Nutritional Value of Cowpea (*Vigna Unguiculata* L. Walp) Grain Grown Under Different Soil Moisture as Affected to the Dual Inoculation with Nitrogen Fixing Bacteria and Arbuscular Mycorrhizal Fungi

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Abstract

The study was performed to evaluate the nutritional value and antioxidant properties of cowpea grain, where plants were grown under conditions of tripartite symbiosis (cowpea plants, nitrogen-fixing bacteria (*Bradyrhizobium japonicum*) and arbuscular mycorrhizal fungi, (*Glomus intraradices*) without fertilization. Cowpea plants (*Vigna unguiculata* (L.) Walp) cv. Hrisi were grown in a glasshouse until the full maturity of grain on the Alluvial meadow soil (Eutric Fluvisol) at three levels of water-holding capacity (40, 60 and 80%). *Bradyrhizobium japonicum* solely and in combination with arbuscular mycorrhizal fungi increased the content of proteins, soluble sugars and total phenols in the cowpea seeds. When dual inoculation has applied the levels of proteins and soluble sugars in the cowpea grain did not reduce at 40%, nor at 80% in comparison with the optimal water-holding capacity level (60%). Antioxidant capacity at 40% water-holding capacity was determined by the increased content of water soluble antioxidants, flavonoid content and increased ferric reducing power activity and increased antioxidant levels was expressed more distinguished in the treatments with dual inoculation. Slight change of antioxidants in the grain of cowpea was established at an elevated level of water supply. Nutritional value of a grain of cowpea after dual inoculation with *Bradyrhizobium* strains and *Glomus intraradices* was not determined by the water-holding capacity levels.

Key words: Arbuscular Mycorrhizal Fungi, Nitrogen-Fixing Bacteria, Nutritional Value, Soil Moisture, *Vigna unguiculata* (L.) Walp

Abbreviations: AM fungi - arbuscular mycorrhizal fungi; DPPH• - diphenylpycril-hydrazyl; FRAP - ferric reducing power; WHC - water holding capacity

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is a widely grown legume food crop of the tropics and sub-tropics, used in the diets of humans and other mammals. Cowpea seeds are an excellent source of carbohydrate (50–60%) and an important source of protein (18–35%). They also contain an appreciable quantity of micronutrients such as vitamin A, iron and calcium (Prinyawiwatkul et al., 1996). The crude protein from the seed and leaves ranges, respectively between 23 and 32% (Diouf, 2011). Polyphenolic compounds are also found. They can interact with proteins and reduce their digestibility, as well as alter organoleptic and functional properties of the seed flour (Okafor et al., 2002). Polyphenolic compounds also have beneficial effects due to their antioxidant activity which is fundamental to the life. (Rice-Evans et al., 1997). Recently, the ability of phenolic substances including flavonoids and phenolic acids to act as antioxidants has been extensively investigated (Rice-Evans et al., 1997). On the other hand vitamin C, a-tocopherol and phenolic compounds, which are present naturally in vegetables, fruits and grains, possess the ability to reduce oxidative damage associated with many socially significant diseases, including cancer, diabetes and cardiovascular diseases (Lee et al., 2000).

During the last years, the role of microorganisms in the conservation of soil fertility increased. Vessey (2003) pointed out that the soil microorganisms were successfully applied as biofertilizers, useful to substitute chemical fertilization. The arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing bacteria have an important role in plant nutrient uptake, not only for the availability of P and N but also of other nutrients such as K, Ca and Mg (Clark and Zeto, 2000). The effectiveness of the tripartite symbiosis – AM fungi, *Bradyrhizobium*, and legumes plant, depends on the competition of the three symbionts for carbon.

Therefore, the present study was aimed at evaluating the nutritional compounds (crude protein and sugars) of the grains of cowpea, grown under conditions of tripartite symbiosis without fertilization, their phenolic constituents, antioxidant compounds and free radical scavenging capacity.

