6	16.2/7.7	25.2/7.5	14.6/5.8
A/E (2º)	18.1/8.5	17.1/7.6	16.2/7.7	15.2/6.8	16.6/6.3	14.3/6.4	15.7/6.1	14.8/6.6	15.2/7.9	15.1/6.4
A/E (3º)	17.1/7.6	14.9/7.5	13.9/8.2	14.8/8.4	16.6/9.1	11.64/7.2	16.6/7.8	14.1/7.5	14.2/6.8	10.2/4.6

Means between planting times, considering the same cultivar and dose (ml / hectare), followed by the same capital letter in a column, do not differ from each other by the Scott-Knott test with 5% of probability; Means between cultivars, considering the same planting time and dose, followed by the same lowercase letter in line, do not differ from each other by the Scott-Knott test with 5% of probability. Means between doses, considering the same cultivar and the same planting time, followed by the same number, do not differ from each other by the Scott-Knott test with 5% of probability. A/E– statement of photosynthesis rates and transpiration, considering cultivars, doses and planting times.

deficit and higher temperatures occurred during flowering and grain filling periods (Figure 1). This differential result for the USA in relation to the seasons and cultivars demonstrates the efficiency of *A. brasiliense* in adverse conditions. This suggests its use in crops in the Cerrado as a palliative to the damages caused to the production due to drought.

In plants subjected to hydric deficit and high temperatures during the reproductive stages, some physiological change such as stomatal closure was observed in this study. In addition, we also observed a reduction in atmospheric CO_2 availability at the carboxylation site, promoting reduction in photosynthetic activity and transpiration (Flexas et al., 2008; Hatfield et al., 2011; Ku et al., 2013). This resulted in winding and falling of leaves, flowers and pods abortion, leading to low productivity (Hatfield et al., 2011; Ku et al., 2013).

Studies have shown that reduction in stomatal conductance under adverse conditions may restrict CO_2 fixating rate, decrease the concentration in the substomata cavity and intercellular spaces, which would result in low photosynthetic rate (Soares et al., 2013; Lauteri et al., 2014). The photosynthetic rate reduced in this work. This confirms the above in relation to the cultures of the second and third seasons, due to drought periods that cause the decrease of the US values when comparing the three growing seasons.

According to Serraj and Sinclair (2002), WUE for water stress (dry) may be associated with osmotic adjustment. It contributes to water absorption and cellular turgor maintenance in a way that physiological processes, such as photosynthesis and cell enlargement, are not interrupted. In plants subjected to different water level availability on soil, it was found that WUE index increased, resulting in stomata closure as plants responded to hydric stress, seeking to reduce transpiration (Mencuccini et al., 2000; Tatagiba et al., 2008; Maes et al., 2009; Roza, 2010). These variations are also important for the maintenance of production in regions that have drought periods during the crop cycle. Examples are crops in the Brazilian Cerrado, that serve as reference in the selection of cultivars that present a better response to drought.

Bulegon et al. (2016) reported that transpiration tends to be lower in non-co-inoculated plants and photosynthesis tends to be stable with the use of *A. brasiliense*, influencing the water use efficiency of soybean. In this work the obtained results present higher values for transpiration and photosynthesis in plants that did not receive *A. brasiliense* as compared to the plants that received different doses of the inoculant (Table 3).

Antony and Singandhupe (2004) studied the irrigation influence on WUE in sweet pepper crops and verified a negative linear relationship with net photosynthesis. In other words, photosynthesis rate increases are associated with a WUE reduction, due to the stomatal opening. Hungria, (2011) reported increase in plant tolerance to abiotic stresses due to the production of phytohomones when they were associated with *Azospirillum* bacteria genera. Rodrigues and Fioreze (2015) also reported similar effects with further discovery that these bacteria identify signs emitted by plants under stressful conditions; they trigger responses along with plant, resulting in increased tolerance to different stresses.

Different authors did not obtain an increase in productivity of beans and soybeans using *A. brasiliense* (Gitti et al., 2012; Bassani et al., 2015; Zuffos et al., 2016). Despite this, an increase in yield has been observed when the plants were associated with *A. brasiliense* as N source for soybean crop (Hungria, 2011), and when corn plantations were associated with *A. brasiliense* together with manure as a nitrogen source (Muller et al., 2016). The comparative study including planting time, different doses and within the same cultivar revealed a higher WUE value at the first planting time ($11/18^{th}$), for both cultivars and all *A. brasiliense* doses; yet, do not differ significantly from the second planting

time (12/02nd) in the 200 and 400 ml doses of the commercial product ha-1. However, smaller WUE value was obtained at the third planting time (12/17th).

Under water conditions adequate for the development of the plants such as those found in the first planting season (Figure 1), the WUE values were derived from a greater increase in the liquid photosynthetic rate (A) in relation to the transpiration (E) due to the availability of water for the plants (Table 3). On the other hand, in the second and third planting seasons, where the availability of water to the plants was less favorable to their development, lower WUE values were obtained as a result of a greater decrease of A in relation to E. The variation between the values found from A and E demonstrates the plants' response to water stress caused by periods of drought during the plants' development. Considering that the ratio between these parameters indicates the WUE, the decrease of A and increase of E to the detriment of the water conditions of the plant cause the WUE to be smaller in the second and third planting seasons.

The smallest WUE values in the second (12/02nd) and third (12/17th) planting times, of all different *A. brasiliense* doses, occurred probably as a result of a greater abscisic acid (ABA) accumulation in the crops planted in December as compared to the first planting time (11/18th). This is due to ABA synthesis produced by the plant itself (promoted by the unfavorable environments) and also, to the *Azospirillum* ABA synthesis, which promoted greater reduction in the photosynthetic activity (A) to the detriment of the transpiration (E).

Under hydric stress, plants produce substances like proline, which is regulated by abscisic acid (Sharma and Verslues, 2010) and inorganic ions. These stabilize membrane proteins and lipids structure, preserving enzymes functions while making the water adhere to these molecules structure in dehydrated cells (Cohen et al, 2009; Sharma and Verslues, 2010; Khan et al., 2013; Cohen et al., 2015).

Cohen et al. (2015) evaluated the response of *Arabidopsis thaliana* plants associated with *A. brasiliense* under drought conditions and found that abscisic acid (ABA) produced by the bacterium increased the root system and stomatal closure due to proline synthesis. Besides, the bacteria provided three times more ABA to the inoculated plants than the control plants under similar experimental conditions.

Conclusion

The cultivars show the same behavior in relation to the WUE, showing that they have the same mechanism of response to water stress. The action of *A. brasiliense* on the WUE becomes perceptible under conditions of water stress for the plant due to the effect of the hormones available to the plant that responds to water stress. The doses of inoculant containing *A. brasiliense* present different results due to the bacterial concentration applied in the plant. With the use of *A. brasiliense* there is a greater reduction in the A and E values under water stress as compared to the absence of the bacterium being recommended to crops in places with periods of drought.

In the selection of cultivars that present the best WUE indices, the crop cycle must also be observed. Inoculation with *A. brasiliense* did not promote significant changes in the WUE in crops under favorable conditions. The concept of WUE can be used in genetic improvement through the selection of cultivars that present better responses to the dry period, aiming at maintaining production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Assessment of three cocoa hybrid families' susceptibility and cysteine involvement in defense process against *Phytophthora megakarya*

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Black pod disease (BPD) is the major cocoa pathology constraint caused by an Oomycete, *Phytophthora megakarya* prevailing in African cocoa producing countries. The development of *T. cacao* planting material tolerant to BPD lies on cross-pollination of adequate parental genotypes which generate offspring with desirable traits. We assessed the susceptibility to BPD of offspring derived from three manual crosses (SNK13xUPA143, T79/501xUPA143 and UPA143xSNK64) using leaf discs test (LDT) which evaluates disease scores of hybrid genotypes. Cysteine involvement in *T. cacao* defence process against BPD has been studied for the potential use of this sulphur amino acid profile to identify tolerant cocoa hybrid genotypes. LDT displayed variable disease scores patterns. Within a given family, LDT revealed heterogeneity in disease scores. This heterogeneity may have been derived from polygenic character of *T. cacao* susceptibility to BPD. In SNK13xUPA143 family, 26.13% exhibited disease scores lower than two (tolerant hybrid genotypes). T79/501xUPA143 and UPA143xSNK64 generated 21.18 and 26.46% of tolerant hybrid genotypes respectively. This variability between families may have resulted from deferential susceptibility of parental clones; UPA143 being more susceptible than T79/501 followed by SNK13. SNK64 was the less susceptible to BPD. The percentages of tolerant hybrid genotypes in this study appeared to be consistent hence, SNK13xUPA143, T79/501xUPA143 and UPA143xSNK64 could be used to produce hybrids genotypes tolerant to BPD. Cysteine analysis was performed in none detached healthy and wounded-infected leaves of hybrid genotypes with variable disease scores. It appeared that cysteine was mobilized during the infection. Two main patterns were observed: in tolerant hybrid genotypes (disease score lower than 2), infection was associated to a significant reduction of cysteine content in young leaves while in susceptible hybrid genotypes, infection was associated to an increase (accumulation) of cysteine in young leaves. Cysteine could be involved in the synthesis of effective defence molecules against *P. megakarya* in tolerant hybrid genotypes. This set of finding may indicate that cysteine profile could be used to discriminate tolerant from susceptible hybrid genotypes of *T. cacao* to *P. megakarya*.

Key words: Cocoa tree, black pod disease, hybrids genotypes, tolerance, cysteine.

INTRODUCTION

Chocolate tree (*Theobroma cacao* L.) is a tropical rainforest plant widely cultivated in Africa, America and Asia (Alverson et al., 1999; Whitlock et al., 2001; Acebo-Guerrero et al., 2012; Ngoh Dooh et al., 2015). In Cameroon, cocoa is one of the most economically important crops grown by small farmers for their livelihoods (Sonwa et al., 2008; ICCO, 2013, 2015). However, there is approximately 50 - 80% of cocoa production harvest losses because of an oomycete, *Phytophthora megakarya*, the pathogen of black pod disease in Cameroon (Djocgoue et al., 2010; Mfegue, 2012). Classically, pesticides are used to reduce the incidence of black pod disease (BPD). However, these chemicals appeared to be environmentally unfriendly, expensive, and inefficient (Sonwa et al., 2008). Hence, breeding for tolerance to BPD appeared to be the way out for sustainable cocoa culture (Iwaro et al., 2005; Ramalho et al., 2012; Bohinc and Trdan, 2012). For this, genetic improvement of cocoa through generative strategy is mostly recommended (Tahi et al., 2000; Nyassé et al., 2003; Ondobo et al., 2014; Effa et al., 2015). Generative breeding is based on the aptitude of a giving couple of parents (genotypes or clones) to generate offspring with desirable traits (Eskes and Lanaud, 1997). Obviously, many couples have not yet been assessed in this purpose, such as SNK13xUPA143, T79/501xSNK13 and UPA143xSNK64

SNK64 clone is the less susceptible to BPD, followed by SNK13, T79/501 (obtained from P7xNa32) and UPA143 clones. UPA143 and T79/501 are upper Amazonians clones, known for their high productivity but very low tolerance to *P. megakarya*. SNK13 and SNK64 belong to Trinitario group of cocoa. SNK13 is a moderately susceptible and productive clone while SNK64 is the most tolerant among the four clones but low yielding (Nyasse et al., 2007). Cross pollination between the above clones might generate offspring with interesting agronomic traits (resistance/tolerance to BPD).

Biomolecules associated to *T. cacao* defence against pathogens could also be useful in the development of *T. cacao* hybrid genotypes tolerant to BPD such as sulfur which has been used as a most important fungicide used especially for antifungal treatments of fruits (Tweedy, 1981; Hassall, 1990; Manga et al., 2016).

Nowadays, industries are producing fungicides with specific and diverse modes of action. Elemental sulfur has always been used because of the development of resistance against the compounds with unique site of action (Jolivet, 1993). Many studies have reported the involvement of sulfur in the defense mechanisms of plants. However, sulfur defense mechanisms are still unclear (Saito, 2000; Cooper and Williams, 2004; Hamdan, 2010). This might involve elemental sulfur form, or organic-sulfur-containing compounds directed against microorganisms (Cooper and Williams, 2004; Hamdan, 2010; Stanislaus, 2011). In *T. cacao*, elemental sulfur was reported to be involved in the protection of this plant against *Verticillium dahliae* (Cooper et al., 1996; Cooper and Williams, 2004). Sulfur moiety in organic sulfur containing compounds in plants is mainly provided by cysteine. Cysteine is the first organic molecule in the reduction/assimilation sequence of inorganic sulfur (SO_4^{2}). Cysteine is considered as the hub of sulfur distribution in organic molecules including those involved in the defense system of plants against biotic stress (Saito, 2000). Hence, cysteine profile might be a useful biochemical marker in the selection of *T. cacao* hybrid genotypes tolerant to *P. megakarya*.

This investigation aimed to: (a) assess the susceptibility to BPD of offspring derived from SNK13xUPA143 (KHA), T79/501xSNK13 (AHK) and UPA143♀xSNK64 (AHK3) and (b) study the profile of cysteine (variability in cysteine contents) during *T. cacao* / *P. megakarya* interaction in order to select tolerant hybrid genotypes which could be distributed to farmers.

MATERIALS AND METHODS

Plant material

Leaf samples from the offspring derived from the crosses SNK13 X UPA13 (KHA), T79/501 X SNK13 (AHK) and UPA143 X SNK64 (AHK3) were used for the study. Manual pollination was conducted in the seeding farm of the Institute of Agricultural Research for Development (IRAD) at Barombi-Kang (Kumba, South-West Region, Cameroon, Africa).

T. cacao seeds from mature pods derived from ♀SNK13x♂UPA143, ♀T79/501x♂SNK13 and ♀UPA143x♂SNK64 were used to set up a nursery. Leaves of three to four months old seedlings from SNK13xUPA143, T79/501xSNK13 and UPA143xSNK64 (of the above nursery) were used for the screening for susceptibility using leaf disc test and cysteine profile analysis.

P. megakarya strain culture

The Plant Pathology Laboratory of IRAD (Nkolbison, Yaoundé, Centre region, Cameroon, Africa) provided the strain of *P. megakarya* with moderate virulence. Virulence was maintained by artificial inoculation of pods followed by the re-isolation and transfer in the phenylethyl alcohol agar (PEA) medium. This was also used to induce the release of zoospores, which were used in the leaf discs test.

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Figure 1. Disease score of leaves infected *in plantae* in the nursery. A =0 ; B=+ ; C =++ ; D=+++ ; E= ++++; F ++++++; 1 cm = 4 cm.

Zoospores productions

Zoospores (or inoculums) were obtained according to Tondje et al. (2006) method. Zoospores were produced by the inoculation of pods (with *P. megakarya* mycelia) followed by soaking them in sterile distilled water when sporangia recover infected pods. The solution was placed at +4°C for 5 min then immediately transferred in room temperature to allow sporangia to liberate zoospores. The zoospores concentration was adjusted to $3x10^5$ zoospores/mL with a MALASSEZ hemati-meter.

Screening for susceptibility to *P. megakarya* and experimental design for leaves discs test

Two to three months old leaves were harvested (three leaves/plant) between 6 and 7 am. Using a cork cutter, 8 discs of 1.5 cm diameter were collected per leave. Hence a total of 24 discs were obtained per leave. These discs were placed bottom-side turned upward, on a wet trays and incubated for 24 h in darkness at 25 ± 1°C prior to inoculation. After the 24 h, leaf discs were inoculated by depositing 10 µL ($3x10^5$ zoospores/ml) of zoospores suspension on either side, in the middle of each leaf disc and incubated in darkness (at 25±1°C). The scoring (from 0: tolerant to 5: highly sensitive) of susceptibility (through the necrosis size) of each leaf discs (for each hybrid) was registered on 4th, 5th, 6th, 7th and 8th day after inoculation.

Artificial inoculations of leaves in the nursery

The test of undetached leaves has been used in several studies to evaluate the tolerance of plants against *Phytophthora* (Ahmad Kamil et al., 2004; Djocgoue et al., 2006; Nyadanu et al., 2013). In our investigations, inoculation was made on none detached leaves in the nursery. The underside of the leaf was cleaned with cotton soaked in 70° ethanol. Scarification was made at the midrib, on which, 3 mm diameter mycelia-agar-disc of 10 days old culture was placed. Then covered with sterile cotton, protected plaster and moisturized with sterile water. The incubation time was 7 days. The disease score was arbitrary defined from 0 to 5 (Figure 1).

Extraction and quantitative analysis of the soluble cysteine

The inoculated nursery leaves were harvested 6 days after inoculation (DAI), with the controls (not inoculated) lightly ground in 5 ml acetone (to remove chlorophyll) and dried for 5 min at room temperature on Wattman Nº1 paper. 0.5 g of chlorophyll-free leaves was ground in 2.5 ml of ethanol 80°. Homogenate was subsequently centrifuged for 30 min at 6000 *g*. The supernatant was collected for cysteine quantification.

Cysteine content was determined according to Gaitonde (1967) method. Cysteine extract (0.15 ml) was mixed with 0.35 ml of acidic ninhydrin reagent [1.3 % (w/v) ninhydrin in 1:4 concentrated HCl: CH_3COOH]. The mixture was heated at 100°C for 10 min then cooled in ice bath to allow pink color development. The optical density was read at 560 nm ($\varepsilon = 2.8 \times 10^4$) against the control in which the 0.15 ml of cysteine extract was replaced by equal volume of ethanol 80°. Cysteine content was expressed in µg per gram of acetonic powder (µg/gFP).

Data analysis

Data collected were subjected to descriptive statistics. Analysis of variance (ANOVA) and mean separation by the Student-Newman-Keuls were done with SPSS 17.0 software. Discrimination of hybrid genotypes according to their disease scores (level of sensitivity) were done through direct hierarchical classification of the same software.

RESULTS AND DISCUSSION

Germination rate

Germination of seeds from mature pods derived from ♀UPA143x♂SNK64 (AHK3), T79/501xSNK13 (AHK) and SNK13xUPA143 (KHA) families were monitored during the 12 days after seeding. KHA family germination rate appeared to be lower than AHK and AHK3 families. At day 4 after seeding, 10 and 22% of seeds derived from AHK and AHK3 respectively germinated. At the same date, there was no germination of KHA seeds. At day 12, AHK family seeds exhibited germination rate of 91.49% while AHK3 showed 98.9% and the germination rate of KHA family was 40.35% (Table 1).

Assessment of hybrids susceptibility to *P. megakarya*

The susceptibility of F1 hybrids from the biparental

Table 1. Seed germination rate.

Germination rate	Hybrid family		
	KHA	AHK	AHK3
Germination rate at day 4 (%)	0	10	22
Germination rate at day 12 (%)	40.4	91.49	98.9

crosses (SNK13xUPA143, T79/501xUPA143 and UPA143xSNK64) to *P. megakarya* was monitored through disease score on the fourth, sixth and seventh day after leaf discs inoculation and incubation. The sixth day appeared to be most discriminative (by generating the highest number of phenotypic subgroups of disease scores) compared to the fourth and seventh day of leaves discs inoculation and incubation.

Family UPA143xSNK64 (AHK3)

At the sixth day of leaves discs inoculation, the 44 plantlets from the subfamily ahk3ca (of AHK3) displayed disease scores between 0.0±0.0 and 4.87±0.37. The 44 plantlets were separated in 18 phenotypic subgroups of disease scores using the Student Newman and Keuls test. Direct hierarchic classification regrouped the 18 phenotypic subgroups of disease scores in 7 classes: [0.0; 1], [1; 1.5], [1.5; 2.1], [2.1; 3], [3; 4], [4; 4.5] and [4.5; 5]. Approximately, 38.64% of hybrid genotypes from ahk3ca displayed disease scores in the interval [0.00, 1.9 (Figure 2 and Table 2).

In the same conditions, the subfamily ahk3cb displayed 13 phenotypic subgroups of disease score. The 13 phenotypic subgroups of disease scores were regrouped in fives classes using direct hierarchic classification: [0.0; 1.0 [(6.81%); [1.0; 2](4.54%) [2; 3] (9.10%), [3; 4] (25%) and [4; 5] (54.54%) (Figure 3 and Table 2). Disease scores ranged between 0.0±0.0 and 5.0±0.0. In addition, 11.37% of the offspring exhibited disease scores in the interval [0.00, 1.9].

