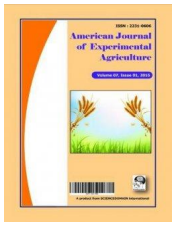

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Effects of Nitrogen Levels and Arbuscular Mycorrhizal Fungi on Biomass Production, Mineral Nutrition, Sugar and Total Phenolic Content of Two *Zea mays* Cultivars

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LNW and ELMN designed the study. Authors RF and APN wrote the protocol and wrote the first draft of the manuscript. Authors JK and FXE reviewed the experimental design and all drafts of the manuscript. Authors RF, APN and PE managed the analyses of the study. Author ELMN identified the plants. Authors RF and SNT performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

The effects of two nitrogen levels and AM fungi inoculation on nutrient assimilation and some biochemical compounds of two *Zea mays* varieties were studied. Plants varieties (CMS8704 and POP66RS) were grown in pots with two nitrogen levels and AM fungi inoculation for 82 days. Plants grown under Low N condition showed significant reduction in biomass production, N and P uptake. POP66RS cultivar proved to be more efficient than CMS8704 under nitrogen deficiency environment, which was reflected by increased chlorophyll rate and carbohydrates content. However, under AM fungi CMS8704 variety show better performance than POP66RS variety. Significant increase in plant dry weights, P and N uptake were recorded following AM inoculation. AM fungi positively influences phenolic compounds while it decreases the soluble carbohydrates and total chlorophyll under low and high N, for the two cultivars. Evaluating such parameters in plant during it growth in fill could be further exploited for maize selection.

Keywords: Nitrogen; maize varieties; mycorrhizal fungi; chlorophyll; phosphorus carbohydrates and phenol compounds.

1. INTRODUCTION

Maize (*Zea mays L.*) is the most important crop in the world after wheat and rice [1]. It is a major item in the diet of many tropical people. In Cameroon, this plant can grow in all the five local ecological zones and is now a cash crop for many farmers [2]. Local demand of maize continuously increases in Cameroon due to the exponential growth of the population and exacerbated by the high demand of feed industry and breweries [1].

The production of this cereal which is dominated by small holder farmers, who always used traditional manual methods generally, is fought with drudgery and a lot of problems such as pest and diseases in a context of the absence of resistant or tolerant local maize varieties. In fact, pest and diseases are important natural factors limiting the production of maize in several cases, accounts for 100% losses [1,3]. Those constraints are highlighted in many local regions by different factors, including erosion, low pH with salt toxicity, loss of soil biodiversity, high phosphorus fixation and N depletion policy [4,5]. Such conditions generally lead to bad feeding of plant and low yield. Nitrogen is one of the few nutrients in terrestrial ecosystems that may limit plants growth [6]. It is necessary for many functions in plant such as chlorophyll, proteins and amino acids synthesis [7]. Nitrogen (N) plays a vital role in the development of crop yield and quality. It is a mobile element in the soil and hence under humid conditions is subjected to leach [8]. N deficiency results in accumulation of carbohydrates (sugars and starch) in the leaves, higher levels of carbon allocated to the roots and an increase in root to shoot biomass ratio [9,10,11]. N deficiency therefore affects, to various extents, primary photosynthesis, amino acid and sugar metabolism [12,13,10]. Nitrogen excess does not increase yield or vegetative growth [14], but negatively affects fruit quality or its derived products [15]. In current horticultural practice, N is often applied in higher amounts to ensure a good productivity [16]. N fertiliser recommendations are traditionally based on soil N status and to a lesser extent on foliar analysis [17].

The availability of nitrogen is directly affected by soil pH. In acid soils, as those encountered in tropical zones [18], the activity of beneficial microorganisms (Bacteria) that decompose soil organic matter are seriously hindered. This prevents organic matter from breaking down, resulting in an accumulation of organic matter and the tie up of nutrients, particularly nitrogen, that are held in the organic matter [19]. These process lead to the limitation of the availability of nitrogen to the plant root system and thus to the plant growth. Under N stress, triggered by its immobilization in organic and inorganic matter, the plants have developed strategies to increase their capacity for N mobilization. One strategy is the symbiotic association with arbuscular mycorrhizal (AM) fungi [20,21].

