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EFFECT OF EXOGENOUS APPLIED HYDROGEN PEROXIDE ON PHOTOSYNTHETIC PIGMENTS AND SOME SECONDARY METABOLITES OF COWPEA UNDER WATER STRESS

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ABSTRACT
In the current study, the effect of exogenous applied hydrogen peroxide (0.1 mM) was examined on the photosynthetic pigments and some secondary metabolites of two varieties of cowpea (Vigna unguiculata) under water stress. The seedlings were exposed to water stress for five days after seedling establishment. The treatments were divided into two regimes which are; H1 – seedlings that were made to receive 200 mL of 0.1 mM of hydrogen peroxide at five days interval; H2 – seedlings that were be made to receive 200 mL of tap water at five days interval (Control). The regimes were laid down in completely randomized design (CRD) with six replicates. At vegetative stage of growth, chlorophyll a, b, total chlorophyll and carotenoid, secondary metabolites such as flavonoid, total phenol and alkaloids, proline accumulation were determined using standard methods. The exogenous application of hydrogen peroxide under water deficit significantly increase chlorophyll a, b, total chlorophyll and carotenoid, secondary metabolites such as flavonoid, total phenol and alkaloids, proline accumulation compared to the control. Exogenous application of hydrogen peroxide triggered accumulation of chlorophyll pigments and secondary metabolites thereby improving cowpea tolerance to water deficit.
KEY WORDS: *acclimation, chlorophylls, flavonoids, proline, total phenols, water deficit*

INTRODUCTION

Hydrogen peroxide is the most stable reacting oxygen species (ROS) that regulates basic processes, such as acclimation, defense and development in plants (Ślesak *et al*., 2007). In contrast to superoxide, hydrogen peroxide is a non-radical reacting oxygen species (ROS) with no net charge (Halliwell, 2006). Hydrogen peroxide is considered a long distance signaling molecule because of its relatively stability and infusibility through membranes (Vranová *et al*., 2002), acting as translocating second messenger triggering Ca^{2+} fluxes, modifying proteins and plays important role in the expression of gene (Bienert *et al*., 2006). In addition, hydrogen peroxide produced endogenously as a result of treatment with various signaling molecules, induce the synthesis or activate various transcription factors, which are associated with the induction of antioxidative enzymes (Agarwal *et al*., 2005).

Endogenous production of hydrogen peroxide in plant cells is increased due to a wide variety of stresses. It was suggested by some authors that hydrogen peroxide is

a key factor mediating acclimation and cross-tolerance in plant under stress (Neill *et al*., 2002). Thus, generation of hydrogen peroxide internally and its application exogenously has been shown to increase tolerance of maize seedlings to chilling stress (Prasad *et al*., 1994). Treatments of maize seedlings with hydrogen peroxide also enhanced multi-resistance to heat, drought and salt stresses (Gong *et al*., 2001). Pre-treatment of rice and maize seedlings with hydrogen peroxide in nutrient solution induces acclimation to salt stress in rice and maize seedlings (Azevedo *et al*., 2005; Uchida *et al*., 2002).

Cowpea one of the food legumes with lower *acclimation capacity has a lower resistance to water stress* (Dadson *et al*., 2005). Therefore, since drought may occur at any stage of growth of plant, cowpea in this work was water-stressed and treated with hydrogen peroxide in other to assess the responses of cowpea as this might provide a basis for the development of strategies to stabilize yields of cowpea under water deficit. Hence the need for this study.

MATERIALS AND METHODS

Experimental Plant.

The seeds of cowpea (*Vigna unguiculata* L.) of two varieties (IT 07 K-292-10 and IT-98K-131-1) were utilized for this experiment and were collected from the Department of Crop Production and Protection, Faculty of Agriculture, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

Raising of Seedlings.

