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**ORIGINAL PAPER**

# EFFECT OF THE PROANTHOCYANIDIN FRACTION FROM *MEDEMIA ARGUN* ON THE *IN VITRO* GROWTH AND ACTIVITY OF SELECTED SOIL MICROORGANISMS*

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**Abstract**

Many substances of plant origin, e.g. alkyloresorcinols, benzoxazinoids, essential oils, isothiocyanates or saponins, have been tested as potential antimicrobial agents to control various pathogenic microorganisms. *Medemia agrun* is a mysterious, not well-known species of the fan palm from the Nubian Desert Oases of Southern Egypt and Northern Sudan. Nuts of *M. argun* have been found to be rich (about 5%) in proanthocyanidins, consisting of afzelechin, catechin and gallocatechin as the main subunits of these polyphenolic compounds. The aim of this work was to assess effects of the proanthocyanidin (PAC) fraction obtained from *M. agrun* nuts on the *in vitro* growth and activity of two soil-borne fungal pathogens of cereals (*Gaeumannomyces graminis* var. *tritici* and *Cephalosporium gramineum*) and a beneficial soil bacterium, *Azotobacter chroococcum*, known to fix atmospheric nitrogen. The fungi were grown on PDA medium and *A. chroococcum* was cultured on Burk's N-free medium supplemented with different concentrations of the PAC fraction. This fraction applied at the highest concentration tested (200 μg $cm^{-3}$) did not affect the mycelial growth of the fungus *Cephalosporium gramineum* on PDA medium but significantly reduced (by 20%) that of *Gaeumannomyces graminis* var. *tritici.* Proliferation and $N_2$-fixation by *Azotobacter chroococcum* in N-free liquid medium containing 500 μg $cm^{-3}$ of the PAC fraction were more intensive than those in the medium with an equivalent concentration of glucose. It has been shown for the first time that this bacterium can use the PAC fraction as the only source of C and energy for $N_2$-fixation.

**Keywords:** *Medemia argun*, nuts, proanthocyanidins, biological activity, soil microorganisms, nitrogen fixation.

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# INTRODUCTION

A new species has been recently added to the numerous plants examined for various aspects of the biological activity demonstrated by their chemical constituents. It is *Medemia argun* (Syns. *Hyphaene argun, Medemia abiadensis, Areca abiadensis*: Coryphoideae: Borasseae: Hyphaeninae*)*, a mysterious and not well-known species of the fan palm from the Nubian Desert Oases of Southern Egypt and Northern Sudan (Boulos 1968, gibbons, spanner 1996, Ibrahim, Baker 2009, Hamed et al. 2012). Gas chromatography (GC) and GC -MS analyses of the essential oil (EO) fraction from fruits and headspace (HS) of the seeds and fleshy mesocarps of this palm have shown that sesquiterpene derivatives were the main volatile compounds in the EO and in the HS (45.0 and 64.0%, respectively), while oxygenated hydrocarbon derivatives were the main constituents in the HS obtained from fleshy mesocarps (96.5%) (Hamed et al. 2012). Results of these analyses indicate that differences in the chemical composition of the headspace obtained from seeds and mesocarps of *M. argun* can be correlated with the different roles that these constituents play in the prevention of dehydration of *M. argun* fruits in the desert region from which the plant was collected. Nuts of *M. argun* have been found to be rich (about 5%) in proanthocyanidins (PACs), consisting of afzelechin, catechin and gallocatechin as the main subunits of these polyphenolic compounds (Hamed et al. 2014). Studies on the biological activity of the PAC fraction extracted from *M. agrun* nuts have shown that they can be considered as important antioxidants and promising protecting factors against oxidative/nitrative damages of blood platelet and plasma components associated with different diseases (Morel et al. 2014).

Many substances of plant origin, e.g. alkyloresorcinols, benzoxazinoids, isothiocyanates or saponins, have been tested as potential antimicrobial agents to control various pathogenic microorganisms (Scalbert 1991, Angus et al. 1994, Zarnowski et al. 1999, Saniewska et al. 2003, Martyniuk et al. 2006). Thus far, the antimicrobial activity of *M. argun* PACs has not been examined. In this work we were particularly interested in assessing effects of the PAC fraction obtained from *M. agrun* nuts on the *in vitro* growth and activity of two soil-borne fungal pathogens of cereals (*Gaeumannomyces graminis* var. *tritici* and *Cephalosporium gramineum*) and a beneficial soil bacterium, *Azotobacter chroococcum*, known to fix atmospheric nitrogen.

