

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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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 its growth in field could be further exploited for maize selection.

Keywords: Nitrogen, Maize varieties, mycorrhizal fungi, Chlorophyll, Phosphorus Carbohydrates and Phenol compounds.

1. INTRODUCTION

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

25 The production of this cereal which is dominated by small holder farmers, who always used
26 traditional manual methods generally is fought with drudgery and a lot of problems such as pest
27 and diseases in a context of the absence of resistant or tolerant local maize varieties. In fact,
28 pest and diseases are important natural factors limiting the production of maize in several
29 cases, accounts for 100% losses [1]. with simple low input technology, resulting in low land
30 and labour productivity[3]. Those constraints are highlighted in many local regions by different
31 factors, including erosion, low pH with salt toxicity, loss of soil biodiversity, high phosphorus
32 fixation and N depletion policy [4,5]. Such conditions generally lead to bad feeding of plant and
33 low yield. Nitrogen is one of the few nutrients in terrestrial ecosystems that may limit plants
34 growth [6]. It is necessary for many functions in plant such as chlorophyll, proteins and amino
35 acids synthesis [7]. Nitrogen (N) plays a vital role in the development of crop yield and quality. It
36 is a mobile element in the soil and hence under humid conditions is subjected to
37 leach. indicating the importance of appropriate N fertilization under such conditions[8]. N
38 deficiency results in accumulation of carbohydrates (sugars and starch) in the leaves, higher
39 levels of carbon allocated to the roots and an increase in root to shoot biomass ratio [9,10,11]. N
40 deficiency therefore affects, to various extents, primary photosynthesis, amino acid and sugar
41 metabolism. and/or carbohydrate partitioning between source and sink tissues [12,13,10].

42 Nitrogen excess does not increase yield or vegetative growth [14], but negatively affects fruit
43 quality or it derived products [15]. In current horticultural practice, N is often applied in higher
44 amounts needed to ensure a good productivity [16]. N fertiliser recommendations are
45 traditionally based on soil N status and to a lesser extent on foliar analysis [17]. A rationalization
46 of fertilization is aprerequisite in the light of the factors up on which productivity depends,
47 namely photosynthesis, translocation of assimilates growth and production.

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

57 It is recognized that ecosystem functioning could be regulated by groups of soil microbes with
58 special functional importances including mycorrhizal fungi [19]. 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 [20]. Despite their implication in phosphorus fertilisation of plant, AM fungi can mediate more than 50% of plant nitrogen uptake [21,22], modify phenolic compounds metabolism [23,24,25], proteins metabolism [26] and amino acids pathway [27]. 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 carbohydrate metabolism as their precursors [28]. 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 [29]. These include effects on nitrogen assimilation, ion uptake, enzyme regulation, membrane organization, photosynthetic carbon dioxide assimilation and nutrient deficiency in plants [30,31,32,33]. Levels of some compounds related to secondary metabolism show a sensitive response to nutrient deficiency in plants [34,35]. 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 [36]. 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 markers involved in plant nutrient stress adaptation.

2. MATERIAL 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 so collected Sand was, sieved at 2 mm and abundantly washed with distilled water to removed organic residual matter. The substrate was then used to fill experimental pot of three liters.

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

104 **2.3 Experimental design**

105 The experiment took place at the National Institute of Agricultural Research for Development
106 (I.A.R.D.) under control conditions. The experimental design was a randomized complete block
107 with 3 factors 5 treatments and 5 replicates. The first factor was N application with two levels
108 equivalent to 20N= 20 kg N ha⁻¹ and 100N= 100 kg N ha⁻¹. The second factor was two maize
109 varieties POP66SR and CMS8704. The third factor was AM fungi application: AM fungi
110 inoculation (M) and none AM fungi inoculation (NM). Plants were daily watered with distilled
111 water. In addition, each treatment received Rorison's solution without nitrogen to supply nutrient
112 deficiency. Rorison's solution is composed with 1.5 KH₂PO₄ 3H₂O; 1.2 MgSO₄ 7H₂O; 1.2 Fe-
113 EDTA; 0.11 Mn SO₄ 4H₂O; 0.019 CuSO₄ 5H₂O; 0.02 Zn SO₄ 7H₂O; 0.14 H₃BO₃; 0.1 MoNa₂O₄
114 and 0.375 CaSO₄ g.l⁻¹. Plants were harvested 82 days after planting for sampling analyses.