Family T79/501xUPA143 (AHK)

The subfamily ahkca (of the family AHK) displayed 14 phenotypic subgroups of disease scores from 46 plantlets when data of disease scores were subjected to Student, Newman and Keuls test. The hierarchic classification test grouped the 47 plantlets in 6 classes: [0; 0.5[, [0.5; 1.75[, [1.75; 2.75], [2.75; 3.25], [3.25; 4] and [4; 4.5] (Figure 4 and Table 3).

The same analysis conducted with the subfamily ahkcb made of 38 plantlets generated 14 phenotypic subgroups of disease scores. The hierarchic classification analysis grouped the 38 plantlets in 9 classes. Disease scores of this subfamily were included in the interval [0.00, 4.38]. About 17.4% of offspring showed disease score value lower than 2 (Figure 5 and Table 3).

Family KHA (SNK13xUPA143)

At day six of leaves disc inoculation and incubation, disease scores values ranged from 0.12 to 5 in khaca subfamily (SNK13xUPA143). The Student, Newman and Keuls test reveals 9 phenotypic classes of disease score. Approximately, 11.76% of hybrid genotypes showed disease scores lower than 2 (Figure 6 and Table 4).

In khacb subfamily (SNK13xUPA143) Student, Newman and Keuls test displayed 8 phenotypic subgroups of disease scores grouped in 6 classes using the hierarchic classification analysis (Figure 7 and Table 4).

Biochemical analysis

Cysteine content was analyzed in undetached healthy (untreated) and wounded-infected leaves in target hybrid genotypes from the three families. For each family, chosen hybrid genotypes differed in their disease scores.

In the AHK3 family, AHK3ca21, AHK3ca24, AHK3ca25, AHK3cb1, AHK3cb20, AHK3ca1, AHK3ca39, AHK3ca35, AHK3cb9, AHK3ca36, AHK3ca18, AHK3ca7, AHK3cb4, AHK3cb7, AHK3ca17 and AHK3ca46 hybrid genotypes leaves (healthy and wounded-infected) were used for cysteine quantification. In healthy leaves, variability in cysteine content was observed between tested hybrid genotypes. When healthy leaves are wounded and infected, two patterns were observed: in hybrid genotypes with disease scores lower than 2.3 (AHK3ca21, AHK3ca24, AHK3ca25, AHK3cb1, AHK3cb20, AHK3ca1 and AHK3ca39), infection appeared to be associated with cysteine content decrease (compared to healthy leaves). Percentages of cysteine contents decrease ranged between 27.94 (AHK3cb20) and 74.90% (AHK3ca24). When disease scores were above 2.3, infection led to significant increase in cysteine contents (Figure 8a and 8b). Moreover, Pearson correlation analysis showed a positive and highly significant correlation between difference in cysteine contents (between healthy and wounded-infected leaves) and disease scores of hybrids genotypes (Table 5).

Cysteine contents in AHK and KHA families showed two patterns as observed in AHK3 family. In AHK, hybrid

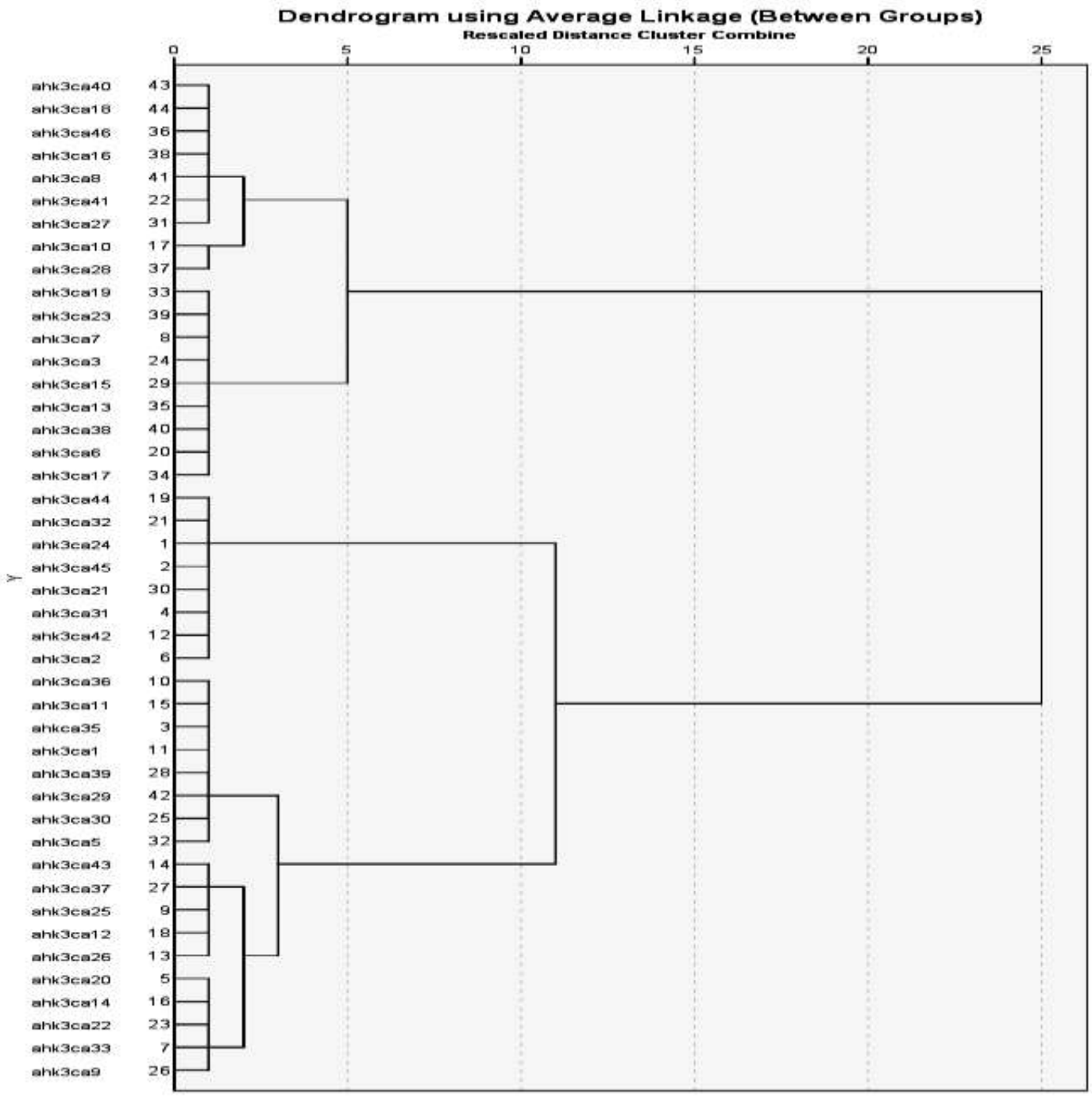

Figure 2. Direct hierarchic classification of hybrid genotypes of the subfamily ahk3ca (UPA143xSNK64).

Table 2. Phenotypic classes of disease scores of the family UPA143xSNK64 (AHK3).

Subfamilies	Phenotypic classes of disease scores (% of hybrid genotypes)						
	0; 1	**1; 1.5**	**1.5; 2**	**2; 3**	**3; 4**	**4; 4.5**	**4.5; 5**
ahk3ca	18.18	11.36	11.36	18.18	20.45	15.91	4.55
ahk3cb	6.82	4.55	0.00	9.09	25.00	18.18	36.36
Means	12.50	7.95	5.68	13.64	22.73	17.05	20.45

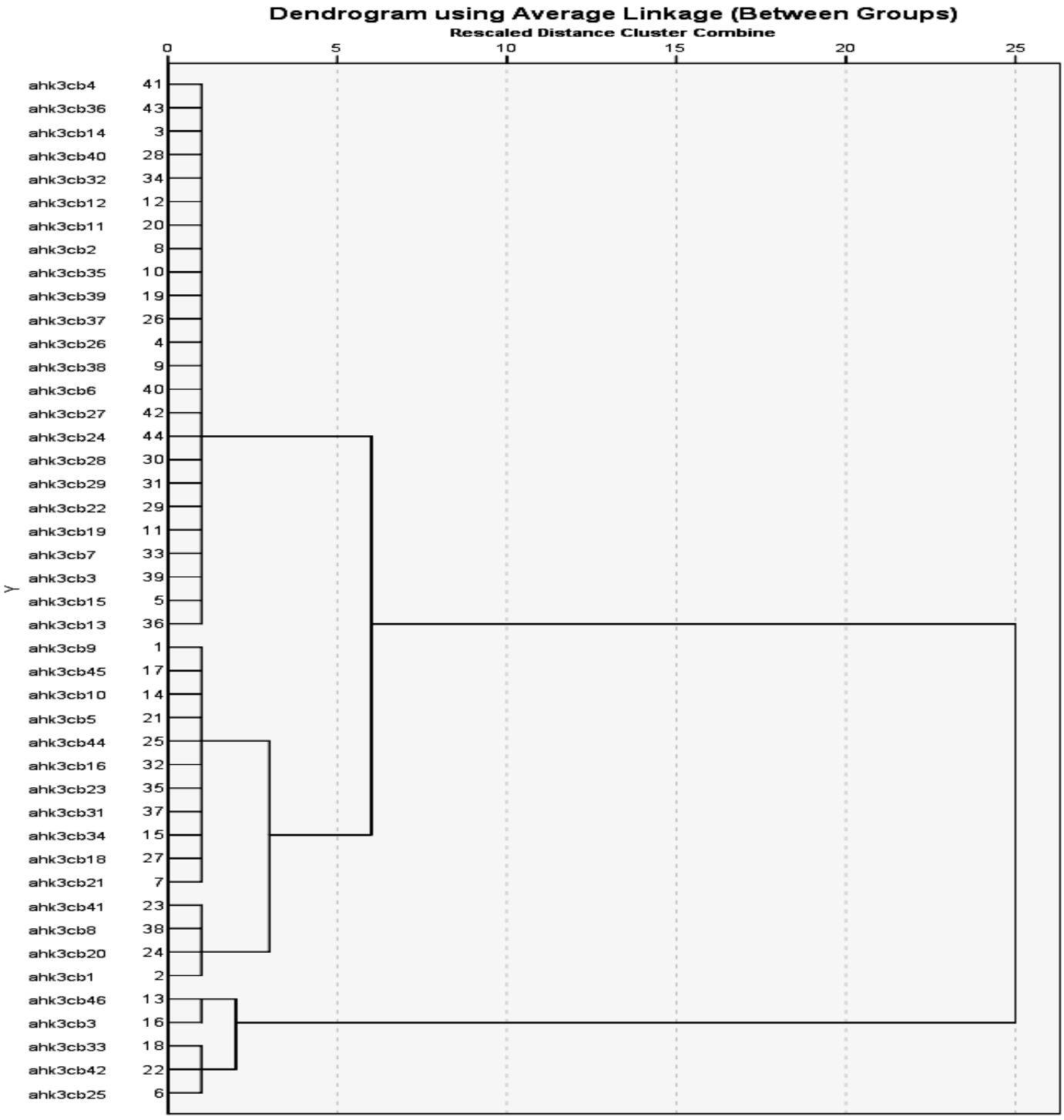

Figure 3. Direct hierarchic classification of hybrid genotypes of the subfamily ahk3cb (UPA143xSNK64).

genotypes with disease scores lower than 3.5; leaves infection led to decrease of cysteine contents (Figure 9a and 9b). In KHA family, hybrid genotypes with disease score under 3.7 exhibited decrease in cysteine content when leaves were infected (Figure 10).

DISCUSSION

A nursery has been established from the crosses pod seeds: SNK13xUPA143, T79/501xSNK13 and UPA143xSNK64. The involvement of cysteine in the

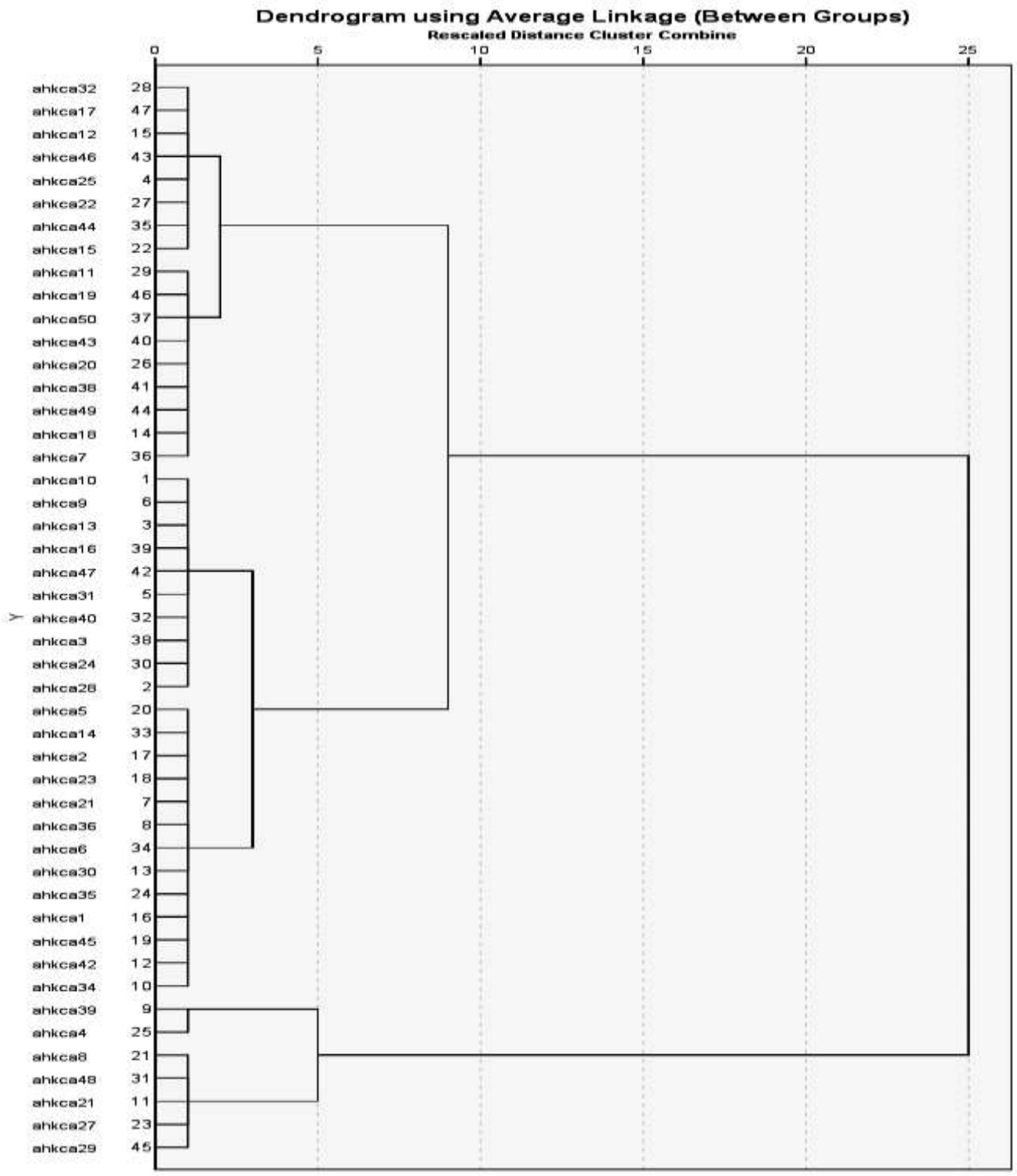

Figure 4. Direct hierarchic classification of hybrid genotypes of the subfamily ahkca (T79/501xUPA143).

defence of this plant was investigated. The assessment of seeds germination, the fourth and twelfth day after planting, showed that the germination rate of KHA family of hybrid genotypes is low compared to those of AHK and AHK3 families. Otherwise, the number of seeds from the pods KHA (27) family was lower than those of AHK (48)

Table 3. Phenotypic classes of disease scores of the family T79/501xUPA143 (AHK).

Subfamilies	Phenotypic classes of disease scores (% of hybrid genotypes)						
	0; 1	1; 1.5	1.5; 2	2; 3	3; 4	4; 4.5	4.5; 5
ahkca	6.38	6.38	4.26	31.91	31.91	19.15	0.00
ahkcb	5.26	7.89	13.16	26.32	31.58	13.16	2.63
Means	5.88	7.06	8.24	29.41	31.76	16.47	1.18

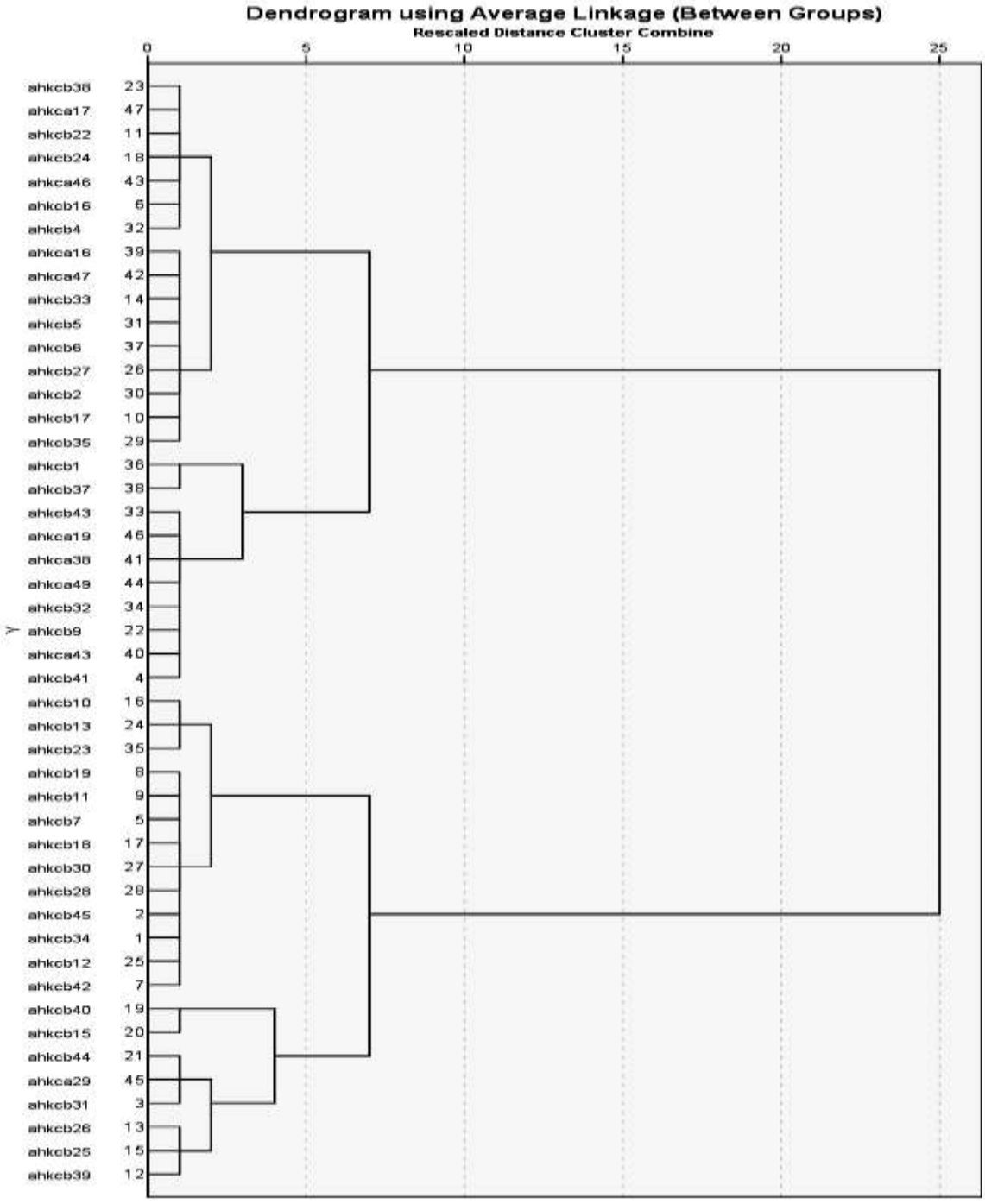

Figure 5. Direct hierarchic classification of hybrid genotypes of the subfamily ahkcb (T79/501xUPA143).