The seedlings were raised under the screenhouse of Botany Department, Obafemi Awolowo University, Ile-Ife, Nigeria, to minimize extraneous factors. 32 pots (of 9 cm in diameter and 7.5 cm in height, conical in shape), 16 for each regimes were used. Each of these pots was filled with 10 kg of collected loamy soil. Ten holes of about 3 mm were bored at the bottom of the pots. This is to allow for proper drainage and prevent water logging during the course of the experiment. The seeds of *Vigna unguiculata* were then sown at a depth of 3 cm below the soil. These seeds were sown at the rate of three seeds per pot to ensured survival and reduce rate of competition of the seedlings. The pots were then supplied with 500 mL of tap water of the pots capacity until the seedlings become fully established. After two weeks of sowing when the seedlings become fully established, the sample pots were divided into two regimes. The first regimes were H1 – seedlings that were made to receive 200 mL of 0.1 mM of hydrogen peroxide at five days interval; H2 – seedlings that were be made to receive 200 mL of tap water at five days interval (Control). The regimes were laid down in completely randomized design (CRD) with 6 replicates.

Determination of Chlorophylls and Carotenoids.

At vegetative stage of growth, 8 g of leaves from each plant were harvested; the leaves were grinded with mortar and pestle. A pinch of sodium bicarbonate was

added to the mixture to prevent the degradation of chlorophyll. 16 mL of 80% acetone was added. The blended materials were then filtered through a Whatman's No 1 filter paper (Coombs *et al*., 1990). The absorbance of the samples was determined on a digital spectrophotometer at wavelengths of 470 nm, 646 nm and 663 nm. A Beer-Lambert equation was used to determine the concentrations of Chlorophyll a and Chlorophyll b and the carotenoid in the leaf extract as follows:

Chlorophyll a (µg/ml) = $12.21A_{663}$- $2.81A_{646}$

Chlorophyll b (µg/ml) = $20.13A_{646}$- $5.03A_{663}$

Carotenoids (µg/ml) = ($1000A_{470}$- 3.27[Chl a] – 104[Chl b])/227

Total chlorophyll (µM) = 7.93 A_{663}+ 19.53 A_{646}

In the carotenoid equation, '[chl a]' and '[chl b]' refer to the calculated concentration of chlorophyll b from the previous equations. A_{663} represent the absorbance at wavelength 663 nm while A_{646} represents the absorbance at wavelength 646 nm.

Determination of Proline.

The method ascribed by Bates *et al*. (1973) was used for the estimation of free proline in leaves. An amount of 0.5 g of fresh leaf was homogenized in 3% solution of sulfo-salicylic acid (10 mL). Then, the 2.0 mL of the homogenate after filtration was mixed thoroughly with 2.0 mL of acid ninhydrin, which was prepared by adding 1.25 g ninhydrin in 20 mL of 6 M orthophosphoric acid and 30 mL glacial acetic acid, along with 2 mL of glacial acetic acid in a test tube. The mixture was then incubated for 60 min at 100^{o}C. The final material was then cooled immediately in an ice bath. Then, the triturate was mixed with 4.0 mL of toluene using a vortexer. The toluene layer containing chromophore was separated and kept at room temperature for a few minutes and the absorbance was read at 520 nm on a spectrophotometer using toluene as a blank. The concentration of proline was estimated using a standard curve prepared from a range of standards (0–50 mg/kg), such as pure proline, and the specific content was calculated on a fresh weight basis as follows:

µmole prolineg^{-1} fresh weight = (µg proline mL^{-1}× mL of toluene/115.5)/ (g of sample)

Determination of Secondary Metabolites. Secondary metabolites were carried out at vegetative stage of growth as follows:

Estimation leaf total phenol.

