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Fungal Tannase Degrading Condensed Tannins of *Camellia sinensis* and Measure of the Enzyme Activity on Quebracho

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Abstract: Condensed tannins are well known for their antinutritional effect because of their ability to combine different nutritive molecules and principally proteins inhibiting thus their assimilation. The current study is based on the separation and quantification of condensed tannins extracted from *Camellia sinensis*; then, on the isolation of ground moulds which were capable of degrading these molecules. The measure of the enzyme activity was carried out on Quebracho. The results showed that the leaves of *C. sinensis* contained mainly oligomeric tannins identified as procyanidins. Among twenty three moulds isolated from ground, only three had a tannase which degraded these procyanidins. *Aspergillus awamori* was the best tannase producer with an enzyme activity of 21,4 U/ ml on Quebracho.

Key words: Tannins · Extraction · Quantification · Tannase · Production · Activity

INTRODUCTION

Tannins are natural aromatic compounds synthesized by plants during their secondary metabolism. Once present in our nutrition, they procure prevention against different diseases: infectious [1, 2], cardiovascular [3, 4], hormone-depending and cancerous diseases [5, 6]. However, the presence of condensed tannins in big amount in foodstuff causes serious malnutrition (antinutritional effect) [7]. In fact, these compounds are able to combine proteins and other nutritive molecules which become inaccessible for digestive enzymes. Also, tannins are able to inhibit the action of these enzymes which are proteins too [8, 9].

Camellia sinensis grow on the Eastern Algerian Sahara. Its leaves are much utilized in the Algerian alimentation.

The aim of this investigation was to extract and quantify condensed tannins of *C. sinensis*. Then, to isolate ground moulds capable of synthesizing tannase which degrade both hydrolysable and condensed tannins. The measure of the tannase activity was realized on Quebracho which is the reference condensed tannin.

MATERIALS AND METHODS

This investigation was realized on the leaves of *C. sinensis*. The plant was harvested from the Illizi Basin (Eastern Algerian Sahara). It was identified as the voucher no. wdr/tspf 200 in the botanical department of the university Mentouri, Constantine, Algeria.

Extraction and Solvent Partitions of Condensed Tannins: Tannins were extracted from the plant material by maceration in aqueous acetone (70 %) [10]. The obtained extract was filtered and evaporated using a rotary evaporator and freeze dryer, respectively to give the crude dried extract.

The Compounds Present in the Acetonic Extract Were Separated According to Their Structure by a Series of Solvent Partitions: First partition was carried out with Petroleum Ether in order to discard out the non phenolic compounds. This phase was thrown away. The second partition was done with Diethylic Ether to extract the monomeric tannins. The third and the fourth partitions were carried out with the Ethyl Acetate and the Methyl-Ethyl-Cetone (MEC) which got up the dimeric and

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oligomeric tannins respectively. The fifth partition was done with n-Butanol and it extracted the polymeric tannins. So, the remaining aqueous phase contained only condensed tannins which were not extracted and nor separated.

Ultraviolet-Visible (UV-Vis) spectrums were recorded for each phase in order to confirm that they contained condensed tannins. Then, each phase was divided into two: First part was used to tannins quantification and the second part to search for moulds producing tannase.

Condensed Tannins Quantification and Identification: The tannins quantification was effectuated for each phase by the n-Butanol / HCl method [11]. The UV-Vis spectrums recorded after this operation allowed the identification of the present tannins and thus their initial structure.

Research and Isolation of Moulds Capable of Degrading Condensed Tannins: The study was carried out on a ground sample taken around cultures of *C. sinensis*. This localization was chosen because of the big content of condensed tannins revealed in the used leaves. Moulds culture and isolation were realized on Malt Agar Blakeskee (MAB) and Sabouraud (S) mediums. The incubation was occurred at 25°C during 72 h.

The identification of the isolated species was done by the traditional methods: microscopic observation and growth evaluation on Malt Agar Blakeskee, Czapeck agar and/or Sabouraud agar mediums.

Two other culture mediums were prepared to select moulds able to produce tannase. The first one contained only tannic acid as a carbon source (TA medium). And the second one contained only polymeric tannins extracted with the n-Butanol partition (tannins revealed as those present in the highest amount) (PT medium).

- TA medium (per liter): tannic acid 10g, $NaNO_3$: 3g, KH_2PO_4 : 1g, $MgSO_4.7H_2O$: 00,5g, KCl: 0,5g, $FeSO_4$: 0,07g, Agar: 30g, pH: 5,5.