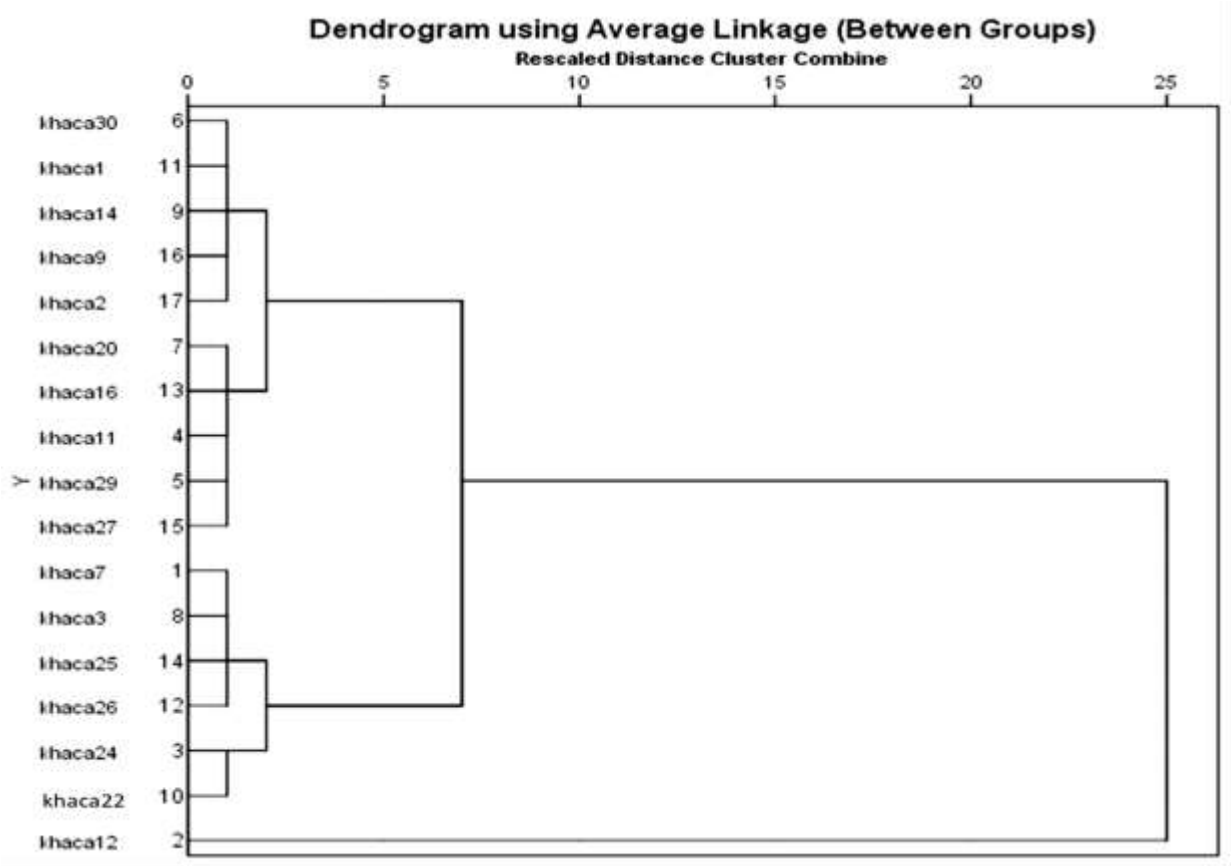

Figure 6. Direct hierarchic classification of hybrid genotypes of the subfamily khaca (SNK13xUPA143).

Table 4. Phenotypic classes of disease scores of the family SNK13xUPA143 (KHA).

Subfamilies	Phenotypic classes of disease scores (% of hybrid genotypes)						
	0; 1	1; 1.5	1.5; 2	2; 3	3; 4	4; 4.5	4.5; 5
khaca	5.88	0.00	0.00	29.41	35.29	17.65	11.76
khacb	11.76	17.65	17.65	5.88	23.53	23.53	23.53
Means	8.82	8.82	8.82	17.65	29.41	20.59	17.65

and AHK3 (46) families. These results are similar to those of some authors who reported that the germination rate and the number of seeds per pod are agronomic traits which guide preferably a crossover ratio to another (Liabeuf, 1967; Despréaux et al., 1989). Thus, considering only this stage of analysis based on these two characters, which are germination rate and number of seeds per pod, AHK and AHK3 families can be recommended to famers due to their high rate germination as compared to the KHA family.

Concerning the analysis of the sensitivity of hybrid genotypes of these three families, results showed the variability within and between families. This variability reflects the genetic heterogeneity of their offspring for the sensitivity character vis-a-vis to *P. megakarya*. Much research has, in fact, reported that the sensitivity of *T. cacao* to *P. megakarya* is a polygenic, non-cytoplasmic and nuclear character (Ndoumbe-Nkeng et al., 2001; Djocgoue et al., 2006; Nyadanu et al., 2013). This would mean that because of parental heterozygosity, no crossing would give rise to a very tolerant or completely susceptible offspring. When families are examined one by one, it was observed that the KHA family presented the highest percentage of sensitive hybrid genotypes compared to AHK and AHK3. These results are in conformity with those obtained by Nyasse et al. *(*2007) who reported that from the sensitivity of both parents (SNK13 and UPA143), SNK13 is moderately susceptible

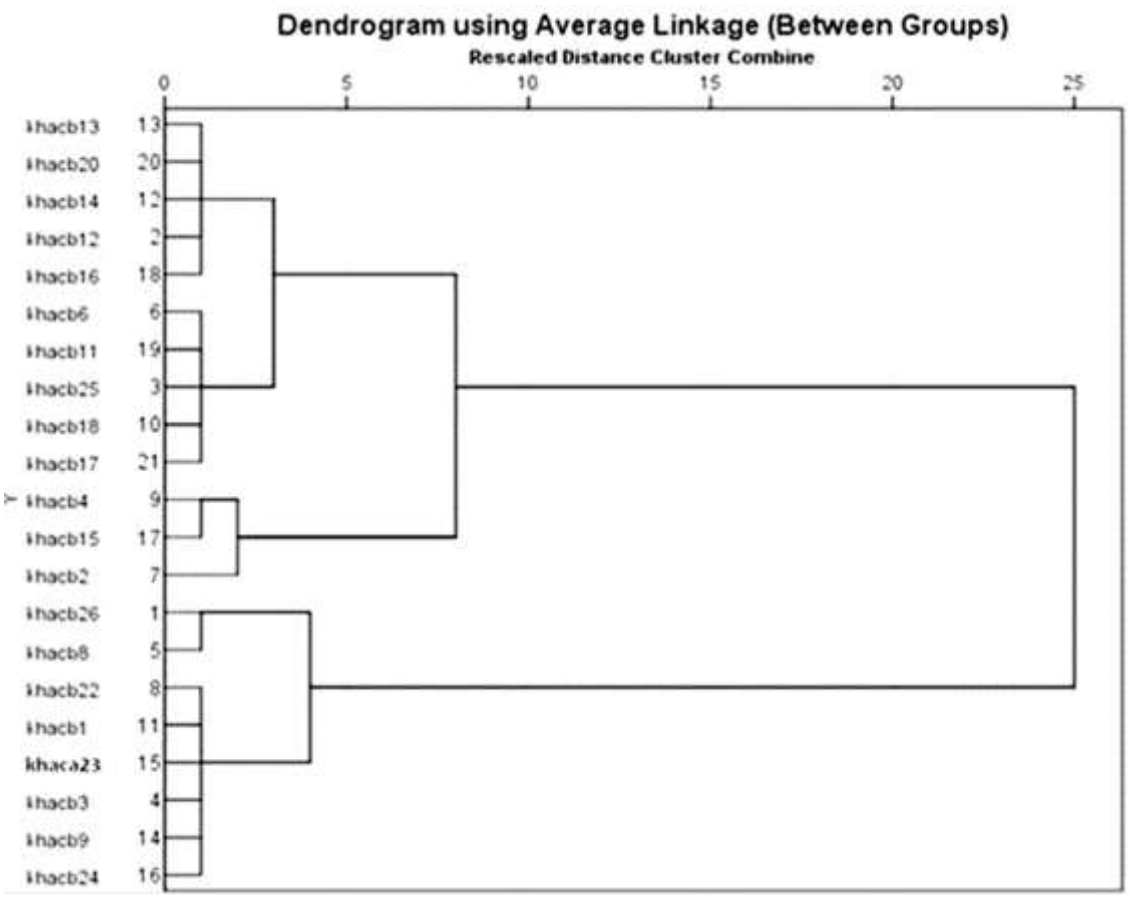

Figure 7. Direct hierarchic classification of hybrid genotypes of the subfamily khacb (SNK13xUPA143).

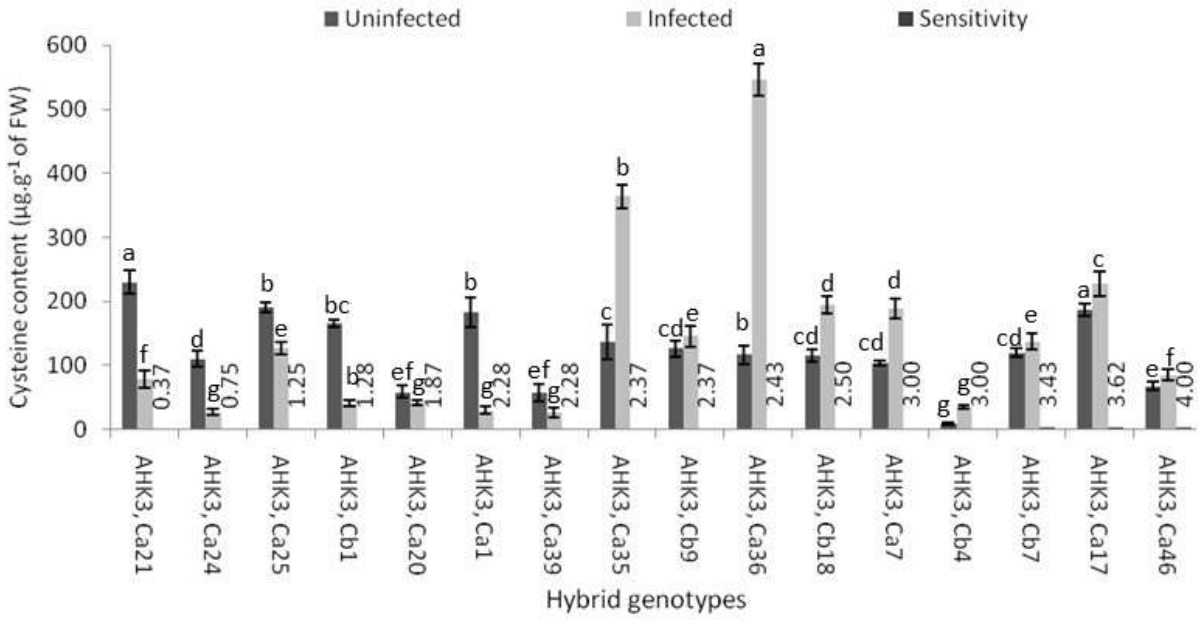

Figure 8a. Levels of free cysteine in leaves of some representatives of AHK3 family with their sensitivity. Values following by the same letter for a giving treatment are not significantly different (P< 0.05).

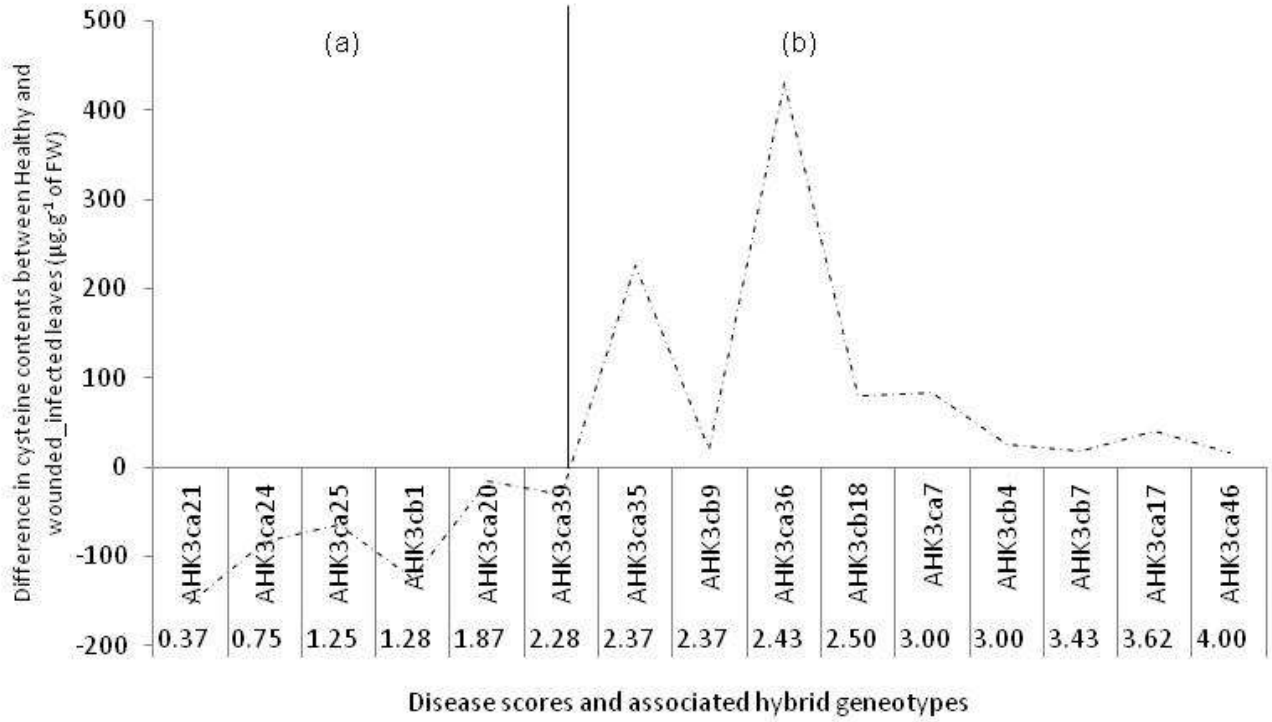

Figure 8b. Difference in cysteine content between healthy (H) and wounded-infected (W_I) leaves as function of disease scores of hybrid genotypes of AHK3 family. (a) = a group of tolerant hybrid genotypes (W_I – H) give negative value in cysteine content); (b) = a group of susceptible hybrid genotypes (W_I – H) give positive value in cysteine content).

and UPA143 has a high susceptibility to *P. megakarya*. It follows that the cross–of susceptible clones leads to a high proportion of susceptible individuals within the offspring. The crossover offspring resulted in three groups which consist of low, medium and high sensitivity. In the family T79/501xSNK13, the tolerant individuals' proportion is higher than the previous family, but lower than the AHK3 family. About the sensitivity of the parents, it has been reported that T79/501 is more tolerant than UPA143. Thus, the moderate sensitivity of SNK13 used as male parent contributes to the improvement of tolerance in the offspring (Nyasse et al., 2007).

The AHK3 family from the cross between UPA143xSNK64 is the one that resulted in the largest percentage of tolerant individuals compared to the previous two families. Certainly, UPA143 used as female parent is very sensitive but SNK64 used as male parent is classified as one of the most tolerant cacao clones to *P. megakarya*. Using SNK64 would be responsible for the high proportion of tolerant individuals observed in this cross. In other words, as SNK64 is the most tolerant clone of our study, we could say that this high rate is due to its ability as a male parent to transfer to the offspring the loci responsible for resistance to *P. megakarya*. According to some authors (Nyasse et al., 1995; Nyasse et al., 2002; Djocgoue et al., 2006; Tahi et al. 2006; Djocgoue et al., 2010), cocoa tolerance to *P. megakarya* is nuclear and not cytoplasmic. This explains that tolerance of cacao to *P. megakarya* is not related to mitochondria or chloroplasts and should therefore be influenced by the male parent. Our results are not in agreement with those of these researchers because during our studies we made direct crossings and not reciprocal crosses. On the other hand, observations in the nursery show that the development of necrosis is faster within the different families when it rains abundantly. These results could be explained by the fact that moisture gives favorable environmental conditions for the development of zoospores, responsible for the black pod disease. These results are similar to those obtained by Ngoh Dooh et al. (2015) who showed that the wide variation in rainfall during the two campaigns revealed an existing relationship between the fluctuations in rainfall and severity of the impact of black pods disease of cocoa.

Apart from the morphological aspect, the analysis of cysteine content in uninfected and infected leaves showed significant variations within representative groups of three hybrid families. In general, it appeared that, prior to infection (uninfected leaves), cysteine content varies from an individual to another. This soluble cysteine content appears to be associated with the level of sensitivity of hybrid genotypes. After infection, decrease in cysteine content was observed in tolerant hybrid

Table 5. Pearson Correlation using data of cysteine contents in healthy and wounded-infected leaves of hybrid genotypes from AHK3 family.

Parameter	Correlation staus	Disease scores of hybrids genotypes	Difference in cysteine content between healthy and wounded-infected leaves	Cysteine content in healthy leaves	Cysteine content in wounded-infected leaves
Disease scores of hybrids genotypes	Correlation	1			
	Sig.				
Difference in cysteine content between healthy and wounded-infected leaves	Correlation	0.670**	1		
	Sig.	0.009			
Cysteine content in healthy leaves	Correlation	-0.268	-0.479	1	
	Sig.	0.354	0.083		
Cysteine content in wounded-infected leaves	Correlation	0.472	0.636*	0.373	1
	Sig.	0.088	0.015	0.189	

* Correlation is significant at the 0.05 level (2-tailed); ** correlation is significant at the 0.01 level (2-tailed).

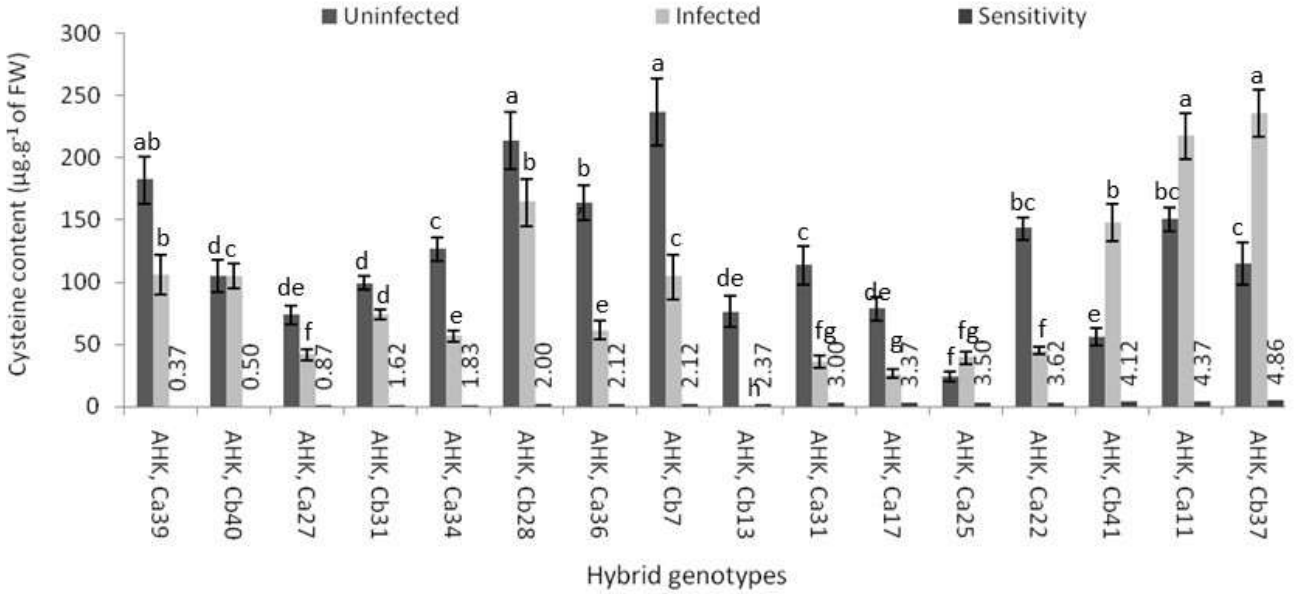

Figure 9a. Levels of free cysteine in leaves of some representatives of AHK family with their sensitivity. Values followed by the same letter for a given treatment are not significantly different (P<0.05).