115 **2.4 Plant analyses**

116 Seventy five days after planting, total chlorophyll (%) was evaluated *in situ* in the third leaf of
117 each plant, by using chlorophyll meter (SPAD-502). Shoots and roots of each plant were
118 weighed separately. One g of fine root was used for evaluation of AM fungi colonization [39].
119 Remaining roots, together with the shoots were dried at 60°C for 72 h. The whole plant was
120 crushed and sub-samples used for the determination of P and N concentration. Sub-samples of
121 plant powder were digested with concentrated sulphuric acid at 480°C and analyzed for N and P
122 content. Analysis of P-uptake was done using the ammonium-molybdate blue method described
123 by [40] while N content was done using method described by [41].

124 **2.4.1 Determination of carbohydrates.**

125 One gram of fresh material was crushed and extracted in 5 ml of 80% ethanol. [42] and titrated
126 using the anthrone method of [43]. Pure analytical grade glucose (1mg/ml) was used as
127 standard.

128 **2.4.2 Determination of total soluble phenol compounds**

129 Total phenols were extracted according to the describe method of [44]. Half g (0.5 g) of plants
130 powder was ground and mixed with 25 ml of 70% methanol at room temperature for 45 min. The
131 methanolic extract was filtered with Whatman n°1 paper and the methanol was evaporated at

132 40°C under vacuum using a rotary evaporator. The aqueous phase obtained was adjusted
133 **to with 25 ml** sterile distilled water, and depigmented by adding 12.5 ml of 40% ammonium
134 sulphate $[(\text{NH}_4)_2\text{SO}_4]$, 0.35 ml of 80% orthophosphoric acid and 25 ml of petroleum ether. The
135 ether phases were discarded and the aqueous phases were extracted four times with 25 ml
136 ethyl acetate. The aqueous phases were discarded and the organic phases of the same sample
137 were combined, dried by addition of five g of magnesium sulphate (MgSO_4) and filtered after 5
138 min with Whatman n°1 paper. The salt residue was discarded and the clear organic phase was
139 dried at 40°C under vacuum using a rotary evaporator. Total phenol content was determined
140 according to protocols established by [45]. Total phenol content was expressed in mg of
141 chlorogenic acid per gram of plants.

142 **2.5 Data analysis**

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

147 **3. RESULTS AND DISCUSSION**

148 **3.1 Results**

149 **3.1.1 Root colonization and biomass production**

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

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

159 Table 1: Effect of N levels and AMF colonization on root colonization, shoot and root dry matter
160 (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

161 Means within columns followed by same letters are not significantly different at $P < 0.05$ NM:

162 none A.M. fungi; M: inoculated with A.M. fungi; POP66SR and CMS8704 maize cultivars; 20N

163 and 100N: lowest and highest N levels.

164 3.1.2 Chlorophyll rate, Nitrogen and Phosphorus uptake

165 Increasing nitrogen level raising chlorophyll content of the CMS8704 variety but causes a
166 reduction of this content for the variety POP66SR. AM fungi significantly reduced chlorophyll
167 content for the two maize varieties **except** for the 100N level of the POP66SR variety (Table 2).

168 Increase nitrogen level significantly **raises** P and N concentration of the two maize varieties. AM
169 fungi inoculation did not influence P and N concentration for the CMS8704 variety however, a
170 significant increase of P content was observed for the POP66SR variety at the low N level
171 following AM fungi inoculation. The N content was significantly affected at the two N levels of
172 the POP66SR variety (Table 2). The lowest P content was **recorded** for the CMS8704 variety at
173 the low nitrogen level without AM fungi inoculation, and the highest at the high nitrogen level for
174 the POP66SR with AM fungi inoculation. The lowest and highest N concentration was **recorded**
175 for the CMS8704 variety at the low nitrogen level without AM fungi inoculation and the high
176 nitrogen level with AM fungi inoculation respectively (Table 2).

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 $P < 0.05$ N.M:

none A.M. fungi; M: inoculated with A.M. fungi; POP66SR and CMS8704 maize cultivars; 20N

and 100N: lowest and highest N levels.