- PT medium (per liter): Polymeric tannins extracted with n-Butanol: 10g, $NaNO_3$: 3g, KH_2PO_4 : 1g, $MgSO_4.7H_2O$: 00,5g, KCl: 0,5g, $FeSO_4$: 0,07g, Agar: 30g, pH: 5,5

The previous isolated moulds were cultivated on these mediums and incubated as 25°C during 72 h.

Production of Extracellular Tannase in Submerged Culture: This production was done only for moulds which had grown in the PT medium, so for those which were able to produce tannase. The spore inoculum was prepared according to the methodology described by Costa *et al.* [12]. Spore suspensions (1 ml with concentration of $5x10^9$ spores) were inoculated in Erlenmeyer flask containing 25 ml of a specific medium constituted with KH_2PO_4 1,0 g ; $Mg\ SO_4.7H_2O$ 2,0 g ; $CaCl_2$ 1,0 g ; NH_4Cl 3,0 g ; yeast extract 1 g and Quebracho 3 g.

Moulds were cultivated during six days in an incubator shaker at 140 rpm and 28°C. Biomass was separated by filtration through the Whatman n° 1 filter paper and the separated cells were then tested for their extracellular tannase activity [12].

Measure of the Enzyme Activity: The tannase activity was estimated by the rhodanin method [13]. The appeared pink color was observed at 520 nm using a spectrophotometer (Shimadzu UV-160A, Japan). The activity was expressed in International units per milliliter.

RESULTS

After extraction and separation of tannins, the UV-Vis spectral analysis realized for each phase gave spectrums characteristic of condensed tannins [14]: each phase spectrum had only one peak in the vicinity of 278 and 280 nm. The industrial catechin used as a control also gave a spectrum with only one peak exactly at 280 nm. These results confirmed that the five phases contained effectively monomer, dimers, oligomers and polymers of catechin.

The quantification by the n-Butanol / HCl method allowed the classification of the phases according to their absorbance and thus to their concentration, like this: n-Butanol > MEC > Ethyl Acetate > Diethylic Ether > H_2O (Figure 1).

The spectral analysis realized for phases after the quantification gave for each one a spectrum with two peaks (at 274 nm and 546 nm respectively) which were characteristic to anthocyans and exactly to cyanidins [14]. So, the extracted tannins were in majority polymeric procyanidins extracted with the n-Butanol.

From the ground surrounding the plant cultures, twenty three moulds were isolated and identified. Among them, eight were identified as *Aspergillus* species: *A. foetidus, A. versicolor, A. aculeatus, A. terreus, A. parasiticus, A. tamarii, A. puniceus* and *A. awamori*.

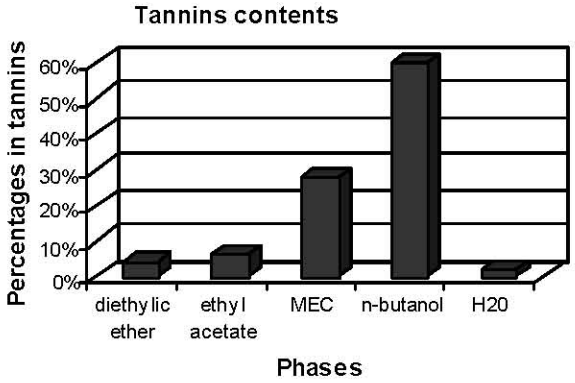

Fig. 1: Tannins contents of the five tannic phases

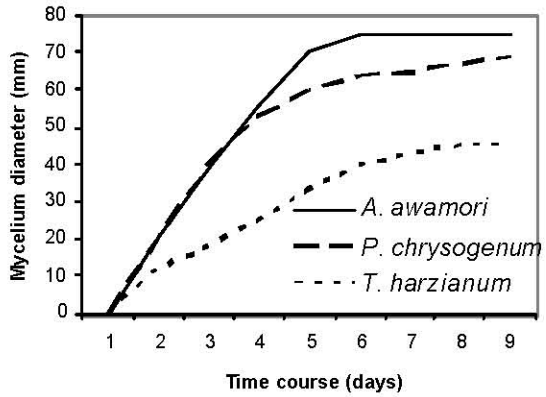

Fig. 2: Difference of the growth speeds on the PT medium

Nine other species were identified as *Penicillium* species: *P. griseofulvum, P. rugulosum, P. chrysogenum, P. variable, P. pinophilum, P. marneffei, P. lilacinum, P. bilaiae* and *P. funiculosum.* And three others as *Fusarium* species: *F. sporotrichioides, F. acuminatum* and *F. proliferatum.* The three last moulds were identified as *Geotrichum clavatum, G. capitatum* and *Trichoderma harzianum.*

Once Cultivated on TA Medium, Only Seven Species Had Grown: *A. foetidus, A. aculeatus, A. awamori, P. chrysogenum, P. funiculosum, A. tamarii* and *T. harzianum.* So, these moulds had produced a tannase degrading hydrolysable tannins.