The method ascribed by Julkunen-Tiitto (1985) was used for the estimation of total phenolic content in leaf samples. Leaf samples (0.5 g) were homogenized in 80% acetone. The supernatant obtained after centrifugation at 1000 µg for 10 min was used. An aliquot (100 µL) was reacted with 2 mL of distilled water and 1 mL of Folin–Ciocalteau's phenol reagent. The triturate was then mixed with 5.0 mL of 20 % Na_2CO_3 solution and the final volume of the mixture was measured to 10 mL with distilled H_2O. After mixing well with a vortexer, the absorbance of the final solution was read at 750 nm using a UV-visible spectrophotometer (Hitachi U-2100).

Test for Alkaloids.

200 ml of 10% acetic acid in ethyl alcohol was added to the leaf extract sample. The mixture was covered and allowed to stand for 4 h. The mixture then filtered, and the extract was allowed to become concentrated in a water bath until it reached to one quarter of the original volume. Concentrated ammonium hydroxide was added until the precipitation was complete. The whole solution was allowed to settle, and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed (Mustapha & Harun, 2014).

Test for Flavonoids.

0.5 mL of leaf extract was added to 1.5 mL methanol and mixed well. After that 0.1 mL of $AlCl_3$ (0.1 mg/mL) and 0.1 ml of 1M CH_3COONa reagents were added to above solution. This reaction mixture was added to 2.8 mL of distilled water, mixed and allows standing for 30 minutes in dark. The absorbance of reaction mixtures was measured at 415 nm. The total flavonoid content was expressed as mg rutin equivalents /100 g d.w. of the extract (Pourmorad *et al*., 2006)

RESULTS AND DISCUSSION

Effect of Hydrogen Peroxide on the Photosynthetic Pigments Accumulation of Cowpea under Water.

From the results obtained in Table 1, a significant reduction ($p \leq 0.05$) was recorded in chlorophyll a, chlorophyll b, carotenoid and total chlorophyll accumulation in the two varieties of cowpea grown under water stress. Exogenous applied hydrogen peroxide significantly improved chlorophyll a, chlorophyll b, carotenoid, total and chlorophyll accumulation of cowpea plants of both varieties under water stress with the highest chlorophyll a, chlorophyll b, carotenoid and total chlorophyll accumulation obtained in cowpea of both varieties supplied with hydrogen peroxide and lowest with those supplied with tap water.

Analysis of variance indicated chlorophyll a, chlorophyll b, carotenoid and total chlorophyll accumulation of the cowpea varieties responded the same way to hydrogen peroxide application. Meanwhile treatments had significant effect on chlorophyll a, chlorophyll b, carotenoid, total chlorophyll and proline accumulation. Treatment and varieties interaction significantly affected chlorophyll a, chlorophyll b, carotenoid, total chlorophyll as well as proline accumulation (Table 2).

TABLE 1: Effect of hydrogen peroxide on the photosynthetic pigments accumulation of cowpea under water stress

	Source	Chlorophyll a	Chlorophyll b	Carotenoid	Total Chlorophyll
Variety 1	Peroxide	17.54a	29.20a	933.3a	46.74a
	Tap water	17.44b	28.56b	931.9b	46.00b
Variety 2	Peroxide	17.42a	27.34a	941.02a	44.76a
	Tap water	9.52b	15.82b	940.3b	25.34b

Values in columns followed by the same letter are not significantly different at $p \leq 0.05$ by Fishers LSD

TABLE 2: Summary of ANOVA result on the effect of hydrogen peroxide on the photosynthetic pigments accumulation of cowpea under water

Source	Chlorophyll a	Chlorophyll b	Carotenoid	Total Chlorophyll
Varieties	ns	ns	ns	ns
Treatments	*	*	*	*
Treatments× Varieties	*	*	*	*

ns, *, ** not significantly or significant at p<0.05 or p<0.01, respectively

Effect of Hydrogen Peroxide on some the Secondary Metabolites of cowpea under Water Stress.