# MATERIAL AND METHODS

## Plant material

Fresh fruits of *M. argun* were collected from the Dungul Oasis (Aswan, Egypt) and dried in dark at room temperature. The plant material was iden-

tified (HAMED et al. 2012) and the voucher specimens were deposited in the Botany Department Herbarium, Aswan Faculty of Science (Egypt).

**Extraction and isolation of proanthocyanidin fraction**

Plant material, extraction and isolation of the proanthocyanidin fraction (PAC fraction) were the same as described in HAMED et al. (2014). Shortly, dried powdered nuts of *M. argun* (450 g) were first extracted with hexane and then with 80% $CH_3OH$. The aqueous methanolic extract was concentrated under reduced pressure to give 50 g of crude extract (*ca* 11% crude extract). An amount of ten grams of the extract was loaded onto a C18 column (4 × 50 cm, 40-63 µm LiChroprep, Millipore Corp, Bedford) and eluted with 50% (v/v) of methanol to give 7 g of the PAC fraction. In order to determine the amount of proanthocyanidins, 15 mg of the PAC fraction were dissolved in 0.25 $cm^3$ of 60% (v/v) $CH_3OH$ and then 1.50 ml of vanillin reagent (4% v/v in methanol) and 0.75 $cm^3$ of concentrated (37%) HCl were added (BROADHURST, JONES 1978). The reaction was carried out in glass cuvettes at the temperature of 22°C and analyzed in a Hewlett-Packard 6453A spectrophotometer, at a 500 nm wavelength. The (ţ)-catechin was used as a standard for the standard curve preparation. The dried PAC fraction from *M. argun* nuts contained 636.88 mg $g^{-1}$ of proanthocyanidins (as an equivalent of (ţ)-catechin).

**Microbial cultures**

The Microbial Culture Collection belonging to the Institute of Soil Science and Plant Cultivation in Pulawy was the source of all microorganisms used in this study. The fungal species: *Cephalosporium gramineum* Nisikado & Ikata and *Gaeumannomyces graminis* (Sacc,) Arx & Oliv. var. *tritici* Walker were obtained from diseased winter wheat plants (MARTYNIUK 1995) and the bacterium *Azotobacter chroococcum* Beij. was isolated from soil in Poland (MARTYNIUK et al. 2013).

**Effect of PAC fraction on fungal growth**

Stock solution containing 5% of the PAC fraction in 50% $C_2H_5OH$ was prepared. Appropriate volumes were taken from the stock solution and added to 200 $cm^3$ portions of autoclaved potato-dextrose agar (PDA, Difco) to achieve concentrations, ranging from 50 µg to 200 µg of PAC fraction per 1 $cm^3$ of the medium. All portions of the media, including the control one, contained the same volume (0.8 $cm^3$) of $C_2H_5OH$. The media in aliquots of 15 $cm^3$ were poured into Petri plates (90 mm diameter) and allowed to solidify. Next day, the plates were inoculated in the centre with a 5 mm disc cut from PDA-cultures of *C. gramineum* or *G. graminis* var. *tritici*. After 72 h, 120 h and 168 h of incubation at 22°C, colony diameters were measured. There were three replicated plates per each concentration tested and the experiment was repeated twice.

**Effect of PAC fraction on A. chroococcum**

Two experiments were performed to assess effects of the PAC fraction on the proliferation and $N_2$-fixation by *A. chroococcum*, and in both Burk's N-free liquid medium was used to culture the bacterium. The medium had the following composition (g $dm^{-3}$): glucose – 10, $K_2HPO_4$ – 0.8, $KH_2PO_4$ – 0.2, Mg $SO_4$ $7H_2O$, – 0.2, NaCl – 0.2, $CaCl_2$ – 0.2, traces of Fe, Mn and Mo. In the first experiment, 20 $cm^3$ portions of this medium were poured into 50 $cm^3$ Erlenmeyer flasks with cotton plugs and autoclaved for 20 min at 121°C. After autoclaving, appropriate volumes of stock solution of the PAC fraction (0.04 $cm^3$ or 0.2 $cm^3$) were added to three replicated flasks so as to obtain the following concentrations of the PAC fraction in the medium: 0 – control treatment, 100 μg $cm^{-3}$ and 500 μg $cm^{-3}$. All treatments contained the same volume (0.2 $cm^3$) of 50% $C_2H_5OH$. All flasks were inoculated with 1 $cm^3$ of 48 h culture of *A. chroococcum* grown in Burk's N-free medium and then incubated on a rotary shaker (100 rpm $min^{-1}$) at 25°C. Proliferation of *A. chroococcum* was monitored by measuring optical density (OD) at 550 nm of the cultures. After 24 h of incubation, intensive flocculation occurred in the cultures with the PAC fraction, making precise OD measurements impossible, therefore in the second experiment proliferation of the bacterium was assessed by inoculating Burk's agar plates with appropriate dilutions of the cultures and counting *A. chroococcum* colonies (CFUs – colony forming units) after 72 h of incubation at 25°C (Martyniuk et al. 2013). For the purpose of this experiment, liquid Burk's medium was modified to contain equivalent amounts (500 μg $cm^{-3}$) of glucose or the PAC fraction. In both experiments, 1 ml samples of the cultures were withdrawn from the flasks at various incubation times to measure amounts of nitrogen fixed by *A. chroococcum* with an automated C/N analyzer (Multi N/C 2100, Analytik Jena, Jena, Germany). One-way Anova was used to test significance of variation at $a < 0.05$.