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 levels (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).

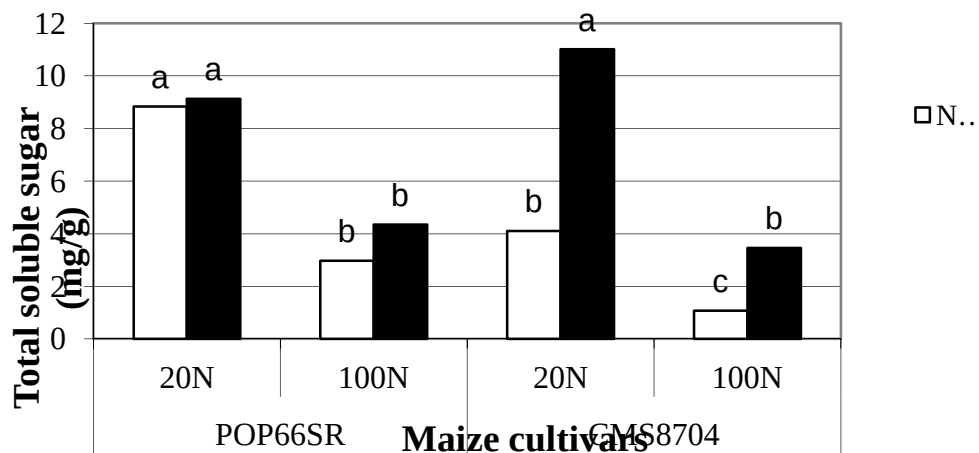


Figure 1: Effect of N levels and AMF colonization on total soluble sugar of two maize cultivars, 82 days after seedlings.

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

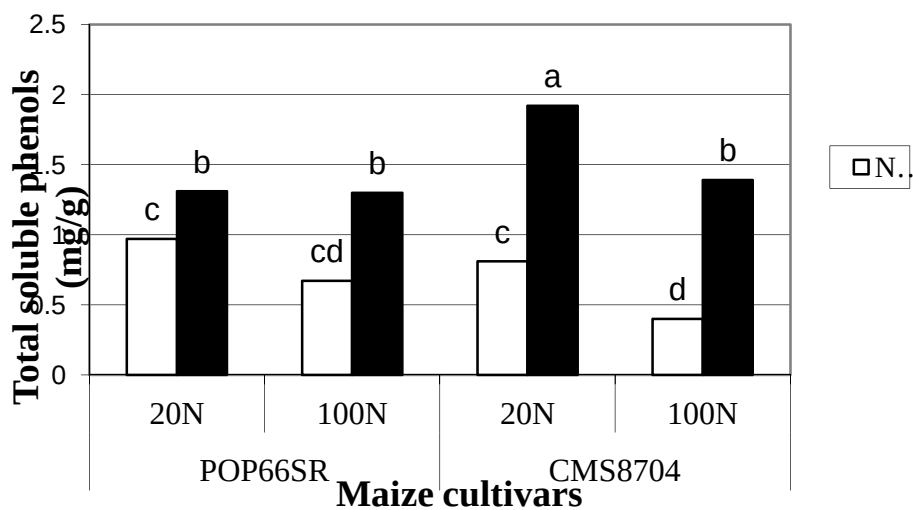


Figure 2: Effect of N levels and AMF colonization on total soluble phenols of two maize cultivars, 82 days after seedlings.

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

205 Soluble phenol concentration of the two maize varieties was significantly influenced by N levels.
206 When nitrogen level increase, the concentration of soluble phenolic decrease simultaneously.
207 AM fungi application increase significantly the total phenolic concentration of all the two maize
208 varieties at any nitrogen levels except for the 20N level of the POP66SR variety (Fig. 2). The
209 lowest phenolic concentration was observed for the CMS8704 variety at the high nitrogen level
210 (100N) without AM fungi inoculation and the highest at the low level (20N) of the same variety
211 after AM fungi application (Fig. 2).