Flavonoid, phenol, alkaloid and proline content of both studied cowpea cultivars also decreased significantly ($p \leq 0.05$) under water deficit. However, exogenous applied hydrogen peroxide improved the flavonoid, phenol, alkaloid and proline content of both cowpea varieties water stressed. For both varieties the highest flavonoid, phenol, alkaloid and proline content was found in those supplied with hydrogen peroxide and lowest in those made to received tap water.

Analysis of variance indicated that flavonoid, phenol, alkaloid and proline content of the cowpea varieties responded the same way to hydrogen peroxide application. Meanwhile treatments had significant effect on flavonoid, phenol and alkaloid. Treatment and varieties interaction significantly affected flavonoid, phenol and alkaloid content (Table 4).

TABLE 3: Effect of hydrogen peroxide on some secondary metabolites of cowpea under water stress.

	Source	Flavonoid	Phenol	Alkaloid	Proline
Variety 1	Peroxide	3.71a	4.61a	2.45a	1.61a
	Tap water	3.29b	4.54b	2.08b	0.75b
Variety 2	Peroxide	3.94a	4.18a	2.46a	1.40a
	Tap water	3.29b	3.15b	1.79b	0.38b

Values in columns followed by the same letter are not significantly different at $p \leq 0.05$ by Fishers LSD

TABLE 4: Summary of ANOVA result on the effect of hydrogen peroxide on some secondary metabolites of cowpea under water stress.

Source	Flavonoid	Phenol	Alkaloid	Proline
Varieties	ns	ns	ns	ns
Treatments	*	*	*	*
Treatments× Varieties	*	*	*	*

ns, *, ** not significantly or significant at p<0.05 or p<0.01, respectively

In the present study, a significant reduction was observed in chlorophyll a, chlorophyll b, carotenoid and total chlorophyll contents of both cowpea cultivars under water deficit. Similar results in the decrease in leaf chlorophyll accumulation due to

water stress have been observed in crops such as soybean (Ishibashi *et al*., 2011), wheat (Stiven, 2006), cucumber (Sun *et al.*, 2016) and maize (Ashraf, 2014). Meanwhile, exogenous application of hydrogen peroxide improved the leaf chlorophyll accumulation of both cowpea cultivars under water stress conditions. This improvement might be due to potential of internal generated hydrogen peroxide to increase the leaf chlorophyll accumulation of crops, its role in enhancing photosynthetic pigments and to protect the chloroplastic membrane containing chlorophyll under water deficit.

Phenols, flavonoids and alkaloids are crucial for plants growth and reproduction, and are produced as a response to environmental stress factors such as light, chilling, pollution etc., and to defend injured plants (Valentine *et al*., 2003, Almășan *et al.* 2021, Sfrangeu *et al.* 2021, Gușiță & Ianovici, 2021). In the present study, increased levels of phenol, flavonoid and alkaloid were observed from exogenous applied hydrogen peroxide in cowpea cultivars under water stress. This stimulation by hydrogen peroxide might be due to its role in the antioxidative defense system (Gao *et al*., 2010, Azevedo Neto *et al*., 2005) acting as signaling molecules (Olowolaju, 2019). This therefore induced a greater protection under water stress.

Exogenous application of hydrogen peroxide also enhanced the proline content of cowpea cultivars under water stress. Proline is a osmolyte that plays a key role in improving cellular water relations under water-stressed conditions (Ali *et al*., 2013, Habib *et al*., 2012). This enhancement might be as a result of the vital role of hydrogen peroxide as a direct scavenger of Reacting Oxygen Species and boosting antioxidation mechanism by playing a key role as a signaling molecule (Hossain *et al*., 2014) in plants under water stress.

CONCLUSION

Overall, exogenous application of hydrogen peroxide triggered increased accumulation of chlorophyll a, chlorophyll b, carotenoid, total chlorophyll, phenols, flavonoids, alkaloids and proline which may improve the drought tolerance of cowpea plants through osmotic adjustment by maintaining better cellular water content leading to better growth and yield of cowpea plants.

Conflicts of Interest. Authors declare no conflicts of interest

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