Material and Methods

Growth conditions and treatments

Cowpea plants (*Vigna unguiculata* (L.) Walp) cv. Hrisi, created in the Bulgarian Institute for Plant Genetic Resources "K. Malkov " were grown in a glasshouse until the full maturity of grain using Alluvial meadow soil (Eutric Fluvisol) (IUSS Working Group WRB, 2006). The agrochemical characteristics of the soil are as follows: pH (H_2O) - 7.4; mineral N - 19 mg kg^{-1} soil; P (P_2O_5) – 99 mg kg^{-1} soil, K (K_2O) – 334 mg kg^{-1} soil. Water was added to make up about 40%, 60% and 80% of water-holding capacity (WHC). An inoculum of AM fungi, *Glomus intraradices* EEZ 01 was used. It was added to the soil (at 2 cm depth under soil surface) before sowing in quantity 0,5 g per pot. The mycorrhizal strain was provided from the AMF collection of Estacion Experimental del Zaidin (CSIC Granada, Spain). The seeds before sowing were inoculated with the bacterial suspension of *Bradyrhizobium japonicum*, strains 273 and 269 at approximately 10^8 cells per cm^3. The tested strains are from the collection of Soil Microbiology Department of Nicola Poushkarov Institute of Soil Science, Agrotechnologies and Plant Protection, Bulgaria.

The following scheme was used per each level of water holding capacity:
1. Control plants
2. Plants inoculated with *Br. japonicum*-273
3. Plants inoculated with *Br. Japonicum*-269
4. Plants inoculated with *Gl. intraradices* EEZ 01
5. Plants inoculated with *Br. japonicum*-273 + *Gl. intraradices* EEZ 01
6. Plants inoculated with *Br. japonicum*-269 + *Gl. intraradices* EEZ 01

Content of total proteins and soluble sugars

The extraction of proteins from cowpea grain was carried out following the method of Mirkov et al. (1994). 0.1 g of finely grounded grains flour were extracted in the extraction buffer (10 mM Tris-HCL (pH 7.5), 500 mM NaCl, 1% 2-mercapto-ethanol, 0.1% Triton-X-100, 2 mM phenylmethylsulphonyl fluoride (PMSF) (1 ml/ml sample) by homogenization followed by incubation at 4°C for 1 h, and then centrifuged at 15,000 rmp for 15 min at 4°C. The supernatant was collected and stored frozen in aliquots. The protein content in the crude extracts was determined by the method described by Bradford (1976) using bovine albumin as standard. The seeds were analyzed for soluble sugars via refractometer.
Soluble sugars were expressed in percents.

Antioxidant capacity and antioxidant metabolites

Free radical scavenging activity was measured from the bleaching of the purple methanol solution of free stable radical (diphenylpycril-hydrazyl, $DPPH^{\bullet}$), according to Tepe et al. (2006). $DPPH^{\bullet}$ is a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant. The percent inhibition of the $DPPH^{\bullet}$ radical (I%) was calculated by the following equation:
$I\% = (A_{blank} – A_{sample}/A_{blank}) \times 100$,
where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound, i.e. cowpea grain extracts.

Ferric reducing power (FRAP assay) The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl), and $FeCl_3$–$6H_2O$ (20 mM) in a ratio of 10:1:1 (Benzie et al., 1996). To perform the assay, 900 mL of FRAP reagent, 90 mL of distilled water and 30 mL of the plant extract were mixed and incubated at 37°C for 15 min. The absorbance was measured at 595nm using the FRAP working solution as a blank. The antioxidant potential of samples was determined from a standard curve, plotted using the $FeSO_4.7H_2O$ linear regression. The results were corrected for dilution and expressed as mmol of Fe^{2+} g^{-1} of dried sample.

For determination of total phenols and flavonoids grain samples (1 g) were ground and exhaustively extracted with 96% (v/v) methanol. Concentrations of phenolic compounds were determined spectrophotometrically using Folin–Ciocalteu reagent and calculated as caffeic acid equivalents (Pfeffer et al., 1998). Flavonoids in plant tissues were measured spectrophotometrically according to Zhishen et al. (1999), using the standard curve of catechin.