It is recognized that ecosystem functioning could be regulated by groups of soil microbes with special functional importances including mycorrhizal fungi [22]. AM fungi are soil fungi, developing symbiotic association with most terrestrial plants under low and high nitrogen conditions. The symbiosis is often mutualistic based largely on exchange of C from the plant and P delivered by the fungi [23]. Despite their

implication in phosphorus fertilisation of plant, AM fungi can mediate more than 50% of plant nitrogen uptake [24,25], modify phenolic compounds metabolism [26,27,28], proteins metabolism [29] and amino acids pathway [30]. Therefore, AM fungi hyphae may contribute substantially to plant adaptation on N poor soil.

Plant phenolics and carbohydrates have received considerable attention in relation to plant stress resistance. In fact, the metabolic pathways of these substances are interconnected. Phenolic pathways use products of carbohydrates metabolism as their precursors [31]. Phenolic compounds are increased in response to adverse environmental conditions, which play an important role in regulation of biochemical, physiological and molecular responses in plants [32]. These include effects on nitrogen assimilation, ion uptake, enzyme regulation, membrane organization, photosynthetic carbon dioxide assimilation and nutrient deficiency in plants [33,34,35,36]. Levels of some compounds related to secondary metabolism show a sensitive response to nutrient deficiency in plants [37,38]. Provision of nitrogen (N), either from organic or mineral fertilizers, has far-reaching consequences on the performance of plants at the biochemical, ecophysiological and ecosystem levels [39]. Since the reaction under stressed condition and the mycorrhizae forming capability depend upon host plant genotype, this study was therefore undertaken to examine the response of AM fungi on the growth of two *Zea mays* varieties under two nitrogen levels as well as the biosynthesis of some biochemical marquers involved in plant nutrient stress adaptation.

2. MATERIALS AND METHODS

2.1 Seed Material, AM Inoculums and N Fertilizer

Seeds of two maize genotypes (CMS8704 and POP66-SR) and a mixture of three AM fungi strains (*Acaulospora tuberculata, Gigaspora margarita, Glomus intraradices*) were obtained from the Maize Breeding Program at the Regional Biocontrol and Applied Microbiology Laboratory of the National Institute of Agricultural Research for Development (IARD), Cameroon. These varieties were selected according to their nitrogen tolerance to poor nitrogen substrate: POP66SR had the highest N assimilation rate while CMS8704 had the lowest. The AM mixture were authenticated on many local grown plants as maize, cowpea, beans and groundnut. Two N rates (20N and 100N) were prepared from urea source, corresponding to 20 kg N ha^{-1} and 100 kg N ha^{-1} respectively. This consisted of adding 0.049 g and 0.246 g per plastic bag for the 20 N and 100 N respectively.

2.2 Substrate Preparation, Plant Culture and Treatment

The experimental substrate was neutral Sand (pH 6.8) collected from Wouri River in Cameroon. The collected Sand was, sieved at 2 mm and abundantly washed with distilled water to remove organic residual matter. The substrate was then used to fill experimental pot of three liters.

Seeds were sterilized by immersion in 5% sodium hypochlorite for 10 min, rinsed four times with distilled water and kept for germination on wet filter paper in Petri dishes at 25°C for 72h. After the germination process, the seed were transplanted into plastic pots. Prior to the transplantation, AM fungi pots were inoculated with 200 g of *Acaulospora tuberculata, Gigaspora margarita* and *Glomus intraradices* (1-1-1) mixture containing approximately 100 spores per type. Non AM pots received the same quantity of autoclaved inoculum. The inoculums were placed adjacent to each seeding root.

2.3 Experimental Design

The experiment took place at the National Institute of Agricultural Research for Development (I.A.R.D.) under control conditions. The experimental design was a randomized complete block with 3 factors, 5 treatments and 5 replicates. The factors were N application (20N= 20 kg N ha^{-1} and 100N= 100 kg N ha^{-1}), maize varieties (POP66SR and CMS8704) and AM fungi application (AM fungi inoculation (M) and none AM fungi inoculation (NM)). Plants were daily watered with distilled water. In addition, each treatment received Rorison's solution without nitrogen to supply nutrient deficiency. Rorison's solution is composed with 1.5 KH_2PO_4 $3H_2O$; 1.2 $MgSO_4$ $7H_2O$; 1.2 Fe-EDTA; 0.11 Mn SO_4 $4H_2O$; 0.019 $CuSO_4$ $5H_2O$; 0.02 Zn SO_4 $7H_2O$; 0.14 H_3BO_3; 0.1 $MoNa_2O_4$ and 0.375 $CaSO_4$ $g.l^{-1}$. Plants were harvested 82 days after planting for sampling analyses.

