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Research Article

Potentiation of antimalarial activity in Artemisia annua cultivated in the presence of fodder peanuts and beneficial microorganisms

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Abstract

Production of *Artemisia annua* chemical active ingredients remains too expensive and unprofitable. Its antimalarial active molecules vary a lot with environmental diversity and are low concentrated in the plant. Research into ways of optimizing their content, extraction and concentration is a current challenge. In this study, we tried to promote an inexpensive and more profitable cultivation procedure to improve Artemisia antimalarial biomass and therapeutic molecules. Seeds of *A. annua* were grown on a non-sterilized soil substrate using non-randomized bloc design. Impact of Arachis pintoï (Ara), special organic matter (MOS), Biochar (Bio), mycorrhizal fungi (CMA) and endophyte bacteria (End) on the parameters of biomass yield in A. annua was evaluated as well as contents of some phytochemical active biomolecules, antioxidant capacity and in vitro PfLDH-based antiplasmodial activity of A. annua. Increased biomass yield in A. annua was higher with Arachis pintoï (Ara) combined to special organic matter. Artemisia annua in "Ara+CMA+End" system was more efficient compare to Artemisia annua without biofertilizer. We had respectively, 86.88±3.38 and 65.77±3.08 mgEqAG/ g of DM for plyphonols; 79.18±5.84 and 67.82±4.38 mgEqAA/ g of DM for antioxidant capacity; regarding antiplasmodial activity we had for 50% Inhibitory Concentration (IC_{50}) in µg/mL: 31±2.05 and 60.68± 13.07 for PfDd2 (multidrug-resistant); 34.14 ± 2.71 and 47.69 ± 6.34 for Pf3D7 (sensitive). Integrated soil fertility management (ISFM) involving a forage legume and microbial fertilizers may be the best option to provide the highest bioactive molecules content and diversity. ISFM improve Artemisia annua biomass production for better active therapeutic ingredients.

Keywords: Microbial biofertilizers, *Arachis pintoï*, malaria active biomolecules, *Artemisia annua*.

Introduction

In Cameroun, malaria is strongly endemic. Every year, we register six million cases of malaria and our health establishments report 4000 deaths around. The WHO estimates that around 11,000 people die of malaria in Cameroon each year. *Artemisia annua*, the source of new drugs Artemisinin and its derivatives have not, to date, induced resistance in Plasmodium. They are therefore widely used to treat malaria, mainly in South-East Asia and Africa[1]. However, the chemical production of *Artemisia annua* biomolecules remains too expensive and unprofitable. Also the antimalarial active biomolecules in *Artemisia annua* plant are low concentrated. Research into optimizing their extraction and concentration is a current challenge[2]. Promoting an inexpensive and more profitable cultivation route is a challenge to take up. A more environmentally sound and productive approach is the use of beneficial microorganisms such as biofertilizers and legume nodulating bacteria (LNB) [3]. Although several studies have investigated the production of secondary metabolites of interest in *Artemisia annua* plants in different cultivation systems, only a few of them have studied the effect of microbial biofertilizers on these plants. The objective of this work was to evaluate the efficiency of microbial biofertilizers associated with a forage legume to improve the biomass and quality of *A. annua.* More specifically (i) evaluate the impact of fodder peanuts and microbial biofertilizers on the parameters of growth, development and biomass yield in *A. annua*, (ii) analyze the major phytochemical compounds of *A. annua,* (iii) determinate anti-oxidant as well as antiplasmodial activities of *Artemisia annua* extracts biofertilized or not.

Materials and methods

Plant Material

Seeds of *A. annua* were grown on a non-sterilized soil substrate using non-randomized bloc design. Two standard treatments were established: control (C) and forage peanut (Arachis pintoi: Ara) alone[4]. With the

latter one we made some combinations: Ara + special organic matter (MOS), Ara + Biochar (Bio) and finally a combination of Ara + mycorrhizal fungi (CMA) + endophyte bacteria (End) as previously described[5, 6, 7]. Infusion was made by steeping 5g of dried leaves and stems in 1L of boiled water for 15 minutes. Whatman paper no. 3 was used to filter. The resulting filtrate was evaporated at 45°C in an oven prior for next experiences.