Spectrophotometric quantification of water-soluble and lipid-soluble antioxidant capacity, expressed as equivalents of ascorbate and α-tocopherols were performed through the formation of phosphomolybdenum complex (Prieto et al., 1999). The assay was based on the reduction of Mo (VI) to Mo (V) by the sample analysis and the subsequent formation of a green phosphate/Mo (V) at acidic pH. 0.5 g plant dry material was ground with pestle and mortar to a fine powder. 3ml dH_2O was added and the suspension was homogenized, transferred to tubes and shaken for 1 h at room temperature in dark. The suspension is filtered and extraction is repeated with 3 ml dH_2O. The pellet was washed again with 2 ml dH_2O. For lipid soluble antioxidant capacity (expressed as α-tocopherols), the procedure is the same except the extraction is carried out with hexane as a solvent. The method has been optimized and characterized with respect to linearity interval, repetitively and reproducibility, and molar absorption coefficients for the quantitation of water-soluble and lipid-soluble antioxidant capacities, expressed as equivalents of ascorbate, and α-tocopherols (Prieto et al., 1999) Absorption coefficients were: (3.4±0.1) x103 M^{-1} cm^{-1} for ascorbic acid and (4.0±0.1) x103 M^{-1} cm-1 for α-tocopherols.

Statistical Analyses

Data are expressed as means ± standard error where $n = 3$. Comparison of means was performed by the Fisher LSD test ($P \leq 0.05$) after performing multifactor ANOVA analysis. A statistical software package (StatGraphics Plus, version 5.1. for Windows, USA) was used.

Results

Under conditions of optimal soil moisture (60% WHC) the protein content as well as the content of the soluble sugars in cowpea grains were affected in a similar way at the experimental treatments. Inoculation with *Br. japonicum*-273 resulted in those parameters increase (Figure 1). On the other hand combined treatment with AM fungi and the other *Bradyrhizobium* strain - 269 affected more favorably protein content and soluble sugars in cowpea grains.

Br. japonicum-273 solely and in combination with AM fungi increased the content of total phenols. Single inoculation with AM fungi significantly increased flavonoids in cowpea grain (Figure 1). Water-soluble and lipid-soluble antioxidant capacity expressed as equivalents of ascorbate and α-tocopherols respectively was measured to be the higher as a result of inoculation with *Br. japonicum*-273. Despite the free radical scavenging activity did not differ among the treatments, ferric reducing power activity (FRAP assay) increased in the grain in plants inoculated with both *Bradyrhizobium* strains as well as at the treatments with dual inoculation (*Bradyrhizobium* strain + *Gl. intraradices).*