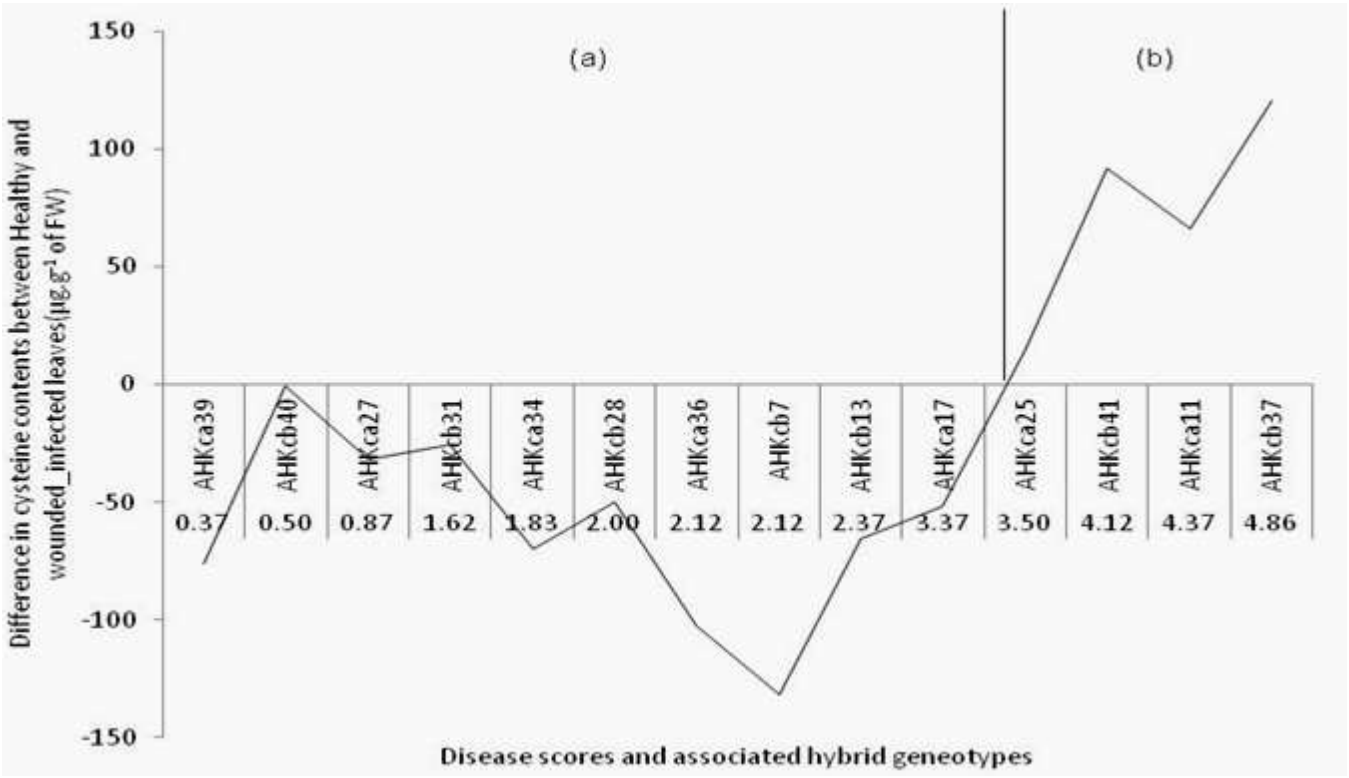

Figure 9b. Difference in cysteine content between healthy (H) and wounded-infected (W_I) leaves as function of disease scores of hybrid genotypes of AHK family: (a) = a group of tolerant hybrid genotypes (W_I – H) give negative value in cysteine content)and (b) = a group susceptible hybrid genotypes (W_I – H) give positive value in cysteine content).

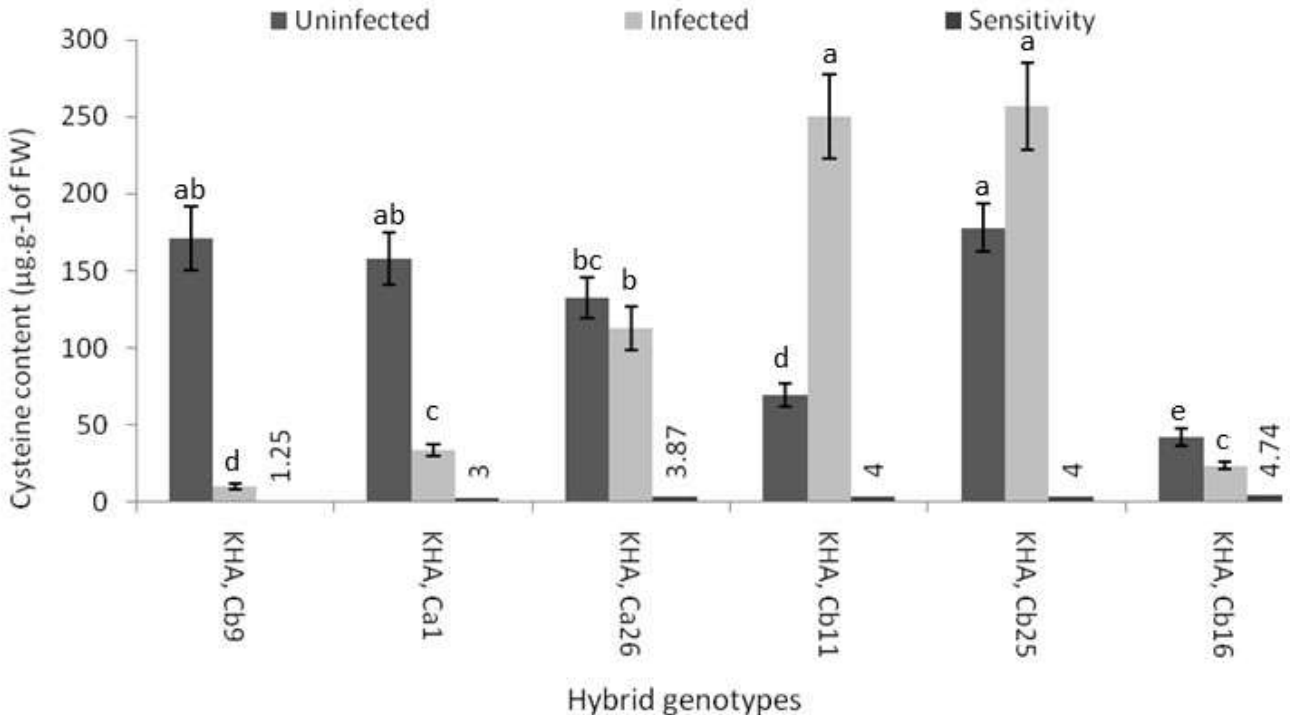

Figure 10. Levels of free cysteine in leaves of some representatives of KHA family with their Sensitivity. Values following by the same letter for a giving treatment are not significantly different (P< 0.05).

genotypes while in susceptible hybrid genotypes displayed a significant increase in cysteine content. Additionally, a positive and highly significant correlation was found between the reduction of cysteine content in infected leaves and disease scores of hybrid genotypes. These results are justified by the fact that in tolerant hybrid genotypes and in infection condition, cysteine is rapidly used by molecules to fight the pathogen. Reversely, in susceptible hybrid genotypes, cysteine synthesized may not be used during the infection. The hybrid genotypes KHACB16 KHA, CB11 and KHA, CB25 from KHA family susceptibility might be due to the fact that this hybrid genotype cannot mobilize cysteine for synthesis of sulfur containing molecules defense molecules during the pathogen attacks to ensure their defense. These observations may justify the high content of cysteine in the three hybrid genotypes after infection. Similar results were obtained from hybrid genotypes from AHK and AHK3 families. These results are in accord with those obtained by Cooper and Williams (2004) who reported that the synthesis of cysteine is triggered when the plant is exposed to parasitic attack. These results are also similar to those obtained by Borgen (2002) and Cerniauskaite (2010) who showed that certain metabolites containing sulfur such as glucosinolates are involved in defense against biotic attacks. In the similar approach, Saito (2000) showed that sulfur of organic sulfured metabolites is exclusively provided by cysteine. Thus, during the infection, dynamic of cysteine might have two phases in *T*. *cacao*: one phase synthesis accumulation, which leads to an increase of the cysteine content in the plant. In the second phase, cysteine is used (decrease of cysteine content) for the synthesis of sulfurous compounds involved in defense. There may exist therefore, an interdependence link between cysteine pools and the susceptibility to BPD of *T*. *cacao* hybrid genotypes when exposed to *P*. *megakarya* attack.

Conclusion

The susceptibility of hybrid genotypes of crosses SNK13xUPA143, T79/501xSNK13 and UPA143xSNK64 was evaluated and the involvement of cysteine in the defense of *T. cacao* against *P. megakarya* was analysed. The present investigation revealed that, crosses T79/501xSNK13 and UPA143xSNK64 gave the best germination rate and the highest number of seeds per pod. Similarly, both crosses generated the greatest number of tolerant hybrid genotypes. However, UPA143xSNK64 is the best of these three crosses.

The results of this study show that cysteine is mobilized during *P. megakarya* attack and quickly used in tolerant hybrid genotypes to fight against the pathogen. This biochemical analysis showed that cysteine pool could be used to discriminate tolerant from susceptible hybrid genotypes of *T*. *cacao*. Additionally, cysteine appeared to be a component of defense mechanism of T. cacao against *P*. *megakarya*. Tolerant hybrid genotypes from this study could be used by the famers to increase yield in their plantations. Moreover, the present finding could allow researchers to use cysteine in cocoa improvement program.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of quercetin nanoparticles on the kidney of the streptozotocin-induced diabetes in male rats: A histological study and serum biochemical alterations

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Diabetes is directly involved in oxidative stress production. Therefore, this study was conducted to investigate the morphological and functional alterations caused by oxidative stress and to evaluate the antioxidant effect of quercetin nanoparticles (QUNPs) in streptozotocin (STZ)-induced diabetic (type II) rats. Seventy two male albino adult rats were randomly distributed in 6 different experimental groups, with 12 animals per group: Normal Control (NC) group, Positive Control (PC) group received one dose of STZ (60 mg/kg body weight [bw]); QUNPs 10 mg/kg bw/day alone group; QUNPs 10 mg/kg bw/day + one dose of STZ (60 mg/kg bw) group; QUNPs 20 mg/kg bw/day alone group; and QUNPs 20 mg/kg bw/day + one dose of STZ (60 mg/kg bw) group. STZ-diabetic rats were treated with QUNPs (10 and 20 mg/kg bw/day) for 7 weeks to analyze their effects on markers of renal enzymes antioxidant [malondialdehyde (MDA), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx)], total protein and albumin, and also on kidney tissues. The results showed that the particle size of QUNPs is 16.13 nm at flow rate 10 ml/min. QUNPs especially at the dosage of 20 mg/kg bw/day gave results close to normal values observed in NC compared to PC. Also, histopathology of kidney sections for QUNPs 20 mg/kg bw/day + STZ and QUNPs (10 and 20 mg/kg bw/day) alone, appeared similar to NC. It can be concluded that QUNPs could become a promising adjuvant in the treatment of diabetes mellitus and can act as an antioxidant agent.

Key words: Diabetic, streptozotocin, free radical, antioxidant.

INTRODUCTION

Diabetes mellitus (DM) or hyperglycemia is a metabolic disorder that develops from cases of insufficient or absence of insulin release from β-cells (Vardi et al., 2003). Chronic hyperglycemia leads to many complications, such as cardiomyopathy, vascular damage, retinopathy, neuropathy, and nephropathy (Review of World Health Organ Tech Rep Ser.,1985). Streptozotocin (STZ), an antibiotic produced by *Streptomyces*

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achromogenes and used as an agent to induced diabetes, damages the insulin-producing β-cells membranes and results in the depletion of intracellular nicotinamide adenine dinucleotide in islet cells (Kanter et al., 2004; Coskun et al., 2004). Also, it is known to induce diabetic kidney injury with hyperlipidemia, inflammation, and hyperuricemia (Hovind et al., 2009; Tone et al., 2005). Kidney injury is the most common pathological disorder predisposing end-stage renal disease worldwide (Ayodele et al., 2004; Donath and Shoelson, 2011; Murea et al., 2010). Diabetic nephropathy is characterized with progression into glomerulosclerosis, interstitial fibrosis, and tubular atrophy by mesangial expansion and thickening of basement membranes, ultimately resulting in renal failure (Tsao et al., 1999; Voziyan et al., 2002; Feliers et al., 2001). Previous studies proposed a wide variety of mechanisms in the pathogenesis of diabetes, including oxidation of renal glycoproteins by reactive oxygen species (Reddy et al., 2002; Natarajan et al., 2002; Chen et al., 2001).

Oxidative stress is caused by highly toxic components, such as the overproduction of reactive nitrogen and oxygen radicals which interact with the lipid bilayer and produce lipid peroxides of cellular membranes and caused toxicity to all the components of the cells (Tatsuki et al., 1997). Higher oxidative stress is one of the factors for impaired antioxidant defense mechanisms or increased levels of free radicals because it is implicated in the progression and development of diabetic complications (Ceriello, 2000; Saxena et al., 1993). Therefore, in recent years, researchers have developed interest to prevent oxidative damage with high oxidative stress in DM by the role and usage of natural antioxidants. Flavonoids have the capacity to promote β-cell regeneration in islets, normalize blood glucose levels, and normal islets from STZ in rats (Un et al., 2006). Individually, the protective effects of polyphenols and flavonoids may exert in a variety of ways: they may scavenge chelating metal ions, reactive oxygen species, also, scavenging lipid peroxyl radicals to act as a chain-breaking antioxidant, or prevent lipid damage by partition into the lipid bilayer (Plumb et al., 1999; Laughton et al., 1991; Robak and Gryglewski, 1988). Moreover, some studies reported that the activity of flavonoids as antioxidant may be dependent on hydroxylation degree (Plumb et al., 1999; Rice-Evans et al., 1996).

Quercetin (3,3,4,5,7-pentahydroxyflavone, QUE) is a lipid-soluble compound (Figure 1). It reduces lipid hydroperoxide production (Coldiron et al., 2002) and is capable of preventing lipid damage, inhibits biomolecule oxidation, radical scavenging, and alters antioxidant defense pathways *in vitro* (Candlish and Das, 1996; Morand et al., 1998). QUE is a well-documented bioflavonoid occurring in many foods and is known to be present in higher concentrations in green tea, red wine, broccoli, apples, and onions (Kiviranta et al., 1998; Weisburer, 2000). Previous studies have focused on the

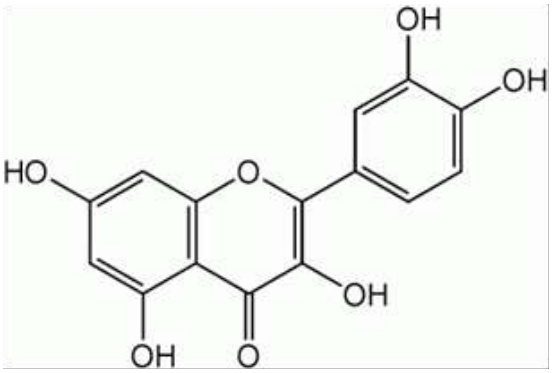

Figure 1. Structure of quercetin.

QUE beneficial properties, namely, anticarcinogenic properties, antioxidant, anti-inflammatory, antiproliferative, and antibacterial (Weisburer, 2000).

On the other hand, QUE is a challenging molecule to be delivered due to its poor water solubility. A water-soluble derivative of QUE has been synthesized but its bioavailability was only 20% (Mulholland et al., 2001) and it has such poor absorption in the gastro-intestinal tract. All these highlight the need for an improved formulation for QUE with enhanced dissolution so that its absorption can be greatly enhanced. Therefore, micro- and nanoparticle preparations are the most important approaches being investigated these days to improve bioavailability (Bilati et al., 2005). Nanoparticles are particularly useful in drug delivery for water-insoluble compounds such as ellagic acid (Bala et al., 2006) and coenzymeQ10 (Hsu et al., 2003), because their size (less than 1000 nm) can increase the absorption and the bioavailability of the delivered drug. Thus, an improved oral formulation of QUE is required with better bioavailability and higher efficacy. Therefore, this study aimed to prepare quercetin nanoparticles (QUNPs) and evaluate the antioxidant effect of QUNPs on renal histopathological and serum biochemical alterations in STZ- induced diabetic (type II) rats.

MATERIALS AND METHODS

In this study, QUE (Sigma-Aldrich, Singapore) was used as received. All reagents used were of technical grade. Absolute ethanol (99.5 to 99.8%) was obtained from J.T. Baker (Avantor Performance Materials, Phillipsburg, NJ).

Preparation of QUNPs

To prepare QUNPs, magnetic stirring (1000 rpm) was used to mix water and ethanol (volume ratio 35:1, fixed flow rate of 10 ml/min) according to the nano participation technique (Kakran et al., 2012; Abd El-Rahman and Al Jameel, 2014). Then, commercial QUE was dissolved in predetermined concentration (5 mg/ml) of ethanol (the solvent). The syringe was filled with the prepared solution and

secured onto a syringe pump. Quickly, drug solution was injected under magnetic stirring into the anti-solvent (deionized water) of definite volume at a fixed flow rate. The QUNPs were filtered and vacuum dried.

Morphology of the particles

Scanning electron microscopy (SEM; Quanta 3D FEG/FEI) with 20 kV, 300 V collector bias was used to observe the samples morphology. Before the SEM observations, the samples powder were spread on a SEM stub and sputtered with gold.

Biological methods

Male albino adult rats (72 animals weighing 170 ± 2 g) were obtained from Vaccination Center, Helwan, Giza, Egypt, then transported to Animal House of Ophthalmology Research Institute, Giza, Egypt. The rats fed on basal diet (casein 10%, salt mixture 4%, corn starch 70%, corn seed oil 10%, vitamins mixture 1% and cellulose 5%) for ten days after being housed in individual cages with screen bottoms. After equilibration and before administration of STZ, rats were divided into six groups (twelve animals per each) and weighted: G1, Negative Control (NC) group; G2, Positive Control (PC) group injected with single dose of STZ (60 g/kg bw); G3, treated group that received QUNPs (10 mg/kg bw/day) only; G4, treated group that received one dose of STZ (60 g/kg bw) + QUNPs (10 mg/kg bw/day); G5, treated group that received QUNPs (20 mg/kg bw/day) only; G6, treated group that received one dose of STZ (60 g/kg bw) + QUNPs (20 mg/kg bw/day) for 7 weeks. Fresh feed was provided every day; also at the beginning and during the experimental period, the animal total body and total feed consumption were weighed and recorded. The heparinized capillary glass tubes were used to collect the blood samples from the orbital plexus according to Schermer (1967). To obtain serum, samples were centrifuged (1500 ×g) at 4°C for 30 min. The study received institutional approval (2016-10-084).

Serum biochemical assays

Serum blood glucose, urea, creatinine, uric acid, albumin, total protein, and MDA were determined by kits obtained from bio diagnostic company (Dokki, Giza, Egypt).

Serum globulin was determined by the following formula:

Serum globulin = Total serum protein – Serum albumin

Catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activity were assayed in serum. Catalase activity was determined in milliunits of enzymatic activity per mg of protein (mU/mg protein) contained in the samples by using Catalase Assay Kit (Cayman Chemical, Michigan, USA). The activity of GPx was determined according to Flohé and Günzler (1984). First, 20 µl of sample was mix with reaction mixture (180 µl) [pH 7.0, 50 mM potassium phosphate buffer, 1 mM glutathione (GSH, Roche, Mannheim, Germany), 0.5 mM EDTA, 1 mM sodium acid, 0.2-mM nicotinamide adenine dinucleotide phosphate (NADPH; Calbiochem) and 0.5 U GR (Roche)]. Then, adding 0.45 mM H_2O_2 (100 µl) to 0.15 mM (a final concentration) to initiated the reaction. The activity of GR was determined by recommended methods (Gutterer et al., 1999). Samples (30 µl) were mixed with reaction mixture (170 µl) (pH 7.0, 100 mM potassium phosphate buffer, 0.2 mM NADPH, 1 mM EDTA). Then, 100 µl of 3 mM GSSG (Roche) was added to 1 mM (a final concentration) to initiate the reaction. For both assays, the absorbance decreases because NADPH oxidation was recorded at 340 nm.

Statistical analysis

Mean (SEM) was used to express the results. One way analysis of variance (ANOVA) followed by Fischer's least significant difference (LSD) test was used to measure the intergroup variation. Statistical significance was considered at ($P \leq 0.05$). Statistical analysis was done using the Jandel Sigma Stat Statistical Software version 2.0.