## RESULTS AND DISCUSSION

The PAC fraction at the concentrations tested had no significant effect on the mycelial growth of the fungus *Cephalosporium gramineum* (Table 1). In the case of *Gaeumannomyces graminis* var. *tritici* (*Ggt*), the PAC fraction at the concentrations of 100 μg $cm^{-3}$ and 200 μg $cm^{-3}$ of PDA medium significantly inhibited the growth of this fungus as compared to the control after 7 days (168 h) of incubation at 22°C (Table 2). The highest concentration (200 μg $cm^{-3}$) of the PAC fraction reduced the growth of *Ggt* also after 3 and 5 days of incubation. In previous studies on the antifungal activity of other chemical constituents of plant origin, like saponins or benzoxazinoids, *C. gramineum* was also less sensitive to these compounds than *Ggt* (Martyniuk, Jurzysta 2005, Martyniuk et al. 2006, Martyniuk, Biały 2008). *Ggt* and *C. gramineum* are soil-borne fungal pathogens of cereals, which can cause

Table 1

Growth (colony diameters in mm) of *Cephalosporium gramineum* on PDA medium supplemented with different concentrations of the PAC fraction

| PAC fraction concentration (μg cm$^{-3}$) in PDA | Incubation time (h) | | |
|---|---|---|---|
| | 72 | 120 | 168 |
| 0 (Ccontrol) | 8,0*a** | 14.8*a* | 17.3*a* |
| 50 | 8.0*a* | 15. 3*a* | 18.0*a* |
| 100 | 9.0*a* | 16.7*a* | 18.3*a* |
| 200 | 9.0*a* | 16.7*a* | 18.7*a* |

* Results in columns assigned the same letter are not significantly different at $\alpha < 0.0$

Table 2

Growth (colony diameters in mm) of *Geumannomyces graminis* var. *tritici* on PDA medium supplemented with different concentrations of the PAC fraction

| PAC fraction concentration (μg cm$^{-3}$) in PDA | Incubation time (h) | | |
|---|---|---|---|
| | 72 | 120 | 168 |
| 0 (control) | 24.7*a** | 61.0*a* | 78.5*a* |
| 50 | 24.0*a* | 60.0*a* | 75.0*a* |
| 100 | 22.8*a* | 58.7*a* | 71.0*b* |
| 200 | 19.7*b* | 55.0*b* | 64.0*c* |

* Results in columns assigned the same letter are not significantly different at $\alpha < 0.05$.

serious grain yield losses when these crops are grown in monocultures or in short rotations (MARTYNIUK 1995, SCHOENY et al. 2001). Both pathogens infect plants through roots and penetrate into the host's vascular system causing root rot (take-all disease – *Ggt*) or stripes on leaves (*Cephalosporium* stripe disease), and finally premature death of whole plants (DOUHAN, MURRAY 2001, COOK 2003). Currently, no effective fungicides to control these pathogens by foliar application are available. Thus, searching for new chemicals, including those occurring naturally in plants, inhibitory to the above mentioned fungi is an important task. In this work, a weak inhibitory effect of proanthocyanidins (condensed tannins), dominating in the PAC fraction extracted from *M. agrun* nuts, was found only in the experiments with *Ggt,* indicating that this group of chemicals cannot be considered as potential antifungal compounds to control *Ggt* and *C. gramineum*. Tannins are known to have antimicrobial properties, but mostly against various bacterial species (SCALBERT 1991). It has been shown, for example, that MICs (Minimum Inhibitory Concentrations) of condensed tannins extracted from *R. apiculata* bark ranged from 1.6 mg cm$^{-3}$ to 12.5 mg cm$^{-3}$ for 13 bacterial species but none of the 12 fungal species tested in this study was inhibited at the concentration of 100 mg cm$^{-3}$ (LIM et al. 2006).