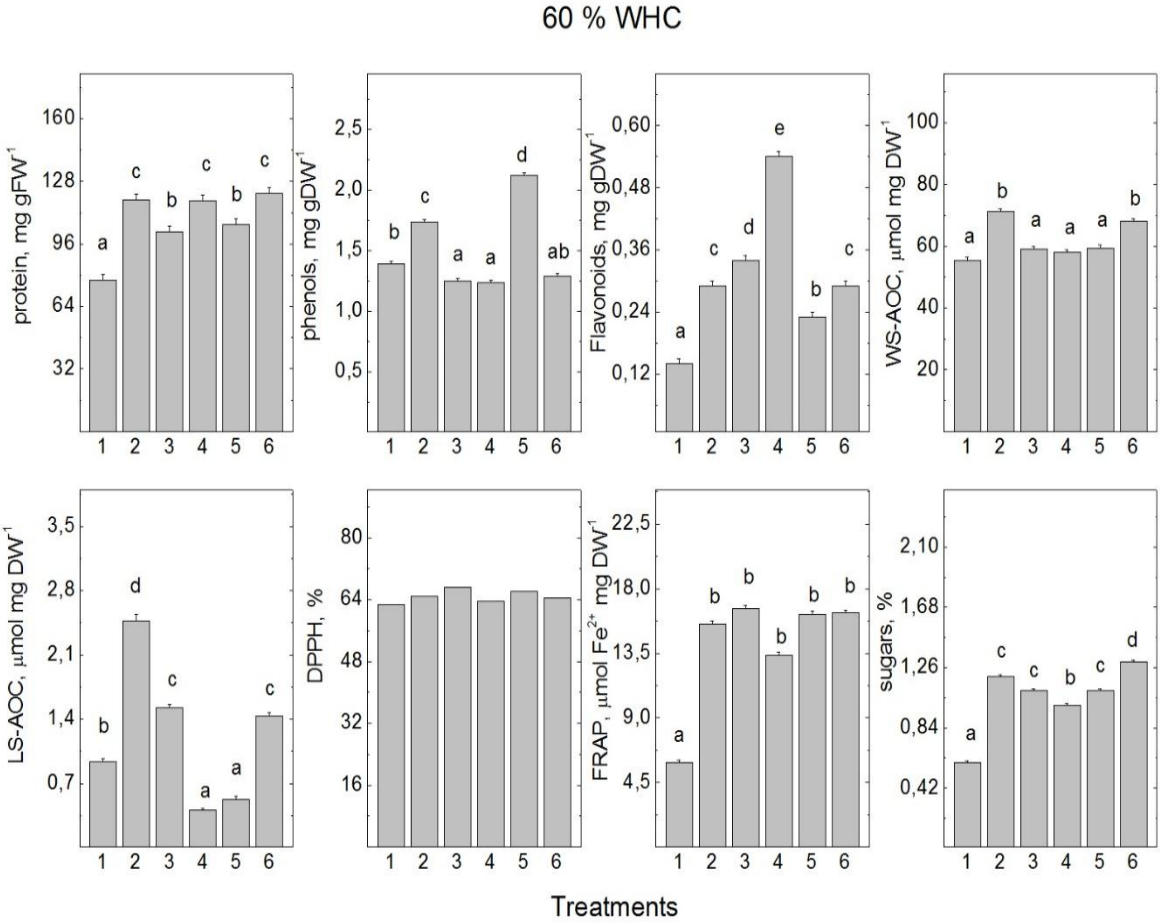

Figure 1. *Nutritional properties and metabolites with antioxidant activity of Vigna unguiculata grain at 60% water holding capacity. Different letters in the values indicate significant differences assessed by Fisher LSD test (P ≤ 0.05) after performing ANOVA analysis*

Under 40% WHC total protein content decreased only in the grain from the treatments with single inoculation with *Br. japonicum*-269 and *Gl. intraradices* EEZ 01 (Figure 2). Dual inoculation with AM fungi and *Bradyrhizobium* strains did not result in protein reduction under drought conditions in comparison with the optimal level of water-holding capacity-60%. Soluble sugars increased in the grain of cowpea grown at 40% WHC. Phenolic content slightly decreased with the exception of the grain from the treatment with AM fungi and *Br. japonicum*-269. Flavonoids rose after inoculation with fungi and combined inoculation with *Bradyrhizobium* strains and *Gl. intraradices*. Under drought conditions, 40% WHC water soluble antioxidants increased while lipid-soluble antioxidants are reduced. Radical scavenging activity was strongly reduced where *Br. japonicum*-269 was applied solely and combination with AM fungi while FRAP activity remained higher in all experimental treatments.