Biochemical analysis

Chlorophyll a and chlorophyll b were measured at wavelengths 645 and 655 nm. Carotenoids were measured at 480. Each sample measurement was performed in three replicates and the pigment content was calculated as previously described by Bulda et al.[8]; Total polyphenols were determined as described by Kouoh et al[9]. Briefly, Gallic acid was used as a standard. The polyphenols content were expressed in mg gallic acid equivalent/g of Dry Matter (mgEqAG/ g of DM). Total flavonoid determination was made as described by Kouoh et al[9]. Quercetin was used as the standard. The flavonoid content was deduced from the calibration line and expressed in mgEqQ/g of DM as described [9]. The total antioxidant capacity (TAS) of the extracts was determined based on the reduction of molybdenum, in the form of molybdate ion M_0O4^{2-}, to molybdenum M_0O^{2+} as previously described[10]. Sulfhydryl groups, or thiols, were determined using spectrophotometric assays, with Ellman's reagent (DTNB - 5,5′-dithio-bis-(2-nitrobenzoic acid)), producing a yellow product measured at 412 nm. The reduction of 5,5'-dithio-bis-2-nitrobenzoic acid (Ellman's reagent, DTNB) by the (-SH) groups of glutathione produces 2-nitro-5-mercapturic acid. This is a yellow colored complex that absorbs at 412 nm[11].

In Vitro Antiplasmodial Activity

The Trager technique[12] was used with slight modification as previously described[13]. Briefly, in a humidified incubator consisting of N (92%), CO_2 (5%), and O_2 (3%), fresh human group O^+ red blood cells at 4% hematocrit in complete RPMI medium ((Gibco, UK) cells were used to culture the chloroquine-sensitive *Plasmodium falciparum* strain 3D7 and the multiresistant *Plasmodium falciparum* strain Dd2. The medium was supplemented with 25 mM HEPES (Gibco, *UK), 0.50% Albumax I (Gibco, USA), 0.1 mM hypoxanthine (Gibco, USA),, and 20* µg/mL *gentamicin (Gibco, China)). The temperature of incubation was 37°C. T*he parasite were synchronized at the ring stage before testing antiplasmodial activity. This was done by treating them with 5% (*w*/*v*) sorbitol for 10 minutes as previously described[14]. The *in vitro* antiplasmodial activity was evaluated according to the method described by Smilkstein et al.[15]. Briefly, a 96-well microplate titer with 90 μL of the parasite suspension at the ring stage of 2% parasitemia and 1% hematocrit was added 10 μL of the various concentrations of extracts, artemisinin, and chloroquine. Then, plates were incubated for 72 hours at 37°C in a CO_2 incubator. This experiment was done in triplicates and the final plant extract concentration ranged from 0.01258 to 200 μg/ml. Prior to 1 hour of incubation in the darkness, 100 μL of SYBR Green was added into each well. At an excitation and emission wavelength of 485 and 538 nm respectively, the result of the antiplasmodial activity was read using an ELISA fluorescence microplate reader (Tecan Infinite M200). Duncan's test allowed the means to be compared with each other at the 5% threshold (risk of error).

RESULTS AND DISCUSSION

Forage peanut and biofertilizers increased biomass yield in *A. annua*. Arachis pintoï (Ara) combined to special organic matter (MOS) is the most efficient treatment (Table 1). However beneficial microorganisms associated with forage peanut may produce bioactive metabolites by acting as signals that activate the expression of genes involved in their biosynthesis[16], increasing the activity of antioxidant enzymes such as superoxide dismutase, catalase and peroxidase (Table 2). It's also known that microorganisms associated with forage peanut can improve the absorption of nutrients favorable to the good growth of the Artemisia plant[3].

Table 1: Influence of peanut and biofertilizers on biomass yield in A. annua. Values in the same column followed by the same letter are not significantly different at the 5% threshold.

Treatments	Fresh aerial biomass (g/plant)	Fresh root biomass (g/plant)	Dry aerial biomass (g/plant)
T0	6.86 ± 0.26^{a}	3.05 ± 0.04^{a}	2.03 ± 0.05^{a}
Ara	8.08 ± 0.30^{b}	4.36 ± 0.04^{b}	3.67 ± 0.11^{b}
Ara+MOS	9.12 ± 0.03^{b}	4.99 ± 0.0.03^{c}	4.26 ± 0.04^{d}
Ara+biochar	8.89 ± 0.10^{b}	4.86 ± 0.05^{d}	4.08 ± 0.04^{d}
Ara +CMA+End	8.43 ± 0.23^{b}	4.54 ± 0.07^{c}	3.90 ± 0.04^{c}

We also noticed an impact of fodder peanut and microbial biofertilizers on photosynthetic pigment, sulfur compound and tripeptide contents (Table 2*)*.