2.4 Plant Analyses

Seventy five days after planting, total chlorophyll (%) was evaluated *in situ* in the third leaf of each

plant, by using chlorophyll meter (SPAD-502). Shoots and roots of each plant were weighed separately. One g of fine root was used for evaluation of AM fungi colonization [40]. Remaining roots, together with the shoots were dried at 60ºC for 72 h. The whole plant was crushed and sub-samples used for the determination of P and N concentration. Sub-samples of plant powder were digested with concentrated sulphuric acid at 480ºC and analyzed for N and P content. Analysis of P-uptake was done using the ammonium-molybdate blue method described by [41] while N content was done using method described by [42].

2.4.1 Determination of carbohydrates

One gram of fresh materiel was crushed and extracted in 5 ml of 80% ethanol [43] and titrated using the anthrone method of [44]. Pure analytical grade glucose (1 mg/ml) was used as standard.

2.4.2 Determination of total soluble phenol compounds

Total phenols were extracted according to the describe method of [45]. Half g (0.5 g) of plants powder was ground and mixed with 25 ml of 70% methanol at room temperature for 45 min. The methanolic extract was filtered with Whatman n°1 paper and the methanol was evaporated at 40ºC under vacuum using a rotary evaporator. The aqueous phase obtained was adjusted with 25 ml sterile distilled water, and depigmented by adding 12.5 ml of 40% ammonium sulphate $[(NH_4)_2SO_4]$, 0.35 ml of 80% orthophosphoric acid and 25 ml of petroleum ether. The ether phases were discarded and the aqueous phases were extracted four times with 25 ml ethyl acetate. The aqueous phases were discarded and the organic phases of the same sample were combined, dried by addition of five g of magnesium sulphate ($MgSO_4$) and filtered after 5 min with Whatman n°1 paper. The salt residue was discarded and the clear organic phase was dried at 40ºC under vacuum using a rotary evaporator. Total phenol content was determined according to protocols established by [46]. Total phenol content was expressed in mg of chlorogenic acid per gram of plants.

2.5 Data Analysis

The data were subjected to analysis of variance (ANOVA) using Statistical Analysis System Software (SAS) version 9.1. The statistical significance of the results was determined by performing Turkey's test at P< .05. Pearson's correlation was used to establish the relationship between pairs of variables.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Root colonization and biomass production

Non inoculated maize plants did not show any structure characteristic of AM fungi in roots for the two cultivars. Colonization rate decreased significantly by increasing N level application. The colonization rate of POP66SR variety, at 20N and 100N nitrogen levels were 60% and 50% respectively and those of CMS8704 variety were 66.7% and 56.7% at the two N levels respectively (Table 1).

Increase N level did not affect significantly shoot and root dry mater of the two maize varieties (Table 1). However, AM fungi inoculation significantly improved shoot and root dry matter of the two maize varieties at each N level. This effect of AM fungi on shoot and root dry matter was better for the POP66SR variety than the CMS8704 variety at the two N levels (Table 1).

Table 1. Effect of N levels and AMF colonization on root colonization, shoot and root dry matter (SDM, RDM) of two maize cultivars, 82 days after seedlings

Parameters	Root colonization				SDM				RDM			
	20N		100N		20N		100N		20N		100N	
	NM	M	NM	M	NM	M	NM	M	NM	M	NM	M
POP66SR	0.0^{c}	60.0^{a}	0.0^{c}	50.0^{b}	2.04^{d}	3.36^{c}	2.92^{cd}	6.06^{a}	1.70^{cd}	2.70^{c}	2.03^{cd}	9.70^{a}
CMS8704	0.0^{c}	66.7^{a}	0.0^{c}	56.7^{b}	1.28^{e}	4.23^{b}	1.75^{de}	2.38^{d}	0.90^{d}	5.40^{b}	1.4^{cd}	4.1^{b}

Means within columns followed by same letters are not significantly different at P < 0.05
NM: none AM fungi; M: inoculated with AM fungi; POP66SR and CMS8704 maize cultivars; 20N and 100N: lowest and highest N levels

3.1.2 Chlorophyll rate, nitrogen and phosphorus uptake

The increment of the nitrogen level significantly raised chlorophyll content of the CMS8704 variety. Inversely, a reduction of this content was found with the POP66SR variety. AM fungi significantly reduced chlorophyll content for the two maize varieties except for the 100N level of the POP66SR variety (Table 2).