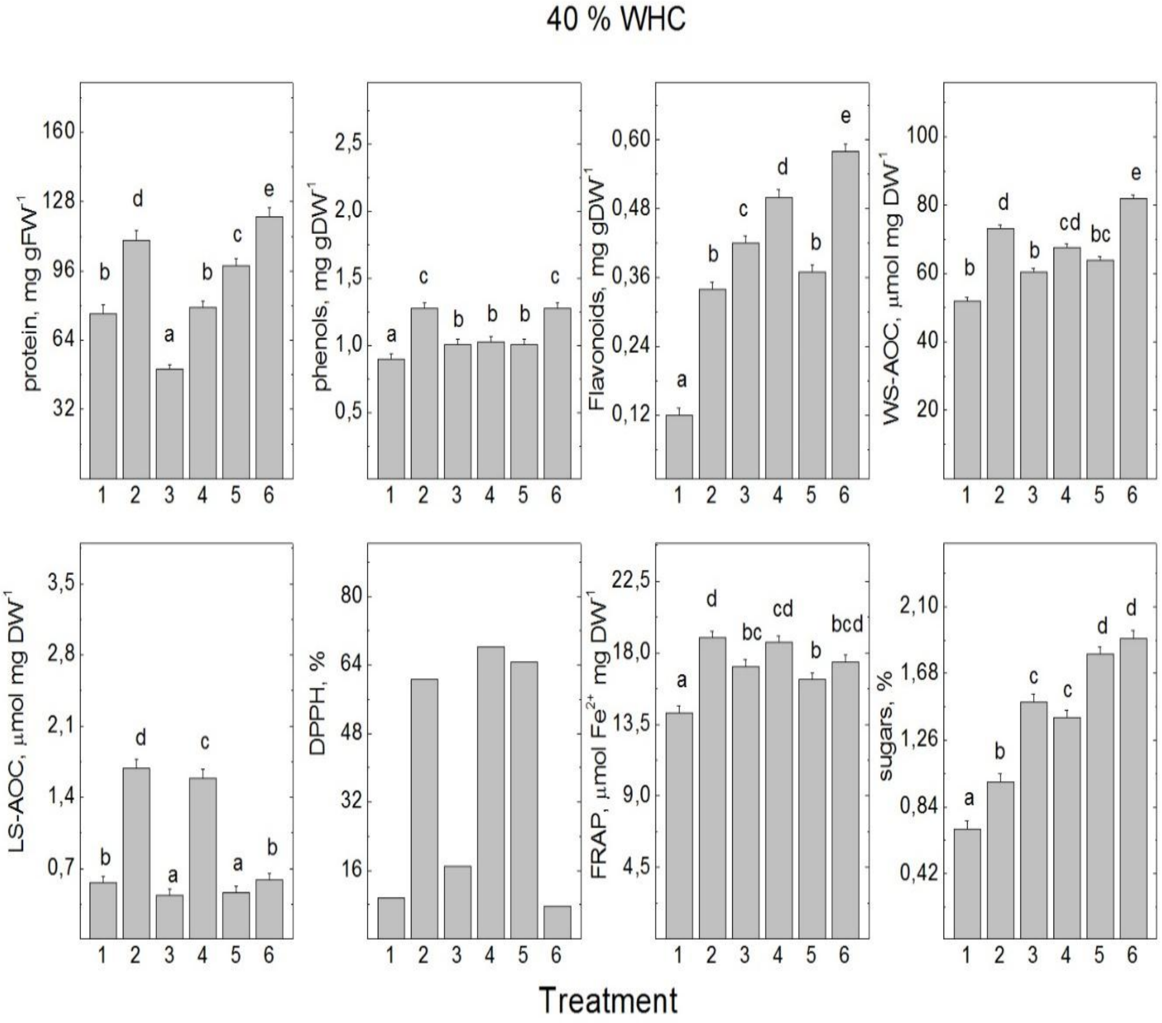

Figure 2. *Nutritional properties and metabolites with the antioxidant activity of Vigna unguiculata grain at 40% water holding capacity. Different letters in the values indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA analysis*

At increased water holding capacity level (80% WHC), protein content increased significantly compared with the treatments from the optimal level as a result of *Bradyrhizobium* strains application solely and in combination with *Gl. intraradices* (Figure 3). Soluble sugars also increased in the grain of cowpea after mycorrhization and dual inoculation. Phenols decreased as compared to the treatments with optimal water supply, but flavonoids remained higher as a result of mycorrhization and dual inoculation. Water - soluble and lipid - soluble antioxidants did not rise under waterlogging conditions.