212 **3.2 Discussion**

213 The colonization rate declined with increasing N level for the two maize varieties, indicating that
214 increase N level affect the growth of AM fungi (Table 1). It has been reported that addition of
215 various N application to soil inhibits hyphal growth with a subsequent decrease in the spread of
216 mycorrhizal colonization [46]. A number of other workers reported that root colonization
217 decreases in rich nitrogen soil [47, 48]. Our results further demonstrate that, under reduced N
218 fertilization (20N), inoculation with AM fungi significantly improved shoot and root dry weight of
219 the two maize varieties. The best response was observed with the CMS8704 variety (Table 1).
220 This observation is consistent with many others indicating the promotion of plant growth by AM
221 fungi under low nutrient availability [49, 50, 51]. We also noted that under high N level (100N),
222 maize SDM and RDW was significantly increased following AM fungi inoculation, with the best
223 performance observed for the POP66SR variety (Table 1). It seems possible that genetic factors
224 related to the maize varieties affect their aptitude to undergoing symbiosis with AM fungi and
225 subsequently their growth. This contrasted observation go in line with [52] and [46] who
226 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 that, 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 [53] and leaf photosynthetic capacity [48].

The findings of this study showed that, nitrogen fertilization significantly influence chlorophyll content, N and P uptake of the two maize varieties. Increase N level significantly increase leaves chlorophyll rate of CMS8704 variety while it decreases the rate in POP66SR variety (Table 2). Inoculation with AM fungi significantly influence 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 [53] and that N fertilization significantly affects Chlorophyll concentration of plants [54, 55, 56]. The N SDM content vary 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 [53, 57]. 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 parameter 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.

Decreased plant biomass production due to N shortage was associated with reductions in both nutrient assimilation [53] and leaf photosynthetic capacity [58]. There is also clear evidence that N deficiency induces decreased growth due to limitation of N and P nutrition within the whole plant [59 60, 61].

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 [21, 62]. It has also been indicated that AM fungi can also influence the uptake of other

253 nutrients necessary for plant growth and yield production including N [21, 62]. [22] indicated that
254 more than 50% of plant N requirement was supplied by mycorrhizal association.

255 Our investigations also reveal that, N fertilization level has significant effects on carbohydrate
256 and phenol leaves content. The leaves soluble carbohydrate and phenolic content was higher in
257 limited N fertilization (20N) than in high N treatment (100N) for the two maize cultivars (Fig. 1).
258 Many studies showed that under inappropriate growth conditions such as drought, high
259 salinities, low temperatures [63], nitrogen deficiency [26, 53, 64], there is a large accumulation
260 of soluble sugars generally interpreted as an adaptive response of plant to such conditions [63].
261 The findings have also shown the increase of phenylalanine ammonia lyase (PAL) activity in
262 plant leaf following N depletion resulting to the synthesis and accumulation of phenolic
263 compound [65]. Probably those two compounds are used by plant to develop adaptation in
264 environmental stress conditions, as they are higher in CMS8704 variety than POP66SR,
265 suggesting the best adaptation capacity of POP66SR under low N condition. Inoculation with
266 AM fungi stimulates the synthesis and accumulation of soluble carbohydrate under the limited N
267 fertilization with remarkable effect on the CMS8704 variety (Fig.1). Positive significant
268 correlation was observed between AM fungi colonization and phenolic compounds of the
269 CMS8704 variety the two nitrogen levels ($r^2=0,96$; $P= .002$ and $r^2=0,98$; $P= .00$). This
270 observation goes in line with other study showing the contribution of AM fungi to plant
271 adaptation [66]. AM fungi also increase significantly phenol content at the two N levels for the
272 CMS8704 variety at the two N levels while it affect less the content of this compound for the
273 other variety. This is an additional argument supporting the high dependency of CMS8704
274 variety under low N fertilization condition.

275 4. CONCLUSION

276 In this study, N deficiency caused a decrease in leaf Chlorophyll, N and P content of maize
277 plants, resulting a lower dry matter accumulation. inversely, this condition resulted in an
278 increase in sugars and phenols content. These results clarifies properly that micorrhizae can

279 increase dry matter, accumulation of sugars, phenols and absorption of N and P in plants under
280 nitrogen stress. The parameters used in this experiment suggest that CMS8704 variety is more
281 efficient than POP66SR variety under N deficiency, which are highly AM fungi dependent. The
282 field experiment has to be investigated, looking for relationship between the results obtain under
283 controled conditions with natural environment. So that this variety could be proposed to farmers
284 working under nitrogen poor field.

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