The nitrogen level was positively related with P and N concentration of the two maize varieties. AM fungi inoculation did not influence P and N concentration for the CMS8704 variety however, a significant increase of P content was observed for the POP66SR variety at the low N level following AM fungi inoculation. The N content was significantly affected at the two N levels of the POP66SR variety (Table 2). The lowest P content was recorded for the CMS8704 variety at the low nitrogen level without AM fungi inoculation, and the highest at the high nitrogen level for the POP66SR with AM fungi inoculation. The lowest and highest N concentration was recorded for the CMS8704 variety at the low nitrogen level without AM fungi inoculation and the high nitrogen level with AM fungi inoculation respectively (Table 2).

3.1.3 Soluble carbohydrate, and total phenol contents

Total soluble carbohydrates of the two maize varieties leaves were significantly influenced by N levels application and AM fungi. There were neither varietal nor interaction effects observed in the study. The accumulation of soluble carbohydrates in our maize varieties was high under low nitrogen level (20N) and low under high N level (100N). The same tendency was observed after AM fungi application (Fig. 1). The lowest sugar concentration was observed for CMS8704 maize variety at the high N level without AM fungi inoculation while the high soluble carbohydrate concentration was observed for POP66SR at the low N level and the CMS8704 at the same N level after AM fungi application. AM fungi application tends to increase soluble sugar concentration in all the varieties with significant effect in the CMS8704 variety (Fig. 1).

Soluble phenol concentration of the two maize varieties was significantly influenced by N levels. When nitrogen level increased, the concentration of soluble phenolic decreased simultaneously. In addition, AM fungi application significantly increased the total phenolic concentration of all the two maize varieties at any nitrogen levels except for the 20N level of the POP66SR variety (Fig. 2). The lowest phenolic concentration was observed for the CMS8704 variety at the high nitrogen level (100N) without AM fungi inoculation and the highest at the low level (20N) of the same variety after AM fungi application (Fig. 2).

3.2 Discussion

The colonization rate declined with increasing N level for the two maize varieties, indicating that N affects the growth of AM fungi (Table 1). A number of other workers reported that rich nitrogen soil inhibits hyphal growth with a subsequent decrease in the spread of mycorrhizal colonization [47,48,49]. Our results further demonstrated that, under reduced N fertilization (20N), inoculation with AM fungi significantly improved shoot and root dry weight of the two maize varieties. The best response was observed with the CMS8704 variety (Table 1). This observation is consistent with many others indicating the promotion of plant growth by AM fungi under low nutrient availability [50,51,52]. We also noted that under high N level (100N), maize SDM and RDW significantly increased following AM fungi inoculation, with the best performance observed for the POP66SR variety (Table 1). It seems possible that genetic factors related to the maize varieties affect their aptitude to undergoing symbiosis with AM fungi and subsequently their growth. This contrasted observation go in line with [53,47] who hypothesized that, when plants are grown under optimal conditions growth promotion by AM fungi is unlikely, whereas under suboptimal conditions enhanced growth can be achieved. Besides, N deficiency suppressed maize shoot growth and dry matter accumulation in both cultivars (Table 1). Decreased plant biomass production due to N shortage was associated with reductions in both leaf area [54] and leaf photosynthetic capacity [49].

The findings of this study showed that, nitrogen fertilization significantly influenced chlorophyll content, N and P uptake of the two maize varieties. The increment N level significantly increased leaves chlorophyll rate of CMS8704 variety while it decreased the rate in POP66SR variety (Table 2). Inoculation with AM fungi significantly influenced leaves chlorophyll rates of the two maize varieties. Intrinsic genetic factors