Histopathological assay

For microscopic evaluation, the kidneys were first fixed in neutral phosphate buffered formalin solution (10%). After dehydration in an ascending series of ethanol (70, 80, 96, and 100%), the samples' tissue was cleared in xylene and embedded in paraffin. Tissue sections (5 µm) were stained with hematoxylin-eosin (H-E). Fields (10, a minimum) for each kidney slide were examined and assigned for severity of changes by pathologist blinded to the treatments of the animals.

RESULTS AND DISCUSSION

Scanning electron microscope (SEM)

Morphology of original QUE and QUNPs were studied using SEM tool. As shown in Figure 2, QUNPs showed a particle size of 16.13 nm at flow rate 10 ml/min. The powder of original QUE (Figure 2a) exhibited particles lacking uniformity in size which was relatively much larger than the QUNPs. While, QUNPs prepared by syringe pump, exhibited less crystallinity, absence of larger particles, and particles uniformity in size (Figure 2b) (Abd El-Rahman and Al Jameel, 2014).

These results indicated that QUE made by syringe pump gave particle size more uniform and significantly smaller than the commercial QUE that was more evidenced in the case prepared sample at 5 mg/ml (lower drug concentration). This behavior can be explained by considering two factors: the concentration influence on the viscosity and the nuclei number formed in the interface of solvent/anti-solvent (Kakran et al., 2012).

Effect of QUNPs on blood sugars, urea, creatinine and uric acid

As shown in Table 1, STZ diabetic rats showed increases in blood sugars, urea and creatinine and decreased uric acid as compared to NC. Treatment of STZ diabetic rats with QUNPs (10 and 20 mg/kg bw/day) resulted in decreased serum blood sugars, urea and creatinine and increased uric acid levels in those treated rats as compared to PC. QUNPs (10 and 20 mg/kg bw/day) alone gave results close to normal values observed in NC. Also, STZ + QUNPs (20 mg/kg bw/day) gave similar results. The antihyperglycemic effect of quercetin might be due to its property of antioxidant, which inhibits the peroxidation of lipid by scavenging the free radicals produced by STZ and prevents oxidative stress induced

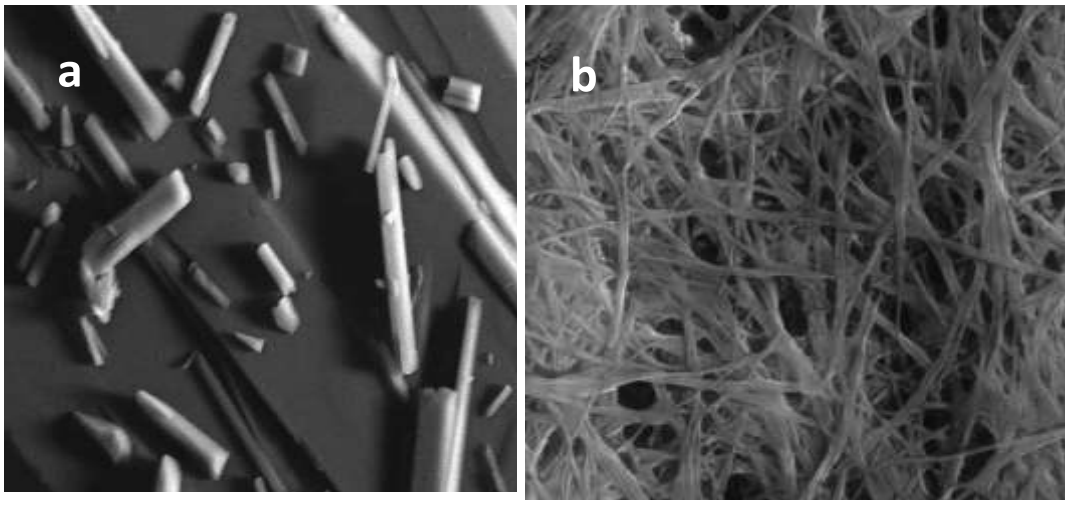

Figure 2. SEM photographs of (a) original quercetin (QUE) and (b) quercetin nanoparticles (QUNPs).

Table 1. Effect of QUNPs on blood sugars, urea, creatinine and uric acid in serum of control and experimental rats.

Group/Parameter	Blood sugars (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
Control	$76.40^{a} \pm 4.50$	$24.70^{a} \pm 2.60$	$1.03^{a} \pm 0.048$	$7.845^{a} \pm 0.014$
Diabetic	$219.55^{d} \pm 3.60$	$30.02^{b} \pm 2.68$	$1.77^{b} \pm 0.20$	$5.365^{c} \pm 0.010$
QUNPs 10	$77.62^{a} \pm 1.80$	$21.89^{a} \pm 2.17$	$1.2^{a} \pm 0.17$	$6.335^{b} \pm 0.019$
Diabetic QUNPs 10	$121.66^{c} \pm 3.02$	$20.36^{a} \pm 1.76$	$1.45^{ab} \pm 0.30$	$7.225^{ab} \pm 0.004$
QUNPs 20	$76.73^{a} \pm 2.90$	$22.78^{a} \pm 2.11$	$1.12^{a} \pm 0.12$	$7.803^{a} \pm 0.010$
Diabetic QUNPs 20	$85.16^{ab} \pm 3.30$	$22.04^{a} \pm 0.75$	$1.27^{a} \pm 0.12$	$7.888^{a} \pm 0.011$

Each value is mean ± SD for twelve rats in each group. Values that have a different superscript letter (a, b, c, d) differ significantly with each other ($p \leq 0.05$).

by STZ, and also, helps the surviving β-cells to secrete more insulin and proliferation. Additionally, QUE enhances the sensitivity of insulin, leading to increased glucose utilization by the extrahepatic tissues and thereby decreasing the levels of blood glucose (Babujanarthanam et al., 2010). Vessal et al. (2003) reported that supplementation has proven to be beneficial in decreasing the concentration of blood glucose, promoting the pancreatic islets regeneration and increasing release of insulin in STZ treated diabetic rats; thus exerting its beneficial antidiabetic effects (Formica and Regelson 1995).

This is supported by the previous literature reports where these types of flavonoids enhance release of insulin up to 70% by its effect on function of islet at least in part, metabolism of cyclic nucleotide, and via alteration in Ca^{+2} fluxes (Yen et al., 2009; Hii and Howell, 1985a; Hii and Howell, 1985b). Blood glucose levels were significantly increased after 72 h following STZ injection compared to control group, while all QUE treatment groups had significantly decreased blood glucose concentrations compared to the diabetic group after 30 days (Elbe et al., 2015).

Previous studies reported that the administration of STZ decreased levels of insulin and increased levels of plasma glucose, while treatment with QUE resulted in decreased glucose in plasma and increased levels of insulin. Diabetic groups treated with QUE suspension showed significantly lower glucose levels as well as QUNPs as against diabetic control group (Babujanarthanam et al., 2011; Sinha, 1972).

Serum urea and creatinine levels are the most important indicators of kidney functions. Lu et al. (2007) and Maciel et al. (2013) reported that urea and creatinine levels were increased in diabetic rats as compared to the control group. Also, urea level was increased in diabetic rats when administrated with 5, 25 and 50 mg/kg of QUE than healthy groups treated with the same QUE dosages ($P < 0.05$).

Effect of QUNPs on total protein, globulin and albumin

Data in Table 2 showed the total protein levels, albumin (A), globulin (G) and A/G ratio after treatment with STZ

Table 2. Effect of QUNPs on serum total protein, albumin and globulin of control and experimental rats.

Group/Parameter	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G Ratio
Control	$7.315^{a} \pm 0.014$	$4.155^{a} \pm 0.014$	$3.160^{a} \pm 0.014$	$1.316^{a} \pm 0.014$
Diabetic	$4.158^{d} \pm 0.010$	$2.200^{e} \pm 0.010$	$1.958^{c} \pm 0.010$	$1.124^{c} \pm 0.010$
QUNPs 10	$7.268^{ab} \pm 0.010$	$4.073^{b} \pm 0.010$	$3.195^{a} \pm 0.010$	$1.275^{b} \pm 0.010$
Diabetic QUNPs 10	$6.115^{c} \pm 0.019$	$3.800^{d} \pm 0.019$	$2.315^{b} \pm 0.019$	$1.641^{b} \pm 0.019$
QUNPs 20	$7.288^{a} \pm 0.011$	$4.138^{a} \pm 0.011$	$3.150^{a} \pm 0.011$	$1.314^{a} \pm 0.011$
Diabetic QUNPs 20	$6.785^{b} \pm 0.004$	$4.003^{c} \pm 0.004$	$2.782^{a} \pm 0.004$	$1.439^{b} \pm 0.004$

Each value is mean ± SD for twelve rats in each group. Values that have a different superscript letter (a, b, c, d) differ significantly with each other ($p \leq 0.05$).

Table 3. Effect of QUNPs on MDA, CAT, GR and GPx of control and experimental rats.

Group/Parameter	MDA (nmol/ml)	CAT (nmol/mg protein)	GR (nmol/mg protein)	GPx (nmol/mg protein)
Control	$1.28^{a} \pm 0.11$	$63.873^{a} \pm 0.014$	$14.618^{a} \pm 0.014$	$5.003^{ab} \pm 0.014$
Diabetic	$2.05^{d} \pm 0.20$	$35.043^{e} \pm 0.010$	$7.908^{d} \pm 0.010$	$2.343^{d} \pm 0.010$
QUNPs 10	$1.31^{a} \pm 0.10$	$64.960^{d} \pm 0.019$	$15.268^{c} \pm 0.019$	$5.358^{c} \pm 0.019$
Diabetic QUNPs 10	$1.53^{c} \pm 1.19$	$63.298^{c} \pm 0.004$	$14.425^{b} \pm 0.004$	$4.985^{b} \pm 0.004$
QUNPs 20	$1.43^{b} \pm 0.08$	$66.863^{a} \pm 0.010$	$15.635^{a} \pm 0.010$	$5.818^{a} \pm 0.010$
Diabetic QUNPs 20	$1.31^{a} \pm 0.15$	$63.808^{b} \pm 0.011$	$14.643^{a} \pm 0.011$	$5.010^{ab} \pm 0.011$

Each value is mean ± SD for twelve rats in each group. Values that have a different superscript letter (a, b, c, d) differ significantly with each other ($p \leq 0.05$).

(60 mg/kg bw), QUNPs (10 and 20 mg/kg bw) and QUNPs (10 and 20 mg/kg bw) + STZ for 7 weeks. PC group showed decreased levels ($P \leq 0.05$) of total protein, albumin, and globulin in relation to NC group. Diabetic rats treated with QUNPs (10 and 20 mg/kg bw) presented increased concentration of total protein, albumin and globulin to near NC levels ($P \leq 0.05$) and these results agree with several studies (Maciel et al., 2013; Arya et al., 2014). The albumin level was significantly decreased in the diabetic group, which might be due to albumin leakage due to glomerular basement damage of membrane combined with an increase in the pressure of trans glomerular filtration or impaired reabsorption of tubular (Maciel et al., 2013). Also, the results should a significant reduction in concentration of serum albumin in diabetic rats, except the group of rats treated with QUE at 50 mg/kg (Naoum, 1999)[(50)]. However, the QUE treatment significantly increased the serum albumin towards the negative levels, which was reflected in the reduction of kidney damage due to STZ-induced hyperglycaemia. Consistent with our results, Kandasamy and Ashokkumar (2012) reported that in diabetic nephrotoxic rats, flavonoids restore the reduced level of albumin. Thus, QUE has a beneficial pharmacological effect on diabetic, especially at a dose of 50 mg/kg on hepatic, protein levels, diabetic, and functional markers.

Effect of QUNPs on MDA, CAT, GR and GPx

Oxidative stress is an imbalance between the production of free radicals and antioxidants defense capacity (Hamadi et al., 2012). It is well known that hyperglycaemia increases mitochondrial ROS production and impairs cellular antioxidant enzymes, which could represent a key event in the development and progression of the complications of diabetes (Hamadi et al., 2012; Chang et al., 2012).

Glutathione reductase, catalase and glutathione peroxidase are among those enzymes that metabolize endogenous free radicals and reactive oxygen species, often with the concomitant oxidation of reduced glutathione (GSH) to its oxidized form (GSSG) (Josephy, 1997). Reduced glutathione deficiency is also seen in tumorigenesis aminoaciduria, nephropathy (Meister, 1988) and cataract genesis (Nagasawa et al., 1996; Walsh and Aleo, 1997).

QUNPs significantly increased the serum activity of MDA, CAT, GR and GPx enzymes in normal rats after treatment for 7 weeks (Table 3). On the other hand, STZ had an opposite effect on the activity of serum

malondialdehyde (MDA), catalase (CAT), GR and GPx enzymes, but treatment with QUNPs ameliorated its effect (Table 3). Similar results were noted by Elbe et al. (2015) where they found that QUE was beneficial in reducing diabetes-related alterations. QUE has a free radical scavenger, transfer electrons, chelate metals and superoxide radical inhibitor properties (Vessal et al., 2003; Ferrali et al., 1997). Beneficial effects of QUE are attributed to its antioxidant effects as well as protective effects on β-cell integrity. Also, Vessal et al. (2003) noted its increasing effect on the islets number of Langerhans in pancreas. Also, there is a significant decrease in the plasma level of glucose.

MDA is the most commonly used indicator of lipid peroxidation. Decreases in cellular antioxidant enzymes and increase in tissue level of MDA emphasize oxidative stress. Previous studies reported that QUE treatment significantly decreased diabetes-related oxidative damage in various organs by increasing the activities of antioxidant enzyme but decreasing the levels of MDA (Dias et al., 2005; Sirovina et al., 2013; Edremitlioglu et al., 2012).

Babujanarthanam et al. (2011) indicated that QUE decreases the levels of thiobarbituric acid reactive substances (TBARS) in plasma in STZ-induced diabetic rats. H_2O_2 may be an important mediator for tissue damage in STZ induced diabetes (Yanardag et al., 2005). CAT protects the cell from oxidative damage induced by H_2O_2, because it is localized in the microperoxisomes or the peroxisomes, which catalyzes the decomposition of H_2O_2 to water and oxygen (Abolfathi et al., 2012). If it is not decomposed by GSH peroxidase or CAT, it causes production of reactive hydroxyl radicals. Excess amounts of free radicals damage nucleic acids and cellular proteins by attaching to them and causing lipid peroxidation. STZ significantly increased the ROS and significantly decreased the activity of antioxidant enzyme. The activities of plasmatic GSH levels were increased significantly and the activities of antioxidant enzyme [CAT and superoxide dismutase (SOD)] were decreased significantly in STZ-diabetic rats. The diabetic rats treated with quercetin showed an increased in CAT, GSH and SOD activity. Quercetin directly scavenges free radicals and ROS; therefore, it is an important flavonoid which is known to be a potent antioxidant (Annapurna et al., 2009; Boots et al., 2008; Jeong et al., 2012). When treated with 50 mg/kg of QUE, the levels reverted close to normal values observed in control group ($P < 0.05$) (Maciel et al., 2013; Stanley and Menon, 2001). Also, Elbe et al. (2015) reported that in diabetic group, CAT activity was decreased significantly compared with the control group and were significantly increased in treated group compared with the diabetic group. QUE increases CAT activity and reduces lipid peroxidation, thus, it prevents oxidative stress (Elbe et al., 2015; Maritim et al., 1991).

GPx react with GSH, thus, it serves to detoxify peroxides (Sen, 1997). Low GPx activity in diabetic might be due to low GSH content, since GSH is a substrate and cofactor of this enzyme (Dominguez et al., 1998). In the process of converting H_2O_2 to water, GPx converts GSH to GSSG, which is reduced back to GSH by GRx (Maritim et al., 2003). Glutathione may contribute to antioxidant defense by networking with the other major antioxidants (Babujanarthanam et al., 2011).

STZ could decrease pancreatic GSH-Px and CAT, but QUE could enhances pancreatic GSH-Px and CAT activity and consequently antagonizes STZ effect on these antioxidant enzymes (Abdelmoaty et al., 2010). These results together suggest that QUNPs is effective to protect kidney against oxidative stress induced by STZ-induced diabetes.

Histopathological studies

As shown in Figure 3, the rat kidney sections were stained with hematoxylin and eosin. The NC rat kidney presents a normal glomerulus surrounded by Bowman's capsule, distal convoluted and tubules proximal, normal podocytes (Pc) and normal capillaries (CP) (Figure 3A). Figure 3B shows the kidneys of the STZ-treated rats, which present a glomerular hypertrophy (Gh), thickening of the basement membrane and mesangial expansion. Also, degeneration of glomerular capillaries both tubular and proximal convoluted tubule exhibited edematous changes. Figure 3D shows the effect of QUNPs 10 mg + STZ treatment and the findings present less expansion of the glomerulus, features of healing, mildly dilated capillaries, and little damage of proximal and distal tubules. Moreover, the histopathological of kidney sections for QUNPs 20 mg/kg bw/day + STZ and QUNPs (10 and 20 mg/kg bw/day) alone, appeared similar to NC (Figure 3F, C and E).

Our results agree with Elbe et al. (2015) who reported that diabetic group showed severe tubular and glomerular alterations. Cellular swelling, tubular changes including tubular basal membrane thickening, peritubular infiltration, epithelial desquamation, mesangial matrix expansion within glomerulus and capillary and intracytoplasmic vacuolization were obvious. Eddy (1996) and Babujanarthanam et al. (2011) suggested that tubular and glomerular changes were reduced in QUE administered groups. Also, Arya et al. (2014) and Bashir et al. (2014) reported that the diabetic rats treated with QUE demonstrated a recovery of the normal structure of kidney with intact tubules and glomerular epithelial cells.

Conclusion

The morphological and serum biochemical findings suggested that the administration of QUNPs to diabetic rats causes beneficial effect in terms of regeneration of cells in damaged kidney. Thus, it is concluded that

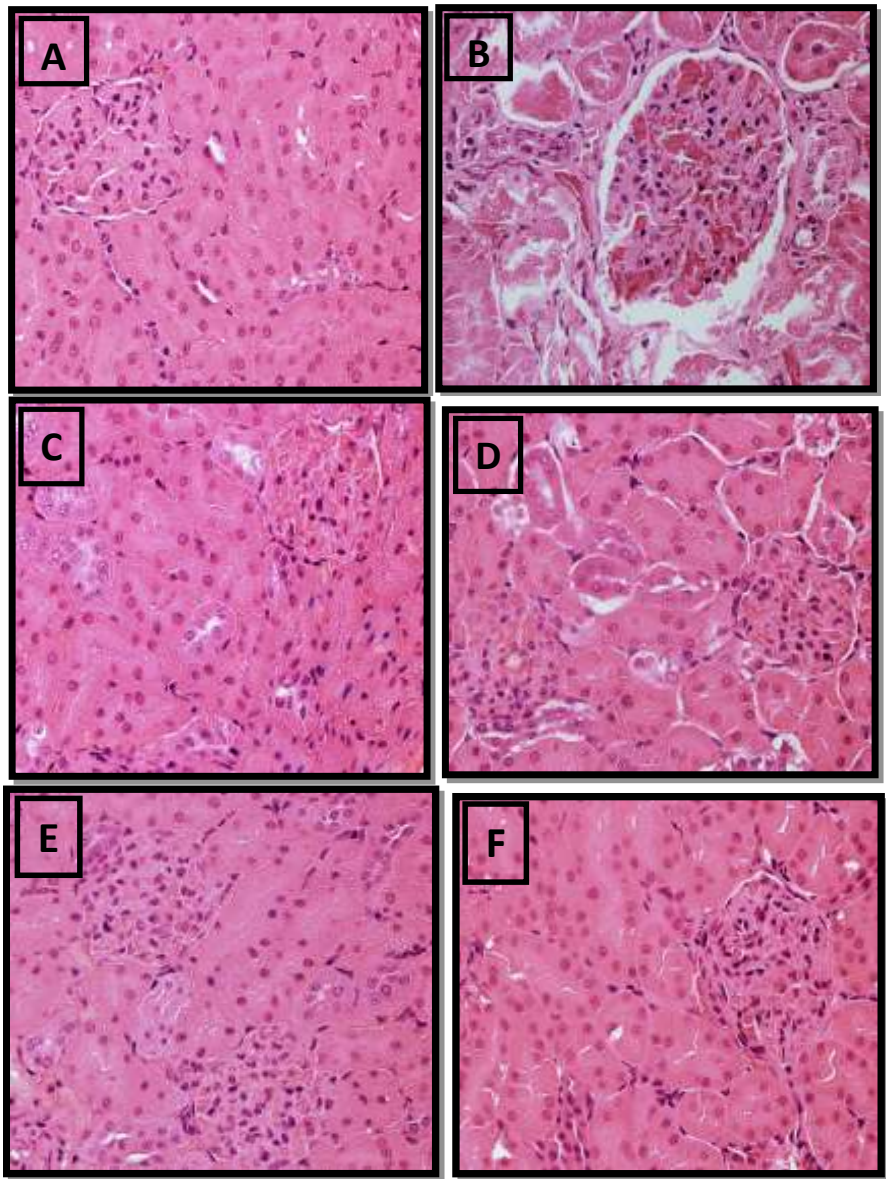

Figure 3. Light micrograph of kidney sections of (A) normal control (NC), (B) positive control (PC), (C) QUNPs (10 mg), (D) QUNPs (10 mg) + STZ, (E) QUNPs (20 mg), and (F) QUNPs (20 mg) + STZ H&E ×400.