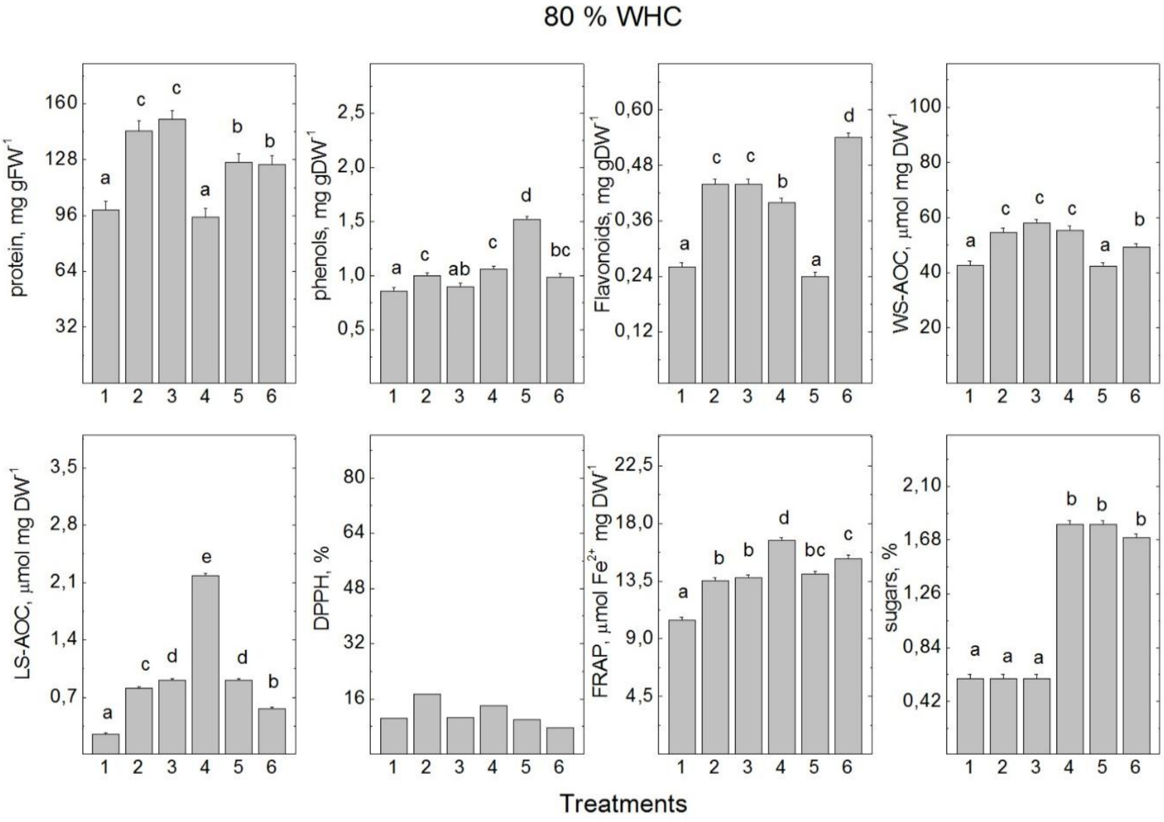

Figure 3. *Nutritional properties and metabolites with the antioxidant activity of Vigna unguiculata grain at 80% water holding capacity. Different letters in the values indicate significant differences assessed by Fisher LSD test (P ≤ 0.05) after performing ANOVA analysis*

Discussion

At 60% WHC protein content of cowpea grain was in the range 77.31 -121.96 mg.g^{-1} which is 7.7-12.2%. The level of soluble sugars in the cowpea grain at 60% WHC was in the range 0.6-1.3%. Cowpea seeds are an excellent source of carbohydrate (50–60%) and an important source of protein (18–35%) (Mune et al., 2008). Rivas - Vega et al. (2006) reported that crude protein content of whole raw cowpea is within the range of values reported for other varieties of cowpea - 26.14%). The protein content of cowpea grain obtained from intercropped plots (23.7 to 26.3%) in South Africa was similar to that from sole plots (23.7 to 25.7%) (Sebetha et al., 2010).