related to plant maize variety could justify this observation. It has been shown that the response of leaf photosynthesis is largely dependent on the leaf N content [54] and that N fertilization significantly affects Chlorophyll concentration of plants [55,56,57]. The N SDM content varied according to N application level for both maize varieties, with low amount obtain under low N level and the higher under high N level (Table 2). Shoot N increase is the result of the increase of N level application [54,58]. A negative correlation (r^2 = -0,89; p = .016) was shown between AM fungi colonization and nitrogen content for POP66SR variety at 20N rate, however there were no correlation between the two parameters at 100N level. There was no correlation between the two parameters at the two nitrogen levels for the CMS8704 variety. This observation can explain the implication of AM fungi and plant variety in the adaption of plant to low nutrient environment especially nitrogen. The decrement plant biomass production due to N shortage was associated with reductions in both nutrient assimilation [54] and leaf photosynthetic capacity [59]. There is also clear evidence that N deficiency induces decreased growth due to limitation of N and P nutrition within the whole plant [60,61,62].

Different research works have indicated that, AM fungi can substantially enhance the uptake of different nutrients, especially phosphorous (P) by the host plant through the extension of hyphal network [24,63]. It has also been indicated that AM fungi can also influence the uptake of other nutrients necessary for plant growth and yield production including N [24,63]. [25] indicated that more than 50% of plant N requirement was supplied by mycorrhizal association.

Table 2. Effect of N levels and AMF inoculation on chlorophyll rate, nitrogen (N) and phosphorus (P) uptake of two maize cultivars, 82 days after seedlings

Parameters	Chlorophyll (%)				N(mg/plant)				P(mg/plant)			
	20N		100N		20N		100N		20N		100N	
	NM	M	NM	M	NM	M	NM	M	NM	M	NM	M
POP66SR	28.10^{a}	9.50^{e}	25.80^{bc}	27.0^{bc}	1.50^{d}	2.25^{bc}	2.09^{c}	2.60^{ab}	3.0^{d}	8.38^{c}	9.09^{b}	11.86^{ab}
CMS8704	18.50^{d}	10.86^{e}	24.05^{c}	19.13^{d}	1.37^{d}	1.55^{d}	2.38^{ba}	2.75^{a}	0.80^{e}	1.74^{de}	1.46^{de}	2.21^{de}

Means within columns followed by same letters are not significantly different at P < 0.05
NM: none AM fungi; M: inoculated with AM fungi; POP66SR and CMS8704 maize cultivars; 20N and 100N: lowest and highest N levels

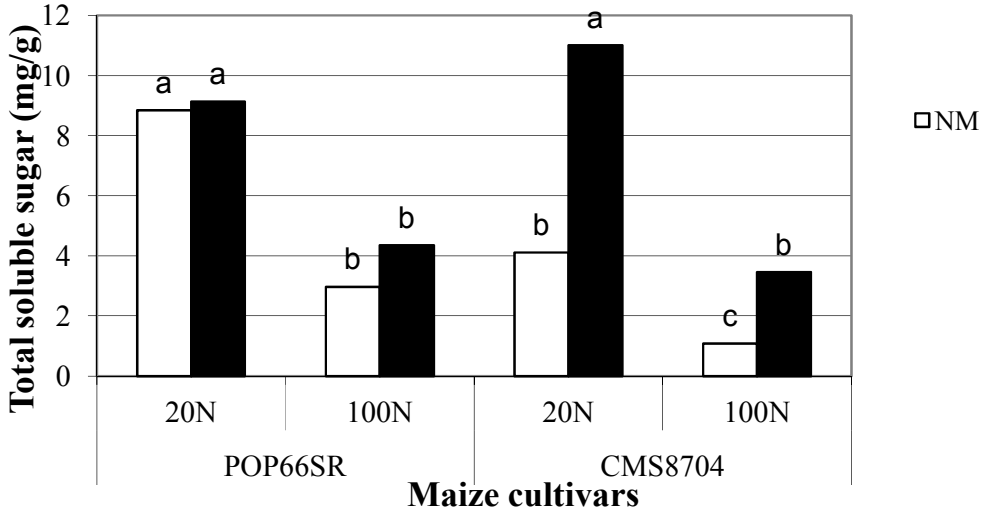

Fig. 1. Effect of N levels and AMF colonization on total soluble sugar of two maize cultivars, 82 days after seedlings

Barres with the same letters is not significantly different at P <0.05. NM: none AM fungi; M: inoculated with AM fungi; POP66SR and CMS8704 maize cultivars; 20N and 100N: lowest and highest N levels