A. awamori, P. chrysogenum and *T. harzianum* showed a speed growth similarly to their culture on the MAB medium. This indicated that these species used tannic acid as easily as the common glucose source present in the MAB medium. Contrary to some others, particularly *A. foetidus* and *P. pinophilum* which had a lower growth speed on the TA medium. These species poorly degraded tannic acid.

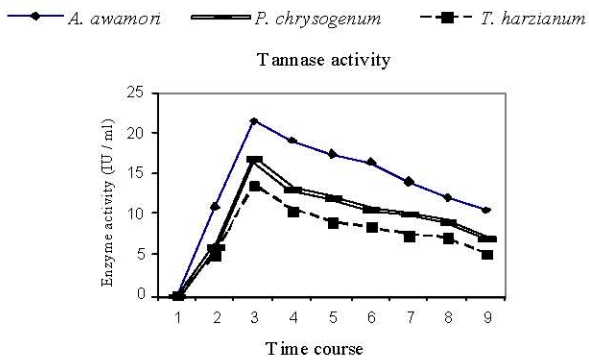

Fig. 3: Difference of the tannase activities on Quebracho

On the PT Medium, Only Three Moulds Had Grown: *A. awamori, P. chrysogenum* and *T. harzianum.* So, these species had a tannase which degraded procyanidins as well as tannic acid. *A. awamori* had shown the biggest diameters of the mycelium. It showed practically the same growth speeds on the three used mediums showing thus that it degraded procyanidins as easily as the glucose and the tannic acid.

P. chrysogenum gave a lowest growth on the PT medium compared to the TA one. This indicated that its tannase degraded procyanidins with more difficulty than tannic acid. But, *T. harzianum* had the slowest growth speed on the PT medium: its tannase degraded procyanidins with more difficulty than *P. chrysogenum* (Figure 2). So, *A. awamori* was the Best User of the Procyanidins Extracted from *C. sinensis.*

The Tannase Activity and the Biomass Production Were Determined as the Beginning of the Stationary Growth Phase: Among the three procyanidins degraders and also able to degrade condensed tannins, *A. awamori* had given the fast growth speed, followed by *P. chrysogenum* then *T. harzianum.* So, these results had shown the big potential of *A. awamori* to produce a tannase degrading condensed tannins.

In a recent study realized on different species of *Aspergillus* and *Penicillium,* it had been obtained that the highest levels of the tannase production were rather given by *A. versicolor.* But the produced tannase were only tested on tannic acid [15].

For the three moulds, the optimum of the enzyme production was obtained after two days of the culture. The highest level of production was given by *A. awamori* with a rate of 21,4 U / ml. *P. chrysogenum* and *T. harzianum* had given 16,9 and 13,5 U / ml respectively (Figure 3).

DISCUSSION

Tannins extracted from the leaves of *C. sinensis* were identified in the majority as polymeric procyanidins. Among the twenty three isolated moulds, nine were able to degrade tannic acid but only three degraded the extracted procyanidins. This indicated that the condensed tannins remained used with difficulty by the moulds.

Some previous works had also showed that the tannases of *P. chrysogenum* and *T. harzianum* degraded hydrolysable tannins like gallotanins, gallic acid and tannic acid [16, 17, 18], but no work had reported that its were also able to degrade condensed tannins.

A. awamori had shown growth speeds very similar on the three used mediums, this could be explained by the fact that this mold was an excellent tannase producer [19]. The measure of the tannase activity confirmed this information. Until now, no work had been found on the tannase production of this species for degrading condensed tannains. This investigation is the first one.

Also, it had been reported previously that the tannase was produced by *A. foetidus, A. aculeatus, A. tamarii* and *P. lilacinum* [20, 21, 22]. But this investigation clearly demonstrated that the tannases produced by these species were only able to degrade hydrolysable tannins.

Moreover, this is the first work which demonstrated that *P. funiculosum* was able to produce tannase and that the latter degraded only hydrolysable tannins too.

CONCLUSION

The leaves of *C. sinensis* principally contained procyanidins. The majority of the isolated moulds could not degrade these molecules except three. *P. chrysogenum* and *T. harzianum* degraded them with difficulty, but *A. awamori* was able to degrade these molecules with easiness. So, the latter species was considered as the best user of the condensed tannins. This was confirmed by the fact that the tannase of *A. awamori* had given the higher activity on the Quebracho which was the condensed tannin reference.

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