Addition of 100 μg cm$^{-3}$ or 500 μg cm$^{-3}$ of the PAC fraction to Burk's

N-free liquid medium resulted in a significant stimulation of the proliferation of *A. chroococcum* and nitrogen fixation by this bacterium after 24 h of incubation (Table 3, Experiment I). Low amounts of N detected in the cultures at the beginning of the experiment (incubation time 0) originated probably from *A. chroococcum* inoculum (1 $cm^3$) added at the onset of the incubation. In this experiment, multiplication of the bacterium was monitored by measuring $OD_{550}$ of the cultures, but after 24 h of incubation intensive flocculation occurred in the cultures with the PAC fraction, making further OD measurements impossible and for this reason the experiment was terminated after 24 h of incubation. In the second experiment (Table 4), proliferation of the bacterium was assessed by counting of *A. chroococcum* colonies (CFUs – colony forming units) on Burk's agar plates following inoculation with appropriate dilutions of the cultures. In this experiment, *A. chroococcum* was cultured in two liquid media: Burk's medium containing 500 μg $cm^{-3}$ of glucose (control) or 500 μg $cm^{-3}$ of the PAC fraction as the only sources of C and energy. The proliferation of *A. chroococcum* in both media was similar at the beginning of the incubation time, but after 48 h of incubation it was significantly more intensive in the medium containing the PAC fraction than in the control medium with glucose (Table 4, Experiment II). Interestingly, the medium with the fraction contained substantial amounts of fixed nitrogen

Table 3

Proliferation ($OD_{550}$) of *Azotobacter chroococcum* and $N_2$-fixation (N content in μg $cm^{-3}$) by the bacterium in Burk's N-free liquid medium as influenced by the PAC fraction concentration

| PAC fraction concentration (μg $cm^{-3}$) | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | 24 | | 48 | |
| | proliferation | N cont. | proliferation | N cont. | proliferation | N cont. |
| 0 (control) | 0.022*a** | 6.3*a* | 0.14*a* | 53.4*a* | n.d. | n.d. |
| 100 | 0.025*a* | 6.4*a* | 0.19*b* | 65.9*b* | n.d. | n.d. |
| 500 | 0.030*a* | 6.8*a* | 0. 26*c* | 72.6*c* | n.d. | n.d. |

* Results in columns assigned the same letter are not significantly different at $\alpha < 0.05$; n.d. – not determined

Table 4

Proliferation (CFUs numbers $cm^{-3}$) of *Azotobacter chroococcum* and $N_2$-fixation (N content in μg $cm^{-3}$) by the bacterium in Burk's liquid medium containing 500 μg $cm^{-3}$ of glucose or the PAC fraction

| Burk's medium with: | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | 24 | | 48 | |
| | proliferation | N cont. | proliferation | N cont. | proliferation | N cont. |
| Glucose | $1.7 \cdot 10^3$*a** | 5.1*a* | $1.9 \cdot 10^7$*a* | 55.0*a* | $3.1 \cdot 10^8$*a* | 184.0*a* |
| PAC fraction | $2.0 \cdot 10^3$*a* | 6.7*a* | $2.5 \cdot 10^7$*a* | 74.5*b* | $1.5 \cdot 10^9$*b* | 242.5*b* |

*Results in columns assigned the same letter are not significantly different at $\alpha < 0.05$.

and these amounts measured after 24 h and 48 h of incubation were even significantly higher than those in the glucose medium (Table 4). To the best of our knowledge, this is the first report showing that the PAC fraction (condensed tannins) can be utilized by *A. chroococcum* as the only source of C and energy for growth and $N_2$ fixation. Earlier, it had been demonstrated that *Azotobacter vinelandi* could grow on a medium containing condensed tannins (Lim et al. 2006) and *Azotobacter* sp. SSB81 on a medium supplemented with tannic acid as the only source of C and energy (Gauri et al. 2012), but $N_2$ fixation by the bacteria had not been elucidated.

## CONCLUSIONS

Proanthocyanidins (the PAC fraction) extracted from *M. agrun* nuts possess weak antifungal activity. These compounds applied at the highest concentration tested (200 μg $cm^{-3}$) did not affect significantly the mycelial growth of the fungus *Cephalosporium gramineum* on PDA medium and reduced (by 20%) that of *Gaeumannomyces graminis* var. *tritici.* The PAC fraction at the concentration 500 μg $cm^{-3}$ beneficially affected the proliferation *Azotobacter chroococcum* in N-free liquid medium and, interestingly, this fraction could also serve as a source of C and energy for $N_2$-fixation by this bacterium.

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