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# Apyap1 affects aflatoxin biosynthesis during *Aspergillus parasiticus* growth in maize seeds

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## Abstract

In fungal cells grown in synthetic media we have demonstrated that the *Apyap1* gene is implicated in the modulation of aflatoxin biosynthesis following the perturbation of the redox balance. In this study, we suggest that an association between oxidative stress and aflatoxin biosynthesis occurs also in maize seeds. We used Δ*Apyap1*, a strain in which the gene *Apyap1* was disrupted, to verify whether this oxidative stress related transcription factor, by affecting cell redox balance, can have a role in the modulation of aflatoxin synthesis. The amount of hydroperoxides (ROOH) produced by wild type (WT) and Δ*Apyap1,* both grown in potato dextrose broth, was assayed in the filtrate. In maize seeds (30 g), which were inoculated with WT and Δ*Apyap1*conidia, and incubated at 30°C for 15 days, we analysed lipoxygenase activity (LOX), lipoperoxides (LOOH) production, fungal growth and aflatoxin biosynthesis. It was observed that Δ*Apyap1* releases more hydroperoxides in the culture media and more aflatoxins in seeds, likely through a stronger stimulation of LOX, which, in turn led to a greater LOOH production in the seeds. On the basis of our results, we formulated hypothesis regarding strategies to control aflatoxin synthesis.

**Keywords:** ***Apyap1, oxidative stress, Aspergillus parasiticus, maize seeds, aflatoxin biosynthesis***

## Introduction

A more detailed knowledge of the organization, regulation, and expression of genes linked to aflatoxin biosynthesis is important to detect early these mycotoxins and the mycotoxigenic fungi. Additionally, it allows finding rapid prevention strategies to control their presence in different food sources. However, the molecular approach must advantage of information concerning those events that influence the fungal physiology in relation to aflatoxin production.

In the eighties we have demonstrated how lipoperoxidation can intervene in modulating the aflatoxin biosynthesis. This evidence was brought up both *in vitro* studies and in different seeds (Fabbri *et al*.,1983; Fanelli *et al*.,1989). In those systems, various antioxidants and free radicals scavengers affected the aflatoxin production, in different ways (Fanelli *et al*.,1985). Jayashreee and Subramanyam, (2000) further brought up oxidative stress, in particular the increase of lipid peroxidation and free radical generation, as a prerequisite for aflatoxin biosynthesis by *Aspergillus parasiticus*. Other researches have suggested the relationship between aflatoxin production and sporulation process (Guzman De Pena and Ruiz-Herrera, 1997). The formation of conidia is an important factor for the infection onset and for the fungal colony formation on different synthetic and natural media. Calvo *et al*., (1999, 2001) pointed out the sporogenic effect of polyunsaturated fatty acid and their lipoperoxides (LOOH), during the development of *Aspergillus* spp. Hydroxylated derivatives of oleic, linoleic and linonenic acids, whose formation is directed by putative dioxygenase, modulate sporulation (Tsitsigiannis *et al*., 2004). Such factors seemed to regulate the ratio of asexual to sexual development in *A. nidulans* (Champe and El Zayat, 1989).

9-S and 13-S hydroperoxy-linoleic acids (9-S and 13-S HPODE), which are by-products of plant lipoxygenase, affect spore production in *A. nidulans, A. parasiticus* and *A. flavus* (Calvo *et al*., 1999). In particular 9-S-HPODE enhanced spore production and aflatoxin biosynthesis. On the contrary, 13-S-HPODE inhibited both sexual spores and aflatoxin biosynthesis. Furthermore, these

compounds were able to play also a role in the formation of cleistothecia (*A. nidulans*) and sclerotia (*A. flavus*) (Burow *et al*., 1997). The biosynthesis of aflatoxins, like other compounds in the cell, is controlled by a signal transduction cascade that involves a G protein (FadA), a protein kinase (PKA) and other factors regulating the expression of AflR, the aflatoxin cluster regulator.