QUNPs possess preventive and curative effect on STZ induced diabetes in rats and can be used as a natural herbal medicine to protect kidney and pancreatic cells.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Molecular characterization of virus isolates from genus *Potyvirus* infecting *Vigna subterranea* in Burkina Faso

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Bambara groundnut (*Vigna subterranea*) is an African legume with a great nutritional, economic and social potential. However, one of the main constraints to this crop is viral diseases which reduced yields. Indeed, approximately 12 viruses have been reported to infect bambara groundnut. Among these, only four were reported from Burkina Faso, namely, cowpea aphid-borne mosaic virus (CABMV), bean common mosaic virus strain-blackeye cowpea mosaic (BCMV-BlCM), peanut mottle virus (PeMoV), and cowpea mottle virus (CPMoV). This study was carried out in order to identify and characterize the main viruses occurring in bambara groundnut from Burkina Faso using serological and molecular tests. 140 plants were sampled in the three agro-climatic zones of Burkina Faso. Both *Potyvirus* CABMV and BCMV-BlCM were identified in 8.57% of the samples when double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) test was used and 14.29% of the samples when reverse transcription-polymerase chain reaction (RT-PCR) test was used. Phylogenetic tree based on 476 nt in coat protein showed that all Burkina Faso CABMV isolates clustered together with Uganda isolate (KT726938). However, two groups were distinguished within these isolates. Burkina Faso BCMV-BlCM isolates are strengthly clustered with BCMV and BlCMV group. This study reports the first molecular characterization of CABMV and BCMV-BlCM infecting Bambara groundnut in Burkina Faso.

Key words: Bambara groundnut, cowpea aphid-borne mosaic virus (CABMV), bean common mosaic virus strain-blackeye cowpea mosaic (BCMV-BlCM), double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), reverse transcription-polymerase chain reaction (RT-PCR).

INTRODUCTION

Bambara groundnut [*Vigna subterranea* (L.) *Verdc*.] is an indigenous legume that originated from Africa precisely in the northern part of Nigeria and Cameroun (Goli et al., 1997). It is mainly grown for human consumption and

plays an important socio-economic role in tropical Africa (Nadembega, 2016). Its seeds contain an average of 63% carbohydrate, 19% protein and 6.5% oil (Mkandawire, 2007). This well balanced composition makes it a complete food, thus Bambara groundnut could be used to alleviate nutritional problems especially for the rural population (Bamshaiye et al., 2011). In Burkina Faso, it is ranked second seed legume after cowpea [*Vigna unguiculata* (L.) Walp] in terms of production and consumption (Ouédraogo et al., 2008).

However, viral diseases are one of the major constraints to Bambara groundnut production. Indeed, viral disease can cause yield loss of about 60% (Brink et al., 2006). Approximately, 12 virus species were known to infect bambara groundnut in Africa (Thottappilly and Rossel, 1997). These include cowpea mottle virus (CPMoV) (Robertson, 1966; Rossel, 1977; Shoyinka et al., 1978), cowpea aphid borne mosaic virus (CABMV) (Gumedzoe, 1985) from Nigeria, peanut mottle virus (PeMoV) from Kenya (Bock et al., 1978), voandzeia necrotic mosaic virus, voandzeia mosaic virus, southern bean mosaic virus (SBMV) and voandzeia distortion mosaic virus from Cote d'Ivoire (Fauquet and Thouvenel, 1987), bean common mosaic virus strain black eye cowpea mosaic (BCMV-BlCM) formerly known as blackeye cowpea mosaic virus (BlCMV), cowpea mosaic virus (CPMV), cowpea mild mottle virus and cucumber mosaic virus (CMV) from Nigeria (Thottappilly and Rossel, 1997) and an unidentified *Potyvirus* from Togo (Bird and Corbett, 1988). From the listed virus, CABMV and BCMV-BlCM from the genus *Potyvirus*, are reported to be most damageable on cowpea (Udaya Shankar et al., 2009). They are transmitted by seed at the rates of about 40 and 30%, respectively (Frison et al., 1990), by sap inoculation and by aphids in a non-persistent manner (Zettler and Evans, 1972; Bock, 1973; Brunt et al., 1990).

Among these viruses found to occur on bambara groundnut, only four have been reported from Burkina Faso using serological and biological tests. They include CABMV, BCMV-BlCM, PeMoV and CPMoV (Sérémé, 1989; Drabo et al., 1997; Néya, 2011). However, none of the studies have molecular characterized bambara groundnut viruses from this country. Whereas, improving our knowledge in the virus characterization would help to establish some effective control strategies against bambara groundnut viral diseases (Frenkel et al., 1992).

In this study, serological and molecular detection tests were used to screen for the presence of viruses within bambara groundnut leaves samples collected in the Sudan (humid), Sudan-Sahel (sub-humid), and Sahel (dry) agro-climatic zones of Burkina Faso. Virus identified was further characterized for their phylogenetic relationship with GenBank viruses.

MATERIALS AND METHODS

Plant sampling

During the months of September to October of the year 2016, a sampling was carried out on bambara groundnut fields. Symptomatic leaves showing leaf curling, stunting and mosaic diseases were sampled in the three agro-climatic zones of Burkina Faso. Sampling concerned farmers' fields and experimental plots. A total of 140 samples were collected with 5 in the sahel zone, 125 in the sudan-sahel zone and 10 in the sudan zone. Leaves collected were placed on melting ice before storage at -80°C. Each collected sample was divided into three parts. The first was used for double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) serological tests, the second for reverse transcription-polymerase chain reaction (RT-PCR) molecular tests and finally the third part was retained as eventual inoculum.

Serological detection test

The presence of viruses CABMV, BCMV-BlCM, CPMoV, CPMV and PeMoV in the samples collected were detected by using their corresponding polyclonal antibodies from ELISA detection kits. ELISA detection kits were purchased from AC Diagnostics, Inc. (USA) and a DAS-ELISA (Clark and Adams, 1977) was performed following the manufacturer's protocol.

RNA extraction, RT-PCR and sequencing

For molecular characterization, RNA was extracted from collected samples leaves using Trizol reagent (Invitrogen, USA) extraction protocol as described in Longué et al. (2017).

The cDNAs were constructed in two steps. Firstly, 5 µl mixture of total RNA and oligo dT (10 µM) were incubated at 70°C for 5 min and immediately placed on ice for 5 min. Secondly, a mixture constituted of 1.25 µl of dNTPs (10 mM), 5 µl of M-MLV RT Buffer (5x) (Promega, Corp. USA), 0.5 µl of enzyme M-MLV RT RNase (200 U) (Promega, Corp. USA) and H_2O qsp was added to the previous 5 µl mixture. Then, the total volume of 25 µl was incubated at 40°C for 1 h followed by 70°C for 15 min to generate cDNA.

PCR was performed in a final volume of 25 µL containing: 4 µl of Go Taq 5x Buffer (Promega, Corp. USA), 0.5 µl of dNTPs (10 mM), 0.1 µl of Go Taq G2 polymerase (200 U) (Promega, Corp. USA), 2 µl of cDNA, 0.5 µl of each forward and reverse primers (Table 1) and H_2O qsp. The amplifications cycle was as follow: 94°C for 5 min, 35 cycles of 94°C for 30 s, Ta (°C) for 30 s and 72°C for Ext (s), and a final elongation of 72°C for 7 min.

The degenerated primers pair P077/P078 (Table 1) (Marie-Jeanne et al., 2000) was used for further identification of *Potyvirus* in the samples. To sequence the *Potyvirus* (CABMV and BCMV-BlCM), the whole coat protein gene, primers P105 and P106 were designed based on GenBank available sequences. Then, primers combination P105/P078 and P077/P106 (Table 1) were used in independent PCR. Amplification products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide and then visualized under UV light. Expected amplicons for 12 samples were direct sequenced with forward and reverse primers using the Sanger method (GENEWIZ, UK).

Sequences analyses and phylogenetic construction

Sequences obtained were trimmed and assembled using DNAMA

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Table 1. List of primers used in this study.

Primers pair	Sequences (5'-3')	Ta (°C)	Ext (Sec)	Expected fragment length (bp)
P077 P078	ATGGTHTGGTGYATHGARAAYGG CARATGAARGCMGCAGCA	55	30	327
P105 P078	GCYCCDTAYATHGCRGARWCWGC TGCTGCKGCYTTCATYTG	52.5	60	≈800
P077 P106	ATGGTHTGGTGYATHGARAAYGG CACAGTTAKCRTYTCRYG	52.5	60	≈400

Single letter code: H = A/C/T; Y = C/T; R = A/G; K = G/T; Ta, annealing temperature; Ext, extension time.

Table 2. Potyvirus detection in DAS-ELISA and RT-PCR according to sampling area.

Samples code	Samples name	Detection test used		Agro-climatic zones
		DAS-ELISA	RT-PCR	
E1	L86-E111	+	+	Sudan-sahel
E2	203-KVS246-1	+	+	Sudan-sahel
E3	306-KVS235-100GY	+	+	Sudan-sahel
E4	310-E119	+	+	Sudan-sahel
E5	210-KVS235	+	+	Sudan-sahel
E6	209-E125	+	+	Sudan-sahel
E10	L9-E25	+	+	Sudan-sahel
E15	215-E56 A	+	+	Sudan-sahel
E18	308-KVS246-2	-	+	Sudan-sahel
E51	E56 A-KBS2	+	+	Sudan-sahel
E113	Ech-DED2	-	+	Sudan-sahel
E138	KVS246-KBS2	-	+	Sudan-sahel
E139	L85-E117	+	+	Sudan-sahel
E140	120-KVS235-100GY	+	+	Sudan-sahel
E141	220-KVS235-100GY	-	+	Sudan-sahel
E142	Ech-KBS	+	+	Sudan-sahel
E99	Ech-PO4	-	+	Sudan
E100	Ech-SAP5	-	+	Sudan
E101	Ech-LEO2	-	+	Sudan
E104	Ech-SAP3	-	+	Sudan

+ = Positive response to the detection tool used; - = negative response to the detection tool used.

software version 7.2.0. A consensus sequences of about 476 (partial coat protein) to 950 nt (covered the complete coat protein) were reconstituted with the tree primer pair P077/P078, P105/P078 and P077/P106. Sequences were then compared to Genbank available sequences using Basic Local Alignment Search Tool (BLAST; hhttp://www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1997).

Phylogenetic tree was constructed based on 476 nt obtained from 12 samples. Sequences were aligned using MUSCLE (Edgar, 2004) with default setting. Maximum likelihood phylogenetic tree was performed in MEGA 6.0 (Tamura et al., 2013) using the Tamura Nei parameter (TN93+G+I) nucleotidic substitution model. Bootstrap method at 1000 replicates was adopted to support the branches.

The pairwise nucleotide identities were performed using SDT software version 1.2 (Muhire et al., 2014). Sequences obtained in this study have been submitted to Genbank (Table 3).

RESULTS

Virus detection in DAS-ELISA and RT-PCR tests

DAS-ELISA was efficient to identify only CABMV and BCMV-BlCM from the genus *Potyvirus,* respectively in 7.14 and 1.43% samples to make a total of 8.57% (12/140) positive samples (Table 2). None samples was detected positive to CPMoV, CPMV and PeMoV antibodies. However, RT-PCR tests were more efficient in *Potyvirus* detection (14.29%, 20/140) using primers pair P077/P078 (Marie-Jeanne et al., 2000). Beside positive samples in DAS-ELISA, eight new samples become positives (Table 2). Figure 1 shows the detection

Table 3. List of virus isolates from the genus *Potyvirus* from Burkina Faso and some GenBank accessions analyzed in this study.

Accession number	Plant host	Country	Virus names
MF277031	*V. subterranea*	Burkina Faso	CABMV
MF277032	*V. subterranea*	Burkina Faso	BCMV-BlCM
MF277033	*V. subterranea*	Burkina Faso	CABMV
MF277034	*V. subterranea*	Burkina Faso	CABMV
MF277035	*V. subterranea*	Burkina Faso	CABMV
MF277036	*V. subterranea*	Burkina Faso	CABMV
MF277037	*V. subterranea*	Burkina Faso	CABMV
MF277038	*V. subterranea*	Burkina Faso	CABMV
MF277039	*V. subterranea*	Burkina Faso	CABMV
MF277040	*V. subterranea*	Burkina Faso	BCMV-BlCM
MF277041	*V. subterranea*	Burkina Faso	CABMV
MF277042	*V. subterranea*	Burkina Faso	BCMV-BlCM
AB458596.1	*Cucurbita pepo*	Syria	Zucchini yellow mosaic virus
AF348210.1	-	Zimbabwe	CABMV
AJ132414.1	*V. unguiculata*	Nigeria (Ibadan)	CABMV
AJ312438.1	*V. unguiculata*	China	BCMV
D10053.1	*Passiflora* species	South Africa	South African passiflora virus
DQ666332	*Phaseolus vulgaris*	Columbia	BCMV
EU660586.1	*Cucumis melo*	France	Watermelon mosaic virus
KC777407.1	*Passiflora* species	Brazil	CABMV
KT726938.1	*V. unguiculata*	Uganda	CABMV
AY575773.1	-	Taiwan	BCMV-BlCMV
Y17822.1	*V. unguiculata*	Nigeria (Monguno)	CABMV
Y18634.1	*V. unguiculata*	Morocco	CABMV

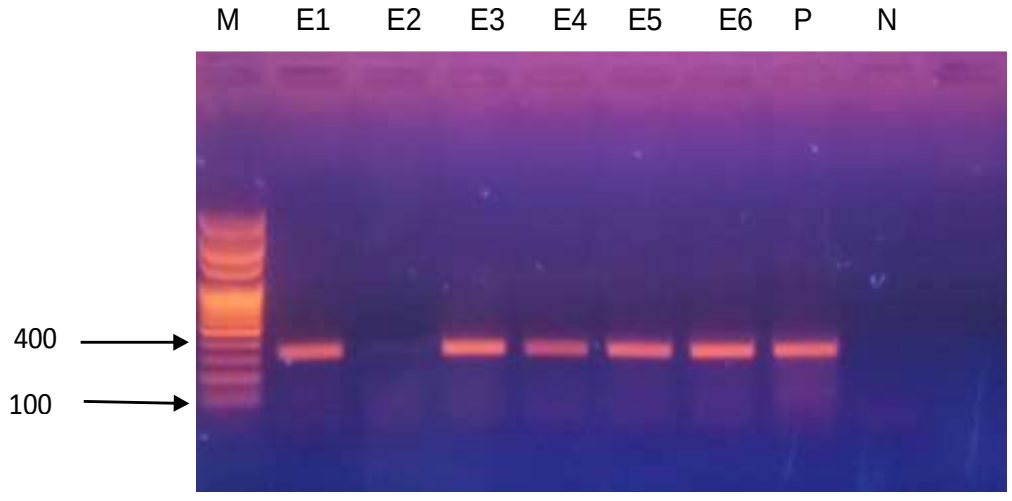

Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) bands in agarose gel with *Potyvirus* primer couple 1 (Marie-Jeanne et al., 2000). M represents marker (100 bp DNA ladder); N represents negative control; P represents positive control; E1-E6 represent RNA of samples tested.

of *Potyvirus* at 327 bp in some samples. Moreover, primers pair P105/P078 and P077/P106 succeeded in amplifying their corresponding fragments (Figure 2).

The BlastN analyses based on consensus sequences

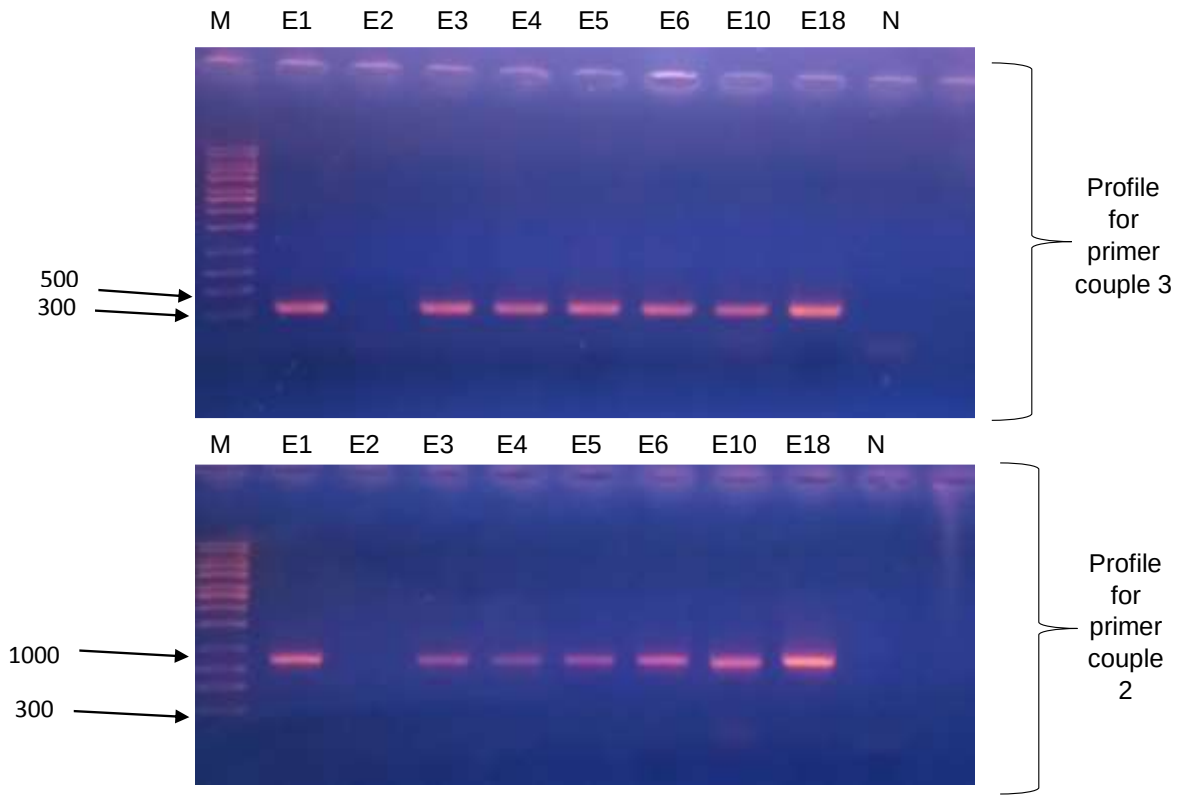

Figure 2. Reverse transcription-polymerase chain reaction (RT-PCR) bands in agarose gel with *Potyvirus* primer couple 2 and 3. M represents marker (1Kb DNA ladder); N represents negative control; E1-E18 represent RNA of samples tested.

(476-950 nt) revealed that nine samples were identified to CABMV Genbank accession number KT726938 at 88 to 98% identity (Table 3) whereas, three samples were identified at 97 to 98% identity to BCMV Genbank accession number AJ312438 (Table 3). These confirmed the identification of CABMV and BCMV-BlCM in DAS-ELISA test.

Among the three agro-climatic zones, sudan zone was the most infected by *Potyvirus* (40%, 4/10), followed by the Sudan-sahel zone (12.8%, 16/125) when none positive sample was detected in the sahel zone (Table 2).

Phylogenetic and nucleotide identity analyses

The maximum likelihood phylogenetic tree (Figure 3) showed that the 5' partial coat protein sequence (476 nt) was sufficient to separate the two *Potyvirus* CABMV and BCMV-BlCM.