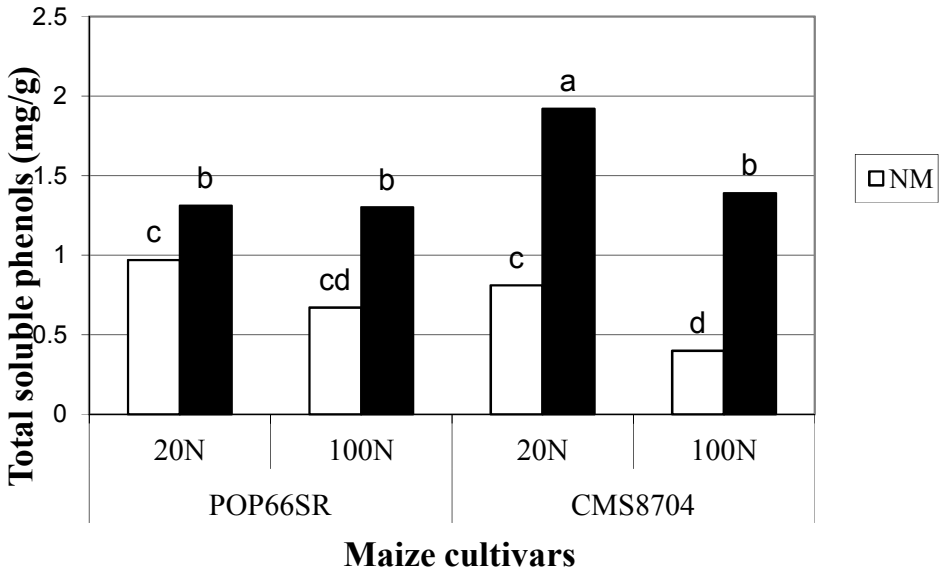

Fig. 2. Effect of N levels and AMF colonization on total soluble phenols of two maize cultivars, 82 days after seedlings

Barres with the same letters is not significantly different at P <0.05. NM: none AM fungi; M: inoculated with AM fungi; POP66SR and CMS8704 maize cultivars; 20N and 100N: lowest and highest N levels

Our investigations also reveal that, N fertilization level has significant effected on carbohydrate and phenol leaves content. The leaves soluble carbohydrate and phenolic content was higher in limited N fertilization (20N) than in high N treatment (100N) for the two maize cultivars (Fig. 1). Many studies showed that under inappropriate growth conditions such as drought, high salinities, low temperatures [64], nitrogen deficiency [29,54,65], there is a large accumulation of soluble sugars generally interpreted as an adaptive response of plant to such conditions [64]. The findings have also shown the increase of phenylalanine ammonia lyase (PAL) activity in plant leaf following N depletion resulting to the synthesis and accumulation of phenolic compounds [66]. Probably those two compounds are used by plant to develop adaptation in environmental stress conditions, as they are higher in CMS8704 variety than POP66SR, suggesting the best adaptation capacity of POP66SR under low N condition. Inoculation with AM fungi stimulates the synthesis and accumulation of soluble carbohydrates under the limited N fertilization with remarkable effect on the CMS8704 variety (Fig. 1). Positive significant correlation was observed between AM fungi colonization and phenolic compounds of the CMS8704 variety the two nitrogen levels (r^2=0,96; P= .002 and r^2=0,98; P= .00). This observation goes in line with other study showing the contribution of AM fungi to plant adaptation [67]. AM fungi also increase significantly phenol content at the two N levels for the CMS8704 variety while it affects less the content of this compound for the other variety. This is an additional argument supporting the high dependency of CMS8704 variety under low N fertilization condition.

4. CONCLUSION

In this study, N deficiency caused a decrease in leaf Chlorophil, N and P content of maize plants, resulting a lower dry matter accumulation. Inversely, this condition resulted in an increase in sugars and phenols content. These results further highlight the fact that micorrhizae can increase dry matter, induce the accumulation of sugars, phenols as well as the absorption of N and P in plants under nitrogen stress. The parameters used in this experiment suggest that CMS8704 variety is more efficient than POP66SR variety under N deficiency, which are highly AM fungi dependent. The field experiment has to be investigated, looking for relationship between the results obtain under controled conditions with natural environment. So that this variety could be proposed to farmers working under nitrogen poor field.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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