Compounds derived from ligninolitic fungi (*Lentinula edodes* and *Trametes versicolor*) are useful to inhibit aflatoxin production as reported by Fanelli *et al*., (2000) and Zjalic *et al*., (2006). These findings land basis for innovating biological approaches to control aflatoxin biosynthesis and fungal growth. Antimicrobials and antioxidants compounds such as thioproline, mannitol, α- and β-glucans, caffeic acid and derivatives, have been detected in these basidiomycetes and plants and might be of significance in modulating oxidative stress and aflatoxin formation (Kurashima *et al*., 1990; Kim et al., 2004). In *A. parasiticus* we have observed (Reverberi *et al*., 2006) that the culture filtrates derived from some basidiomycetes affect transcription factors (such as Apyap1) regulating the expression of genes involved in the removal of reactive oxygen species (ROS), such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) enzymatic activities (Harschmann *et al*., 1988). In budding yeasts, Yap1, a transcription factor homologue of AP-1, is involved in the defence response against oxidative stress (Estruch, 2000). Some other regulatory proteins such as Skn7 and Hsf 1-2 are also involved (He *et al*., 2003; Moye-Rowley, 2003). In a culture media conducive for aflatoxin biosynthesis, an increase of lipoperoxides appears in the trophophase followed by an enhancement of Apyap1 mRNA expression. Coincidentally, a rapid increase of SOD, CAT and GPX enzymatic activities accompanied by a decrease of aflatoxin biosynthesis, occurred. In the following phase, there is a decline of antioxidant enzymatic activities, while biosynthesis of lipoperoxides and the aflatoxin occurs (Reverberi *et al*., 2006). From these results it emerges that the transcription factor Apyap1 regulates the natural antioxidant defence mechanisms of the aflatoxigenic fungi modulating the toxin biosynthesis.

In this paper the behaviour of two *Aspergillus* strains inoculated in maize seeds has been showed. The first strain has a functioning *Apyap1* gene, whereas the second one presents a gene inactivated

by homologous recombination. The results obtained suggested that the lipoperoxides produced by maize seeds can affect aflatoxin biosynthesis in both *Aspergillus* strain likely by Apyap1 pathway.

## Materials and methods

*Plant and fungal material and culture conditions*

The fungal strains used were *Aspergillus parasiticus* (NRRL 2999) wild type aflatoxins ($B_1$, $B_2$, $G_1$, $G_2$) producer and a Δ*Apyap1* mutant obtained from the same strain. The isolate was kept on PDA (Potato Dextrose Agar, Difco) for 7 days at incubation temperature of 30°C before the use. The inoculum of *A. parasiticus* was performed with conidia suspension ($5x10^6$/ml in sterile distilled water) in PDB medium The same number of conidia of the two strains was inoculated in 30 g of maize seeds, cv Cecilia, moistened at $a_w$ 0.95. To avoid interferences due to the growth of other fungi, seeds were previously sterilized with γ-rays (8 Kgy, irradiation ratio 2602 $Gy \cdot h^{-1}$. This procedure allowed obtaining micro flora sterilization and maintaining seed germination (about 97 %; data not shown). The inoculated seeds were kept in 100 ml Erlenmeyer flasks for 15 days at 30°C in the dark.

*FOX-1 assay for the determination of hydroperoxides present in the culture filtrate*

The total hydroperoxides (ROOH) produced by *A. parasiticus* grown in PDB were analyzed by spectrophotometric assay by monitoring the oxidation of xylenol orange at 560 nm (Ferrous ion Oxidation Xylenol orange: FOX-1). The sensitivity of the method was increased according to recent modification by the use of triphenylphosphine and stabilizing the reagent at pH 1.7-1.8 as reported by Banerjee *et al*., (2003).

*Assay of LOX activity in maize seeds*

The activity of lipoxygenase (LOX, EC 1.13.11.12) was tested at different time points (0-15 days), in contaminated and not contaminated maize seeds. The assay was performed monitoring the diene conjugates formation at 234 nm as previously reported by Reverberi *et al*., (2005b).