Starch is the most abundant carbohydrate in cowpea, while sugars represent only a small percentage (Longe, 1980). The sucrose concentration of seeds is an important component of the taste in cowpea (*Vigna unguiculata* L. Walp.) (Tchiagam et al., 2011). Stachyose (3.43%), sucrose (2.97%) and raffinose (1.24%) are the predominant sugars in nonfermented (control) cowpea flour. Verbascose was not detected in the control flour even after a 35-min elution time. Akpapunam and Markakis (1979) reported sugars from 13 American cowpea varieties to have 3.4% stachyose, 2.2% sucrose, 1.2% raffinose, and 0.9%

verbascose (dry weight basis), while 2.7% stachyose, 1.6% sucrose, 0.7% raffinose, and 3.6% verbascose were reported for 20 Nigerian cowpea varieties (Longe, 1980).

Zia-Ul-Haq et al. (2013) reported that cowpea is rich in polyphenolic compounds more than other leguminous seeds and pulses and phenolic constituents contained in cowpea may have a future role as ingredients in the development of functional foods. It was known that polyphenolic compounds also have beneficial effects due to their antioxidant activity and the ability of phenolic substances including flavonoids and phenolic acids to act as antioxidants has been extensively investigated (Rice-Evans et al., 1997). On the other hand vitamin C, α-tocopherol and phenolic compounds, which are present naturally in vegetables, fruits and grains, directly associated with antioxidant activity (Zia-Ul-Haq et al., 2008). Some authors (Siddhuraju and Becker, 2007) pointed out that the DPPH radical and ABTS cation radical scavenging activities were well proved and correlated with the ferric reducing antioxidant capacity of the cowpea seed extracts.

Antioxidant defense under drought conditions was determined by the increased content of water soluble antioxidants, flavonoid content and increased FRAP activity and increased antioxidant levels was expressed more distinguish in the treatments with dual inoculation. Consequently when dual inoculation was applied the levels of proteins and soluble sugars in the cowpea grain did not reduce under drought in comparison with the control. AM symbiosis alleviates drought stress via direct water uptake and transport through fungal hyphae to the host plants (Augé et al., 2007). The problems caused from drought and its effect on growth, yield and nutritional values were under taken in several studies (Henry, Mather, 2003; Anitha et al., 2004).

Conclusion

At a global scale, the effects of continuous agricultural practices such as fertilization can cause serious damage to the environment. Inoculation is one of the most important sustainable practices in agriculture, because microorganisms establish associations with plants and promote plant growth by means of several beneficial characteristics.

In conclusion neither reduced nor excess water supply did affect nutritional value of grain of cowpea after dual inoculation with *Bradyrhizobium* strains and *Gl. intraradices*. Nevertheless, the beneficial effects of the microsymbionts, observed in this study, arouse an interest in considering the role of rhizobia-AM-plant tripartite symbiosis in affecting nutritional value of cowpea grain grown under different soil moisture. *Bradyrhizobium japonicum* solely and in combination with arbuscular mycorrhizal fungi increased the content of proteins, soluble sugars and total phenols in the cowpea seeds. When dual inoculation has applied the levels of proteins and soluble sugars in the cowpea grain did not reduce at 40%, nor at 80% in comparison with the optimal water-holding capacity level (60%). Slight change of antioxidants level is a precondition for the lack of increase free radical scavenging activity ($DPPH^•$) and ferric reducing power (FRAP) in the grain of cowpea grown at elevated level of water supply. Finally, the search for beneficial bacteria is important for the development of new and efficient stimulants for agriculture.

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