All Burkina Faso CABMV isolates clustered together with Uganda isolate (KT726938). However, they might form two distinct groups. The first one is most closed to Uganda isolate (KT726938) at bootstrap 86%. Members of this group shared together 99.6 to 100% identity and 97.5 to 97.7% identity to Uganda strain (Table 4). Members of the second group showed in an isolate group and shared together 83.4 to 99.4% identity. However, pairwise identities between Uganda isolate (KT726938) and members of the second group (85.1 to 93.3%) (Table 4) were low. Furthermore, identities between the two groups of CABMV ranged from 84.9 to 92.4%.

In Burkina Faso, all CABMV isolates and Uganda isolate nested to the Nigeria isolate (Y17822) at bootstrap 97 (Figure 3).

Burkina Faso BCMV-BlCM isolates unambiguously nested with BCMV and BlCMV group and particularly clustered with BCMV (AJ312438) Chinese strain at bootstrap 97. These isolates shared high nucleotide identity together (97.3 to 100%), while they shared 97.3 to 97.5% identity with Chinese isolate (AJ312438).

DISCUSSION

Several viruses have been reported to infect bambara groundnut (Thottappilly and Rossel, 1997). This study identified only *Potyvirus* CABMV and BCMV-BlCM infecting bambara groundnut in Burkina Faso both in DAS-ELISA and RT-PCR tests. This may be associated to the high prevalence of these viruses (over 65%)

Table 4. Percentage of nucleotide identity between Burkina Faso isolates of cowpea aphid-borne mosaic virus (CABMV) and blackeye cowpea mosaic virus strain of bean common mosaic virus (BCMV-BlCM) and Genbank related sequences based on 476 nucleotides in the coat protein.

Accession No.	Country	E1	E2	E3	E4	E5	E6	E10	E18	E113	E138	E139	E142
BCMV (AJ312438)	China	67.8	97.3	66.9	68.1	66.9	66.9	66.9	68	66.9	97.3	64.3	97.5
BlCMV (AY575773)	Taiwan	66.7	94.1	64.5	68.3	64.5	64.5	64.5	68.1	64.5	94.1	65.5	94.3
BCMV (DQ666332)	Colombia	67	84	66.7	67	66.7	66.7	66.7	66.8	66.7	84	66.6	84
WMV (EU660586)	France	67.1	68.7	65.5	66.9	65.5	65.5	65.5	68.5	65.5	68.7	66.7	68.5
ZYMV (AB458595)	Syria	66.7	67.7	66	64.5	65.8	66	65.8	67.3	65.8	67.7	66.8	67.7
CABMV (KT726938)	Uganda	93.1	68.5	97.5	93.1	97.7	97.5	97.7	93.3	97.7	68.5	85.1	68.5
CABMV (Y17822)	Monguno	84.2	69.3	84.2	84.5	84.5	84.2	84.5	84	84.5	69.3	78.4	69.3
CABMV (AJ132414)	Nigeria	80.3	67.7	81.8	80.1	82	81.8	82	80.8	82	67.7	81.2	67.7
CABMV (Y18634)	Morocco	80.3	67	81.6	80.3	81.8	81.6	81.8	80.8	81.8	67	81.6	67
CABMV (KF725712)	Brazil	77	69.1	75.2	77.2	75	74.8	75	77.2	75	69.1	77.1	69.1
CABMV (AF348210)	Zimbabwe	77.5	67.4	76.1	77.7	76.3	76.1	76.3	77.9	76.3	67.4	76.1	67.4

E1, MF277031; E2, MF277032; E3, MF277033; E4, MF277034; E5, MF277035; E6, MF277036; E10, MF277037; E18, MF277038; E113, MF277039; E138, MF277040; E139, MF277041; E142, MF277042.

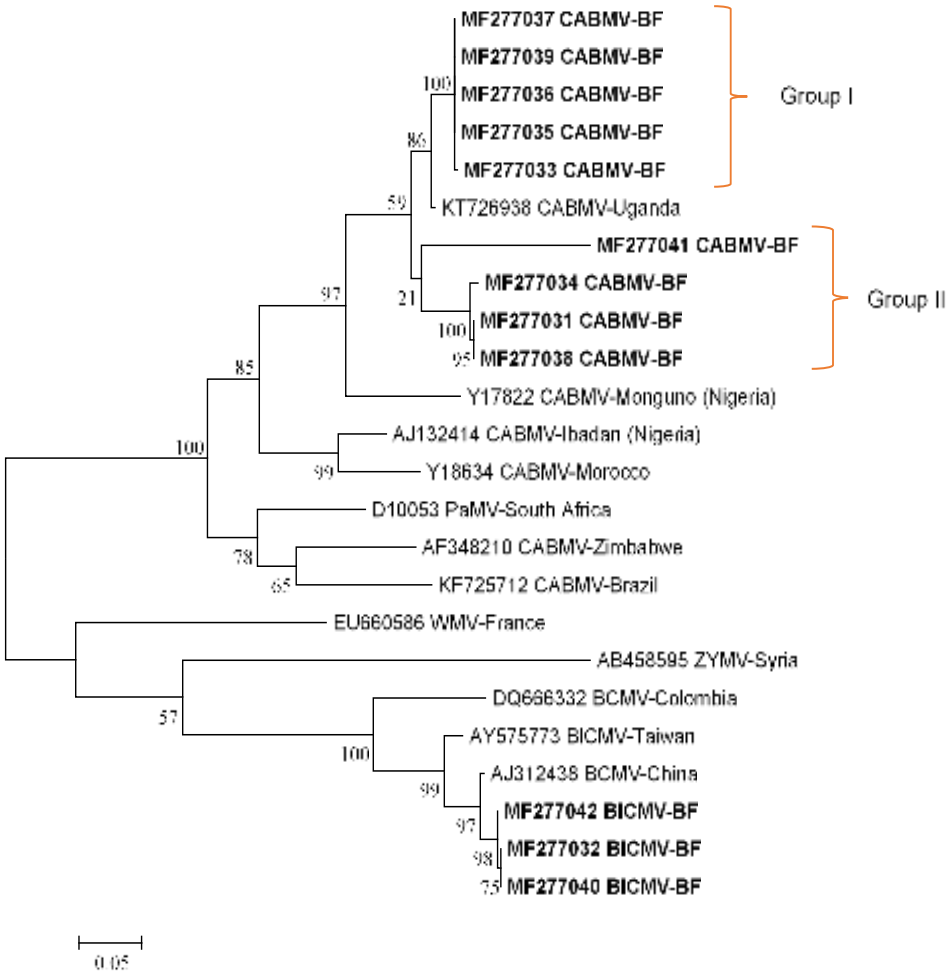

Figure 3. Maximum likelihood phylogenetic tree based on 476 nt in coat protein gene showing relationship between Burkina Faso isolates of CABMV and BCMV-BlCM (MF277031 to MF277042) and Genbank related species (Table 3). Bootstrap method was adopted at 1000 replicates.

reported in the country on cowpea crop (Néya, 2011; Palanga et al., 2016). Bambara groundnut and cowpea are two legume crops cultivated under the same climatic conditions and sometime in association. However, their prevalence in DAS-ELISA (8.57%) was less than in RT-PCR (14.29%). Indeed, some specificity in the coat protein structure of some virus strain may result in their false detection in serological test, whereas primers used in RT-PCR were specific and degenerated (Table 1) to amplified maximum of CABMV and BCMV-BlCM strains. On the other hand, some studies reported the efficiency of ELISA tests in plant virus detection (Konaté and Néya, 1996; Akinjogunla et al., 2008; Lima et al., 2012), but Gillaspie et al. (1999), Sipahioğlu (2005) and Liebenberg et al. (2009) showed that PCR and RT-PCR molecular tests were more sensitive.

Sudan zone (humid) was observed to be the most infected (40%) than sudan sahel (sub humid) (12.8%) and sahel (dry) (0%). Thus, climate might have an influence on *Potyvirus* infection and distribution in bambara groundnut crop. Indeed, Dabiré (1992) and Néya et al. (2008) also showed that the propagation of CABMV epidemic in cowpea crop and aphid population (vectors of these *Potyvirus*) decreased from the sudan zone to the sahel. Elsewhere, Estay et al. (2009) reported that aphids' population increase is a key factor to the spread of viruses and it depend on climate. In other parts, the prevalence of CABMV in cowpea was also found higher in the sudan than the sudan-sahel and the sahel (Néya, 2011; Palanga et al., 2016).

However, this study reports that CABMV was most prevalent than BCMV-BlCMV even in DAS-ELISA (7.14 against 1.43%, respectively) and RT-PCR (10.71 against 2.86%, respectively). This is in agreement with the study of Néya (2011) and Palanga et al. (2016) work on cowpea.

The none identification of CPMV, CPMoV and PeMoV might explain their absence in the 140 bambara groundnut samples tested. However, Sérémé (1989) had already reported CPMoV and PeMoV on bambara groundnut in the country.

The high identity obtained in BlastN identification of CABMV (88 to 98%) and BCMV-BlCM (97 to 98%) is in agreement with the ICTV *Potyvirus* species demarcation (<76% nucleotide identity in the coat protein) (Wylie et al., 2017).

The phylogenetic tree supports the occurrence of the two *Potyvirus* species CABMV and BCMV-BlCM on bambara groundnut in Burkina Faso. The specificity of nucleotide sequence to distinguish these two viruses was reported by Grisoni et al. (2006) and Palanga et al. (2016). Two groups within Burkina Faso CABMV isolates were observed on the phylogenetic tree (Figure 3). When the first group (MF277036, MF277033, MF277035, MF277037, MF277039) shared high nucleotide identity (97.5 to 97.7%) with Ugandan isolate (KT726938), the second group (MF277031, MF277034, MF277038, MF277041) was at 85.1 to 93.3% identity to the same Ugandan isolate. According to the *Potyvirus* strain criteria demarcation proposed by Shukla and Ward (1988) (with nucleotides sequence identity >95% in the coat protein), members of the first group and the Ugandan isolate (KT726938) might be the same strain. However, member of the second group might still be an isolate strain. These results might support the presence of two strains of CABMV infecting bambara groundnut in Burkina Faso. Indeed, previous study based on serological test revealed the presence of four serotypes I, II, III and IV within CABMV species infecting legume crops in Burkina Faso (Néya, 2011). Beside the Ugandan isolate (KT726938), all Burkina Faso CABMV isolates were closed to Nigerian isolate (Y17822) (78.4 to 84.5% identity).

The high seed-transmission of CABMV on cowpea (3 to 100%) was shown by Konaté and Néya (1996), Néya (2002) and Barro et al. (2016). It was considered to be the first source of infection to initiate CABMV epidemic (Néya et al., 2007). Bambara groundnut or other legume crops seeds trade between countries or seeds exchange between research centers could explain the strong relationships between Uganda and Burkina Faso isolates.

Burkina Faso BCMV-BlCM isolates (MF277032, MF277040 and MF277042) shared 97.3 to 97.5% high identity to China BCMV (AJ312438) and 94.1 to 94.3% to Taiwan BlCMV (AY575773). Since Khan et al. (1993) showed that BlCM and BCMV are strain of the same species, our study supports that Burkina Faso BCMV-BlCM and Chinese BCMV isolate (AJ312438) might be the same strain according to (Shukla and Ward, 1988) strain criteria demarcation.

Altogether, our study reports the first molecular characterization of virus from genus *Potyvirus* in bambara groundnut from Burkina Faso.

Conclusion

This study reveals the occurrence of CABMV and BCMV-BlCMV infecting bambara groundnut in Burkina Faso. However, CABMV was the most prevalent. Beside, RT-PCR was the most accurate tool for these virus detection in bambara groundnut; the phylogenetic analyses permit the understanding of evolutionary relationships between Burkina Faso isolates and others Genbank available species. However, further analysis based on the whole coat protein gene in amino acid or nucleotide and increase of samples number is required for better characterization of virus from genus *Potyvirus* occurring in bambara groundnut fields in Burkina Faso.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Effect of physicochemical factors on the biodegradation of phenol by *Pseudomonas putida* ATCC 12842 and *Pseudomonas fluorescens* ATCC 948

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Phenol is a very toxic substance and it can cause a number of environmental problems when it enters a water system. Biological treatment is considered a cost-effective and safe technology and it plays an important role in the remediation of environmental pollutants. In this study, *Pseudomonas fluorescens* ATCC 948 and *Pseudomonas putida* ATCC 12842 were employed to biodegrade phenol, with different culture conditions such as, different incubation periods, initial phenol concentration, temperature, pH, carbon and nitrogen sources) used to examine their effect on phenol degradation. The results showed that phenol degraded completely after 122 h. Different initial concentrations of phenol were added to minimal salts medium and the percentage of phenol degradation decreased as the phenol concentration increased. The optimum temperature for both bacterial strains, *P. fluorescens* and *P. putida,* is 35°C, with the maximum percentage of degradation occuring at pH 7. Glucose is the best carbon source as it increased the rate of biodegradation up to 80%. Ammonium nitrate is the best nitrogen source for *P. fluorescens* to degrade phenol, while ammonium chloride is the best source of nitrogen for degradation of phenol by *P. putida*. The study's results suggest that *P. fluorescens* and *P. putida* are capable of phenol degradation, and thus can be used for bioremediation of synthetic waste water containing phenol.

Key words: Physicochemical factors, biodegradation, *Pseudomonas fluorescens*, *Pseudomonas putida.*

INTRODUCTION

Phenol is present in nature and it is produced in industry. It is an organic compound (Paula and Young, 1998). Phenol and its derivatives are among the most frequently found pollutants in rivers, industrial effluents and landfill run-off waters (Ying et al., 2007; Kotresha and Vidyasagar, 2013). Even if phenol is found in low concentrations of 5 to 25 mg/L, it affects the aquatic environments, leading to bitter taste and bad odour in municipal drinking water. The presence of phenol in water creates significant stress on eco-systems, along with a negative effect on aquatic flora and fauna (Cheela et al., 2014; Sreeremya, 2015).

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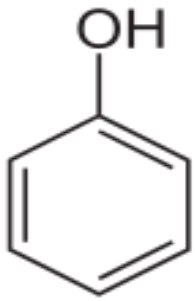
Phenols are used in various industrial applications and industries, such as oil refineries, chemical, petrochemical, pharmaceutical, metallurgical and pesticide products, and in paint and textile industries. Therefore, removal of phenol from industrial aqueous effluents is an important practical problem (Lakshmi et al., 2009). Different types of environmental biotechnologies, such as physical, chemical and biological methods, are used to remove phenol (Mangukiya et al., 2015). Recently developed chemical procedures are specific for phenol removal; these include many expensive methods, such as distillation and liquid-liquid extraction with the use of different solvents, adsorption and membrane filters.

Microorganisms can utilise phenol and its derivatives as a sole carbon and energy source at varying concentrations under optimum conditions (Shweta and Dhandayuthapani, 2013). Biodegradation of phenol occurs in aerobic/anaerobic conditions. Whereas aerobic microbes use oxygen as an electron acceptor, molecular oxygen is a reactant for oxygenase enzymes, and it is incorporated into the final product; in anaerobic conditions, different inorganic electron acceptors are possible, such as NO_3, SO_4, CO_2 and Fe^{3+} (Lakshmi and Sridevi, 2009). Microorganisms and their enzymes can convert phenol to non-toxic intermediates of tricarboxylic acids via meta-pathway (Mohite et al., 2010; Sridevi et al., 2012); however, in higher concentrations, it is difficult to biodegrade phenol and its derivatives because they are toxic to most microorganisms. Phenols can even inhibit the growth of microorganisms that are capable of utilising them. Hence, phenol is used as an antimicrobial agent (Krastanov et al., 2013).

Most types of microbes, including bacteria and fungi, can degrade phenolic compounds and use them as a source of carbon and energy (Michałowicz and Duda, 2007; Supriya and Deva, 2014). Several bacterial species have been studied, including *Acinetobacter calcoaceticus* (Liu et al., 2016), *Pseudomonas sp (*Kafilzadeh et al., 2010), *Bacillus thuringiensis*, *Brevibacterium iodinum* and *Staphylococcus aureus* (Kafilzadeh and Mokhtari, 2013), *Rhodococcus sp*. (Nor Suhaila et al., 2010) and *Nitratireductor aquimarinus, Nitratireductor aquimarius, Marine bacterium* and *Pseudomonas stutzeri* (Boroujeni et al., 2014).

Many studies have recommended using bacteria belonging to the genus of *Pseudomonas* as good degraders of phenol. *Pseudomonas* sp is a Gram–negative bacteria as it is polar flagellated and unicellular. On agar, colonies are circular, have a yellow to greyish colour, 3 to 5 mm diameter, are smooth, glistening and opaque. *Pseudomonas* sp grows well at both 37 and 54°C. No acid or gas is produced from carbohydrates and alcohols, such as arabinose, glucose, fructose, galactose, sucrose, maltose, lactose, starch, inulin, dextrin, glycerol, mannitol and sorbitol. Whereas slight acidity is observed in xylose, nitrate is not reduced, starch is not hydrolysed and gelatin is not liquefied. The catalase, indole and the Methyl Red-Voges Proskauer test results are negative. These organisms do not produce hydrogen sulphide, however, *Pseudomonas* sp grows well in a citrate medium. *Pseudomonas* is aerobic, degrades phenol and resists high concentrations of it (Hamdy et al., 1956; Seker et al., 1997).



Scheme 1. Chemical structure of phenol.

Pseudomonas strains can secret catechol 2, 3-oxygenase, which degrades phenol to catechol (non-toxic intermediate compound). *Pseudomonas putida* can use aromatic compounds such as phenol, as a sole source of carbon and energy. Its optimum microbial growth conditions are 30°C and pH 6.8 (Seker et al., 1997). Also, Hamdy et al. (1956) reported that *Pseudomonas fluorescens* can oxidize phenol to catechol.

Biodegradation is an important process for removing phenolic pollutants. In a biological treatment method it is necessary to select a potential bacterial strain to degrade these pollutants.

Considering the potential of *Pseudomonas* strains, the current study aims to select two bacterial strains, *P. putida* and *P. fluorescens*, and examine their ability to degrade phenol and thereafter, investigate the factors that affect phenol biodegradation.

MATERIALS AND METHODS

Chemicals

Phenol (99% pure, chemical grade), 4-amino antipyrine and all other chemicals were purchased from Merck. Chemical structure of phenol is a white crystalline solid with molecular weight of 94.14 g/mol and formula of C_6H_5OH (Scheme 1) (Sridevi et al., 2012).

Microorganisms

The microorganisms *P. fluorescens* ATCC 948 and *P. putida* ATCC 12842 were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India and maintained separately on nutrient agar medium containing: Beef extract: 1.0, Yeast extract: 2.0, Peptone: 5.0, NaCl: 5.0 and Agar: 20 in g/L. The pH of the medium was adjusted to 7.0 by adding 1 N NaOH. It was incubated at 37°C for further use.

Biodegradation assay

Degradation of phenol was studied on a phenol supplemented-minimal salts medium (MSM) containing: KH_2PO_4, 2.25; K_2HPO_4, 2.25; $(NH_4)_2SO_4$, 1.0; $MgCl_2.6H_2O$, 0.2; NaCl, 4; $FeCl_3.6H_2O$, 0.02; $CaCl_2$, 0.01 and phenol 0.1, in g/L; pH= 7. *P. fluorescens* and *P. putida* were inoculated in a 250-ml flask containing 100 ml of MSM separately. The experimental studies were conducted in shake flasks with agitation at a rate of 150 rpm. Bacterial growth was determined in terms of cell mass by measuring optical density at a wavelength of 600 nm using a UV-1800 UV/VIS Spectrophotometer (RAYLEIGH, Beijing Beifen-Ruili Analytical Instrument (Group) Co., Ltd.) (Reshma et al., 2014). The phenol concentrations in medium were determined by the UV spectrophotometer at a wavelength of 272 nm after incubation period and the percentage of phenol removal were calculated using the following equation (Quintana et al., 1997).

$$\text{Percentage of phenol degradation} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Physicochemical factors

Effect of different incubation periods on the biodegradation of phenol

The efficiency of *P. fluorescens* and *P. putida* to degrade phenol was carried out in 250-ml conical flask containing 100 ml of MSM supplemented with 100 mgl^{-1} phenol and incubated at different periods (24, 48, 72, 96,144 h) under shaking (150 rpm) in a shaking incubator.

Effect of initial concentration of phenol on the biodegradation of phenol

Different initial phenol concentrations (100, 200, 300, 400 and 500 mg/L) were added to the MSM. Flasks were inoculated separately by *P. fluorescens* and *P. putida and* incubated in a shaking incubator (150 rpm) for 96 h. For each concentration of the mixture, three replicates from each treatment were used.