*Analysis of 9- and 13-HODE present on maize seeds surface*

The seeds infected and not infected with WT and Δ*Apyap1* were washed in distilled sterile water, stirring the specimens, to remove mycelia and conidia. Hydroperoxyoctadecadienoic acids (HPODEs) present on the surface of maize seeds were analysed after extraction of the samples with chloroform:methanol 2:1 v/v (20 ml x 3) in presence of 100 µg of butylated hydroxytoluene (BHT) as an antioxidant. The two hydroxyoctadienoic acids (9-HODE and 13-HODE) obtained by reduction of the respective HPODEs, were analyzed by HPLC-APCI-MS as reported by Reverberi *et al*. (2006). Data were collected and analyzed using the Chemstation LC/MSD revision A.09.01 (Agilent Technologies). Authentic 9-HODE and 13-HODE were purchased from Cayman (Ann Arbor, Michigan, USA).

*Analysis of aflatoxins*

The aflatoxins ($B_1$+ $B_2$+ $G_1$+ $G_2$) analyses were performed as previously reported (Fanelli *et al.* 2000) by extracting inoculated maize seeds after homogenisation in chloroform:methanol (2:1 v/v) (60 ml x 3). The extracts were collected after filtration on anhydrous $Na_2SO_4$, concentrated under a $N_2$ stream and quantified by HPLC-UV-DAD.

*Ergosterol analysis for fungal growth determination*

Contaminated and not contaminated maize seeds were extracted with 250 ml chloroform:methanol (2:1 v/v) for 1 h in the dark, in the presence of 100 µg of buthylated hydroxytoluene as an antioxidant. After extraction, the solvents were filtered on anhydrous $Na_2SO_4$, collected and concentrated under a $N_2$ stream. Ergosterol was analysed by HPLC as reported by Fabbri *et al.* (1997), using a Supelco LC-18, 5µm (25 cm x 4,6mm) column, methanol:water (98:2 v/v) as the mobile phase, and detecting at 282 nm.

*Statistics*

All the experiments were performed in triplicate and values, referred to minimum three replicates per sample, were averaged and standard deviations were determined. In all experiments, mean values were compared using Student's *t-test.*

**Results**

ROOH production, measured by FOX-1 assay, of the two *Aspergillus* strains (WT and Δ*Apyap1*) grown in PDB for 168 h at 30°C is reported in Figure 1. Δ*Apyap1* presents a higher ROOH production compared with WT between 36 and 70 h with an evident peak at 42 h. After 60 h both strains show a similar trend of ROOH production. Figures 2 and 3 report LOX activity (U/mg protein) and LOOH formation (ng/g seeds) of maize seeds inoculated or not (MS) with the WT and Δ*Apyap1* strains and incubated for 15 days at 30°C. Δ*Apyap1* promotes a stimulation of LOX activity in maize seeds up to 11 days (stronger at 5 and 11 days) in comparison with WT. Also LOOH were formed at greater extent until the first 7 days of incubation. LOOH and LOX activity of not inoculated seeds followed a trend similar to inoculated ones.

Production of 9- and 13-HODE during the growth of both strains on maize seeds is reported in table 1. In maize seeds, not inoculated (MS) and inoculated with the two strains, a slight prevalence of 13-HODE amount compared to 9-HODE occurred. These differences in the ratio between the two regioisomers in the contaminated and not contaminated maize seeds are not always significant.

The aflatoxin biosynthesis detected in maize seeds inoculated with WT and Δ*Apyap1* for 15 days at 30°C is reported in Figure 4. It was evident that aflatoxins were produced before by the Δ*Apyap1* strain (1.1 ± 0.1 μg/g maize seeds at the 2$^{nd}$ day of incubation) compared with the WT (1.5 ± 0.2 μg/g maize seeds at the 5$^{th}$ day of incubation). In addition the Δ*Apyap1* produces significantly ($p<0.001$) more toxins in the intervals 3-6 days and 8-12 days. No significant differences in fungal growth occurred as evident from ergosterol results (Figure 5).