Table 2: Effect of fodder peanut and microbial biofertilizers on photosynthetic pigment, sulfur compound and tripeptide content. Values in the same column followed by the same letter are not significantly different at the 5% threshold. (Ara): fodder peanut; (MOS): Special Organic Matter; (Bio): Biochar; (CMA): Mycorrhizal Fungi; (End): Endophyte bacteria.

Treatments	Chlorophyll content (mg/l)	Carotenoid content (mg/l)	Glutathione content (µmol/gE)	Teneur en Thiols totaux (mmol/gE)
T0	2.58 ± 0.00^{c}	0.56 ± 0.00^{d}	0.09 ± 0.01^{a}	0.22±0.00^{a}
Arachide	1.92 ± 0.00^{b}	0.78 ± 0.00^{f}	0.26±0.00^{b}	0.34 ± 0.00^{c}
Ara+MOS	3.66 ± 0.00^{f}	0.53 ± 0.00^{c}	0.24±0.01^{ab}	0.27 ± 0.02^{b}
Ara+biochar	1.68 ± 0.00^{a}	0.35 ± 0.00^{a}	0.27±0.01^{b}	0.52 ± 0.00^{d}
A+CMA+End	2.89 ± 0.00^{d}	0.48± 0.04^{b}	0.31±0.01^{c}	0.34 ± 0.00^{c}

Fodder peanut alone or combined with microbial biofertilizers increases biomass yield in *Artemisia annua* and stimulates more antioxidant power, flavonoids, total polyphenols, total thiols and glutathione while organic matter has weaker effects on these parameters. For all these raisons, we have chosen extracts of *A annua* cultivated without biofertilizer and with "Ara+CMA+End" to see what could be the effect of microbial biofertilizers associated with fodder peanut on Plasmodium falciparum. Hence, in Table 3, cultivating *A. annua* in the presence of "Ara+CMA+End" inhibited Plasmodium falciparum Dd2 (*PfDd2)* and 3D7 (*Pf3D7)* strains. Moreover, we had 50% inhibitory effect of PfDd2. Microbial biofertilizers, including "Ara+CMA+End" associated with fodder peanut enhance plant health, nutrient uptake, and soil quality and strengthen agricultural sustainability[17]. This study provide an evidence that biofertilizers applied to fodder peanut could kill or inhibit the malaria-causing *Plasmodium falciparum* parasite.

Table 3: IC_{50} determination in strainds Pf3D7 and PfDd2. AnY = biofertilized Artemisia annua; AnB= none biofertilized *Artemisia annua*; M±ET= Mean ± standard deviation.

Parameters / Samples		CI_{50} M±ET (µg/mL)	
		PfDd2	***Pf3D7***
Artemisia Annua aqueous extracts	*AnY*	31±2,05	34,14 ± 2,71
	AnB	60,68± 13,07	47,69 ± 6,34
Positive Controls	Artemisinin	0,025 ± 0,005	0,035 ± 1E-05
	Chloroquine	0,733 ± 0,09	0,045 ± 0,003

CONCLUSION

Integrated soil fertility management (ISFM) involving fodder peanut and beneficial microorganisms (mycorrhizal fungi and endophyte bacteria) may be the best option to provide the highest bioactive molecules content with the best anti-oxidant and antiplasmodial capacity in *A. annua*. At least, ISFM improve Artemisia annua biomass production for better active therapeutic ingredients.

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Competing interests : The authors declare that they have no personal relationships or known competing financial interests that could have influenced the work reported in this paper.

Author's contributions

JJ Alladoum Kadounia performed the analyses, processed the data and drafted the manuscript.

J O Kuamou Nzoutap collected the samples and performed the analyses

Ferdinand Kouoh Elombo conceived the study, contributed to data processing, analyses and manuscript writing.

M Mbiandjeu Tchoumke performed the analyses

D Nwaga contributed to manuscript writing.

All authors read and approved the final manuscript.

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