Effect of temperature on the biodegradation of phenol

P. fluorescens and *P. putida* were grown in MS medium separately with (100 mg/L) of phenol at different temperatures (25, 30, 35, 40 and 45°C) and incubated in a shaking incubator (150 rpm) for 96 h. Three replicates from each treatment were used.

Effect of pH on the biodegradation of phenol

The pH of the MSM was adjusted accordingly using 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to pH (6, 7, 8, 9 and 10). Flasks were inoculated separately by *P. fluorescens* and *P. putida and* incubated in a shaking incubator (150 rpm) for 96 h. For every different pH, three replicates from each treatment were used.

Effect of carbon source on the biodegradation of phenol

1 g of different carbon sources such as glucose, sucrose, lactose and fructose were added separately to 250-ml elementary flask containing 100 ml of the MSM to make a 1%w/v solution. Flasks were inoculated separately by *P. fluorescens* and *P. putida and* incubated in a shaking incubator (150 rpm) at 35°C for 96 h. Three replicates from each treatment were used.

Effect of nitrogen source on the biodegradation of phenol

1 g of different nitrogen sources such as urea, ammonium chloride, ammonium nitrate and ammonium sulphate were added separately to 250-ml elementary flask containing 100 ml of the MSM to make a 1%w/v solution. Flasks were inoculated separately by *P. fluorescens* and *P. putida and* incubated in a shaking incubator (150 rpm) at 35°C for 96 h. Three replicates from each treatment were used.

RESULTS AND DISCUSSION

Effect of different incubation periods on the biodegradation of phenol

In the current study, physical and chemical factors were tested to study their effects on biodegradation of phenol. Effect of incubation periods on phenol degradation showed that *P. fluorescens* and *P. putida* are capable of degradation of phenol (Figure 1). Bacterial growth (biomass) increases and phenol degraded completely after 122 h.

Effect of initial concentration of phenol on the biodegradation of phenol

The effect of initial concentration (100 to 500 mg /L) on the biodegradation was studied. Figure 2 showed the ability of *P. fluorescens* and *P. putida* in degradation of phenol. The percentage of phenol degradation decreasing according to increase in phenol concentration (Lakshmi et al., 2009) found cultures inoculated with 0.5 mg/L glucose showed the highest rate of phenol degradation, while the cultures inoculated with the higher concentrations showed a decrease in phenol consumption. In Cheela et al. (2014), the substrate with initial concentration (100 mg/L) was degraded in 96 and 60 h by mixed and pure cultures with a lag phase of 12 and 18 h and suggested that increase in the phenol concentration leads to increase in the degradation time and lag phase. Mohite et al. (2010) noticed the decrease in phenol concentration accompanied with increase in biomass. Moghadam et al. (2016) reported that *Rhodococcus pyridinivorans* degraded 250, 500 and 750 mg/L phenol completely in 24 h. Meanwhile, Ying et al. (2007) reported that high concentration of phenol caused the inhibitory effects on microbial growth. Also, Movahedyan et al. (2009) found that the best phenol-degrading bacteria that completely utilized 500 to 600 mg/L phenol after 48 h incubation belong to *P. putida* strains.

Effect of temperature on the biodegradation of phenol

Figure 3 showed that percentage of degradation is affected by temperature degrees. Optimum temperature

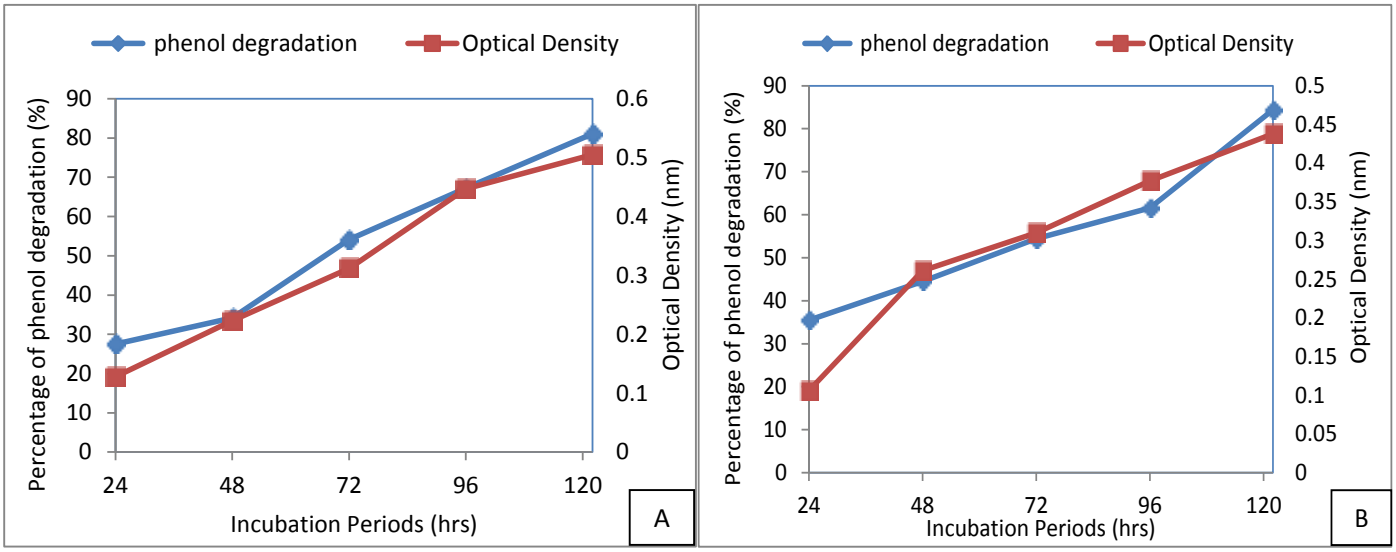


Figure 1. Effect of incubation periods on phenol degradation and growth profiles of the strains (A) *P. fluorescens* and (B) *P. putida.*

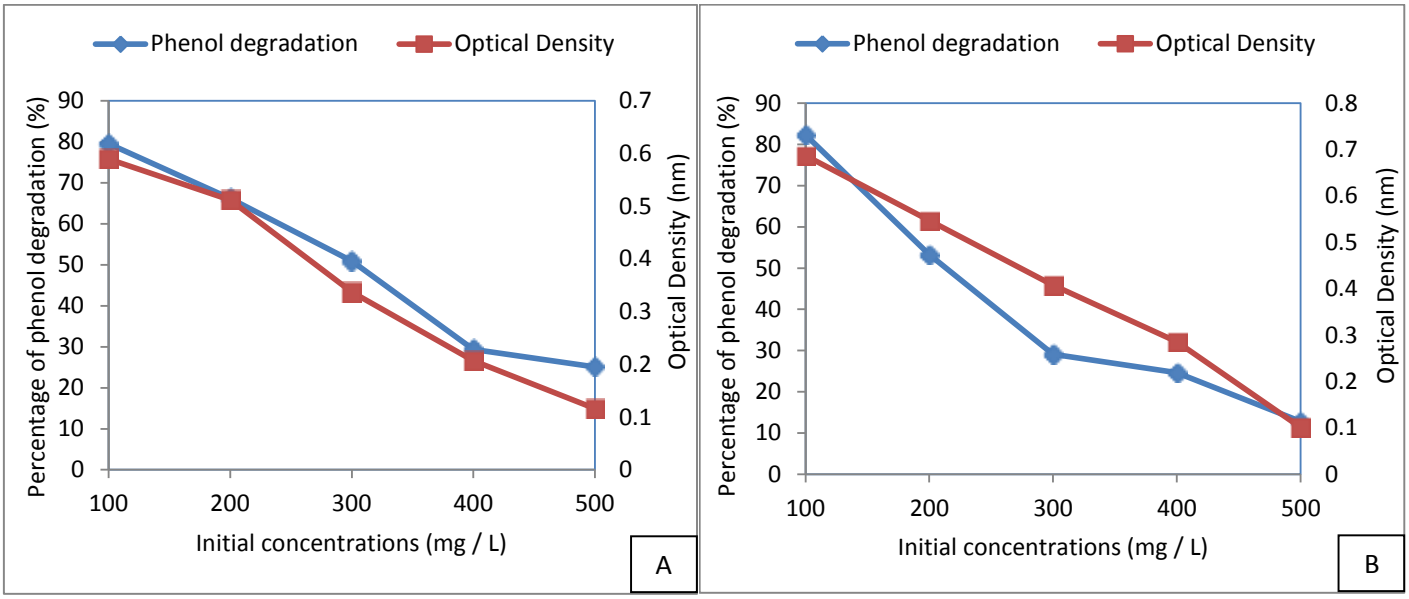


Figure 2. Effect of initial concentrations of phenols on degradation and growth profiles of the strains (A) *P. fluorescens* and (B) *P. putida*.

is 35°C for both *P. fluorescens* and *P. putida.* High temperature degrees (40 to 45°C) led to decrease in the percentage of degradation. Lakshmi et al. (2009) found that *P. aeruginosa* and *P. desmolyticum* degraded phenol rapidly at temperature of 32°C after 24 h of incubation period. Meanwhile, Mohn annd Stewart (2000) reported that temperature plays an important role in affecting petroleum hydrocarbons biodegradation, among the other environmental variables. Shweta and Dhandayuthapani (2013) reported that *P. putida* showed maximum degradation at temperature of 35°C.

Effect of pH on the biodegradation of phenol

Here, the effects of pH values ranging from 6 to 10 were investigated. Figure 4 showed that neutral pH is the best (pH 7). While *P. fluorescens* and *P. putida* recorded a maximum degradation at pH 7, those subjected to extreme alkaline pH (9 to 10) showed a very low percentage of degradation phenol. Rajani and Vijayan (2015) mentioned that when pH decreases, growth also slightly decreases and the degradation does not occur properly. In the case of increasing pH, growth was also

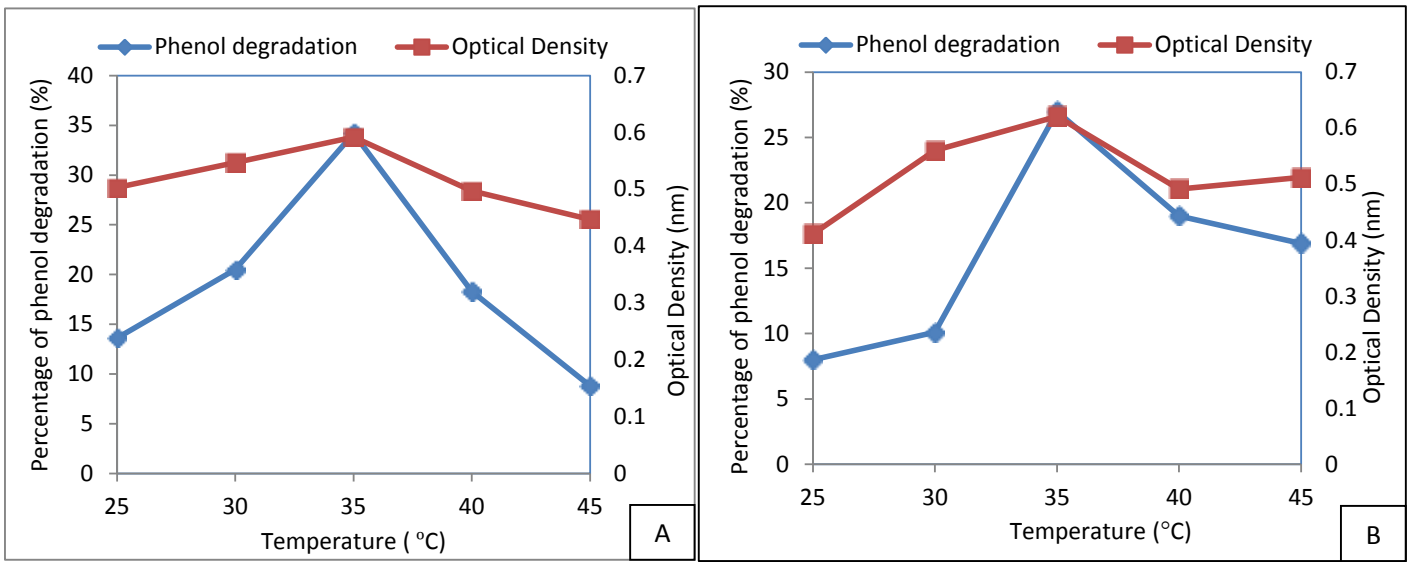


Figure 3. Effect of different temperature degrees on phenol degradation and growth profiles of the strains (A) *P. fluorescens* and (B) *P. putida*.

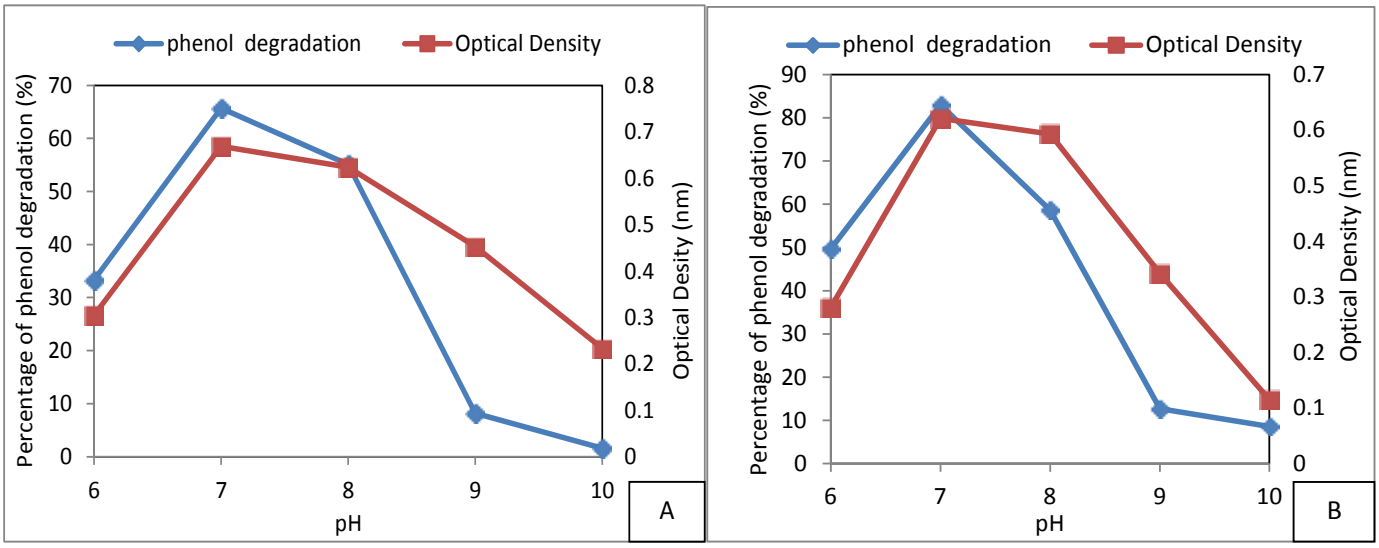


Figure 4. Effect of pH on phenol degradation and growth profiles of the strains (A) *P. fluorescens* and (B) *P. putida.*

increased but degradation rate was very less. Moghadam et al. (2016) found the optimum pH was at 8 for phenol biodegradation by NS1. Mangukiy et al. (2010) found maximum degradation of phenol was at pH 7 by *Candida* spp. Meanwhile, Shweta and Dhandayuthapani (2013) reported that *P. putida* showed maximum degradation at pH 7.

Effect of carbon source on the biodegradation of phenol

Various carbon sources such as glucose, sucrose, lactose and fructose were added separately to MSM. Glucose is the best carbon source which increased the rate of biodegradation up to 80%. Figure 5 showed that glucose is the best source in degradation by *P. fluorescens* and *P. putida.* Medium containing lactose and fructose recorded low percentage in degradation of phenol by *P. fluorescens* while medium containing sucrose and fructose recorded low percentage in degradation of phenol by *P. putida.* Lakshmi et al. (2009) found that the presence of glucose in the culture medium increased the tolerance of the organisms to high phenol concentrations by providing a good source of ready metabolisable carbon to support cell growth.

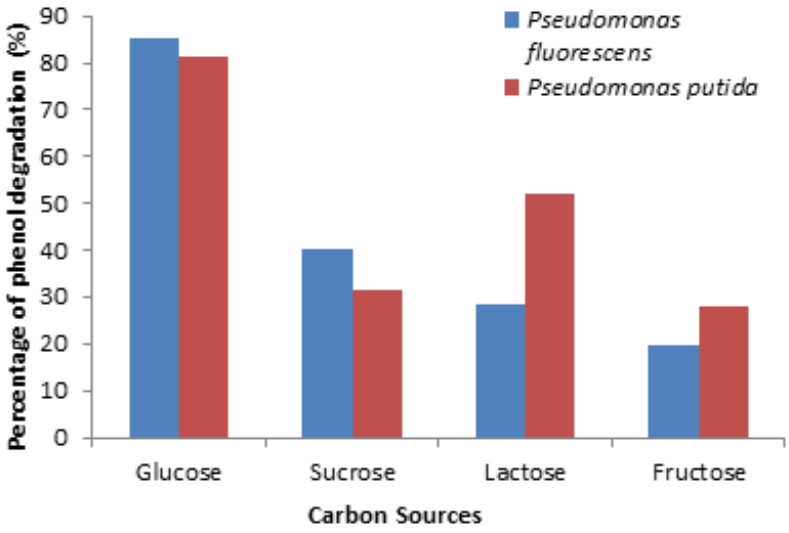


Figure 5. Effect of carbon sources on phenol degradation by *P. fluorescens* and *P. putida.*

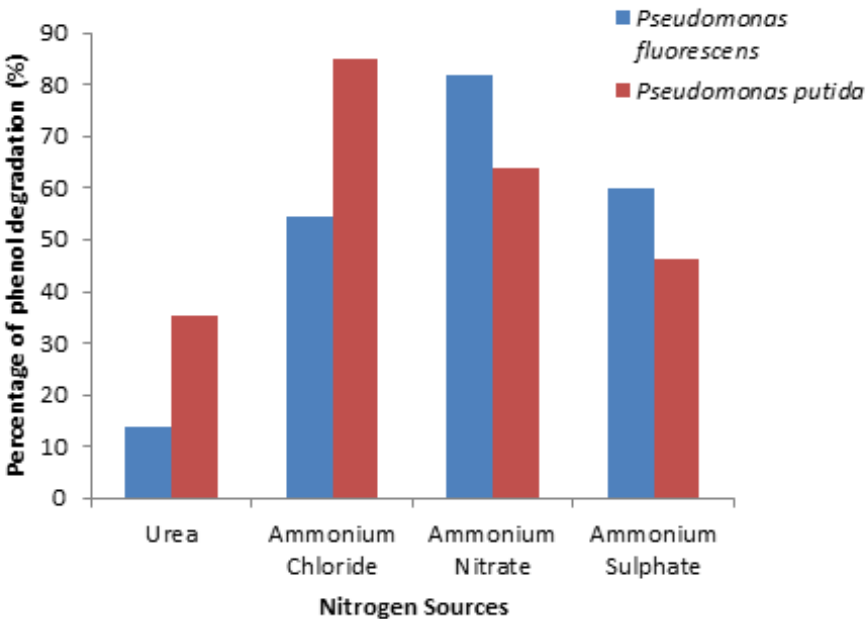


Figure 6. Effect of nitrogen sources on phenol degradation by *P. fluorescens* and *P. putida.*

Effect of nitrogen source on the biodegradation of phenol

Nitrogen sources were tested to examine their ability in degradation of phenol. Figure 6 showed that ammonium nitrate is the best source of nitrogen for *P. fluorescens* to degrade phenol, while ammonium chloride is the best source to degrade phenol by *P. putida.* Medium containing urea recorded the lowest percentage in degradation of phenol. This result agreed with Moghadam et al. (2016) who found that urea showed no significant effects on phenol biodegradation.

Conclusion

This study investigated the biodegradation of phenol using two bacterial strains. It can be concluded that *P.*

fluorescens and *P. putida* have the potential to degrade phenol. Physicochemical parameters can enhance bacterial growth to degrade phenol. The tested strains can remove phenol at optimum temperature 35°C and pH 7 at different initial concentrations. Glucose is the best carbon source as it increased the rate of biodegradation up to 80%. Ammonium nitrate is the best source of nitrogen for *P. fluorescens* to degrade phenol, while ammonium chloride is the best nitrogen source to enhance phenol degradation by *P. putida*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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