## Discussion

Recently we have studied *in vitro* the regulation effect of some transcription factors, in particular Apyap1, hsf2-like and skn7-like, on the intracellular antioxidant defence mechanisms of some aflatoxigenic strains of *A. parasiticus* (Reverberi *et al*., 2006). Notably, there is a direct correlation between levels of oxidative stressors in the cell, such as lipoperoxides, the activity of antioxidant enzymes (i.e. SOD, CAT and GPX), and aflatoxin biosynthesis. In maize seeds inoculated with Δ*Apyap1*, the activity of LOX and the LOOH amount were higher compared with the WT strain. It can be argued that when the seed is infected with Δ*Apyap1* it produces more LOOH probably due to the ROOH produced at high concentration by this strain as suggested by FOX-1 assay. The oxidative stressors present on the surface of the seeds can trigger the activation of LOX and, in turn, lead to the release of LOOH. Thus, the fungus, unable to control the increase of oxidative stressors, such as LOOH, which are an important factor in the enhancement of aflatoxin biosynthesis on the surface of maize seeds, produces a higher amount of toxins and more rapidly. These results suggest that also in *A. parasiticus* - maize seeds interaction, oxidative stress and aflatoxin biosynthesis are correlated. It appears that the antioxidant defences that control the redox balance of fungal cell, also plays a role in the modulation of toxin formation on maize seeds contaminated by Δ*Apyap1*. This defence mechanism is a natural phenomenon that can be improved and controlled with different methods. For example the production of toxins can be modulated, as previously described, by the addition of antioxidants, which, in some cases, show also an intrinsic toxicity against pathogenic fungi (Fanelli *et al.*, 1985, 1986, 2000; Kim et al., 2004). As previously reported (Zjalic *et al*.., 2006), comparable effects can be accomplished with compounds that protect the plant against oxidative stress. The ability of organisms to oppose oxidative stress has been exploited also as a biocontrol mechanism. Some yeasts, used as biocontrol agents (Castoria et al., 2003), successfully compete for space and nutrients over many pathogenic fungi (Droby and Chalutz, 1994). These

agents, by resisting to oxidative stress conditions, colonize wounds and protect the plant from the attack of different pathogens.

The results obtained in this paper suggest that Apyap1 plays a role in the regulation of antioxidant/oxidant balance of the cell in *A. parasiticus* also during maize seeds colonisation. Thus, it can be hypothesised that aflatoxin biosynthesis occurring during the interaction seed/pathogen is affected by their redox status. Some strategies can be advanced on the basis of our results, such as the use of antioxidants to minimise the formation of oxidants both in the pathogenic fungus and in the plant.

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**Figure legends**

**Figure 1.** ROOH detected by FOX-1 assay in culture media (PDB) inoculated with *A. parasiticus* WT or Δ*Apyap1* incubated at 30°C for different periods (10, 12, 18, 21, 24, 30, 36, 42, 48, 60, 72, 96 and 168 h). The results represent the mean ± S.E.M. of three experiments.

**Figure 2.** Lipoxygenase activity of maize seeds not inoculated (MS) or inoculated with *A. parasiticus* WT or Δ*Apyap1* and incubated at 30°C for different periods (0-15 days). The results represent the mean ± S.E.M. of three experiments.

**Figure 3.** Total lipoperoxides (LOOH) originated from linoleic acid, extracted from maize seeds surface not inoculated (MS) or inoculated with *A. parasiticus* WT or Δ*Apyap1* and incubated at 30°C for different periods (0-15 days). The results represent the mean ± S.E.M. of three experiments.

**Figure 4.** Aflatoxin production in maize seeds inoculated with *A. parasiticus* WT or Δ*Apyap1* and incubated at 30°C for different periods (0-15 days). The results represent the mean ± S.E.M. of three experiments.

**Figure 5.** Fungal growth reported as ergosterol content (ng/g seeds) of *A. parasiticus* WT or Δ*Apyap1* inoculated in maize seeds and incubated at 30°C for different time periods (0-15 days). The results represent the mean ± S.E.M. of three experiments.

**Table 1.** 9 and 13 regioisomers of hydroxylated linoleic acid (ng/g maize seeds) produced on the surface of maize seeds at different times (0-15 days) not inoculated (MS) or inoculated with *A. parasiticus* WT and Δ*Apyap1*. The results represent the mean ± S.E.M. of three separate experiments.

| *Time (days)* | WT | | Δ*Apyap1* | | MS | |
|---|---|---|---|---|---|---|
| | 13-HODE | 9-HODE | 13-HODE | 9-HODE | 13-HODE | 9-HODE |
| 0 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.1 ± 0.05 | 0.1 ± 0.05 |
| 1 | 1.1 ± 0.1 | 1.0 ± 0.1 | 1.5 ± 0.2 | 1.4 ± 0.2 | 0.6 ± 0.1 | 0.5 ± 0.1 |
| 2 | 2.9 ± 0.3 | 3.2 ± 0.2 | 3.5 ± 0.3 | 3.7 ± 0.5 | 0.9 ± 0.1 | 0.7 ± 0.1 |
| 3 | 1.8 ± 0.2 | 1.9 ± 0.2 | 3.2 ± 0.4 | 2.9 ± 0.4 | 1.0 ± 0.1 | 1.2 ± 0.2 |
| 5 | 0.6 ± 0.1 | 0.3 ± 0.1 | 2.0 ± 0.5 | 2.0 ± 0.3 | 0.3 ± 0.1 | 0.2 ± 0.1 |
| 7 | 0.9 ± 0.2 | 0.5 ± 0.1 | 1.2 ± 0.2 | 0.6 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 |
| 11 | 2.8 ± 0.3 | 1.9 ± 0.3 | 3.0 ± 0.4 | 1.9 ± 0.5 | 0.8 ± 0.1 | 1.0 ± 0.2 |
| 15 | 4.0 ± 0.5 | 3.0 ± 0.4 | 2.0 ± 0.2 | 1.5 ± 0.2 | 0.7 ± 0.1 | 0.8 ± 0.2 |

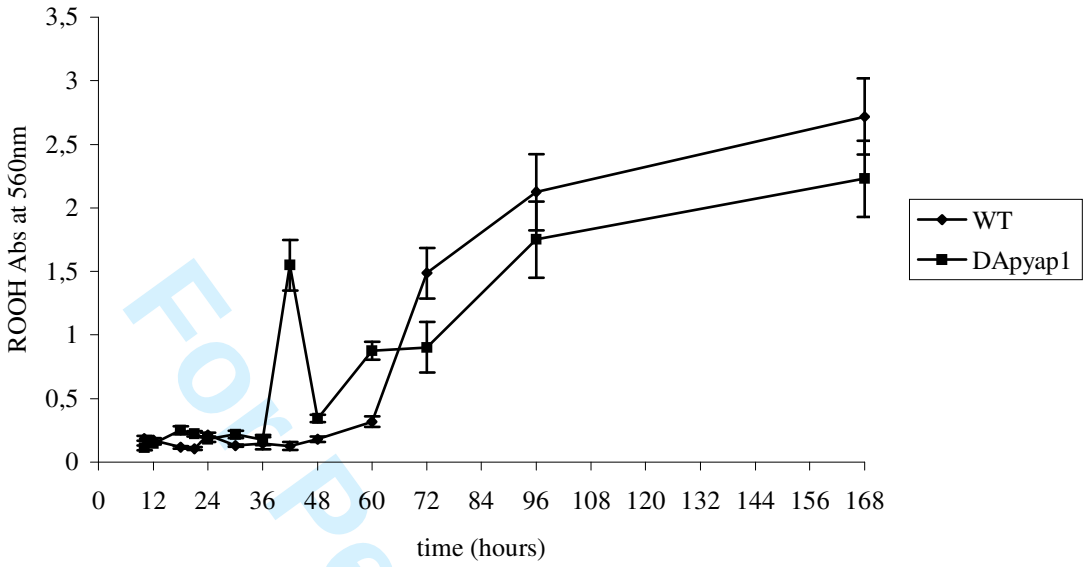




**Figure 1. Reverberi et al.**

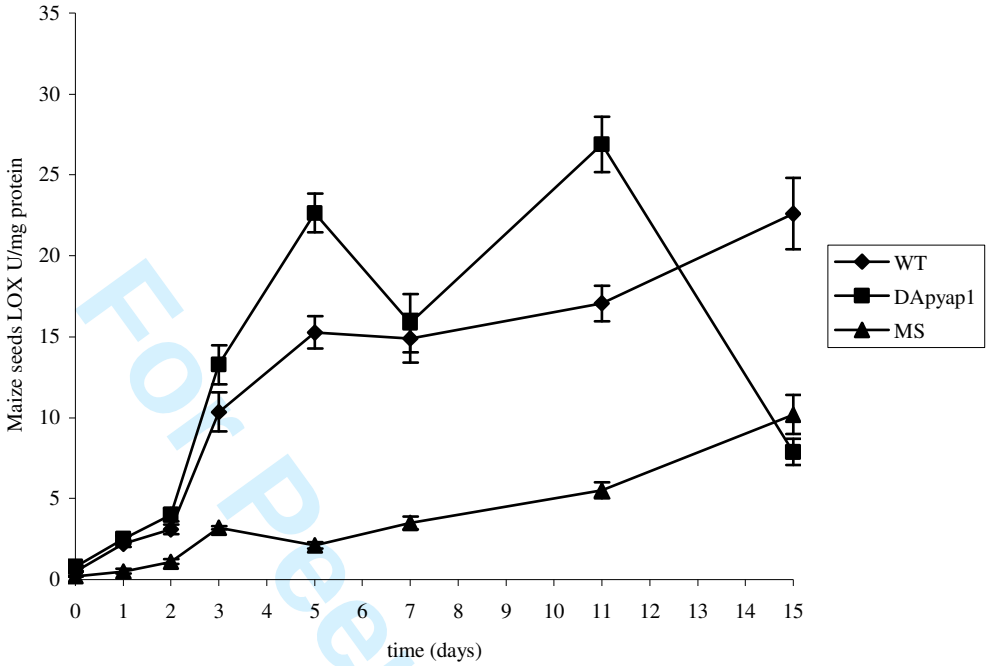




**Figure 2. Reverberi et al.**

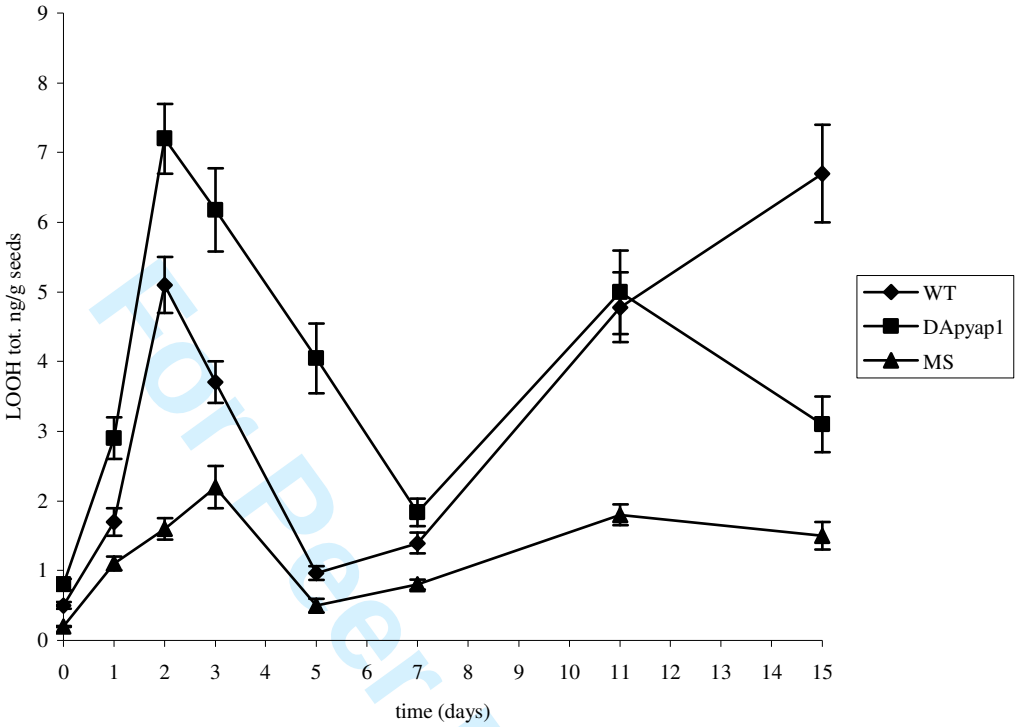


**Figure 3. Reverberi et al.**

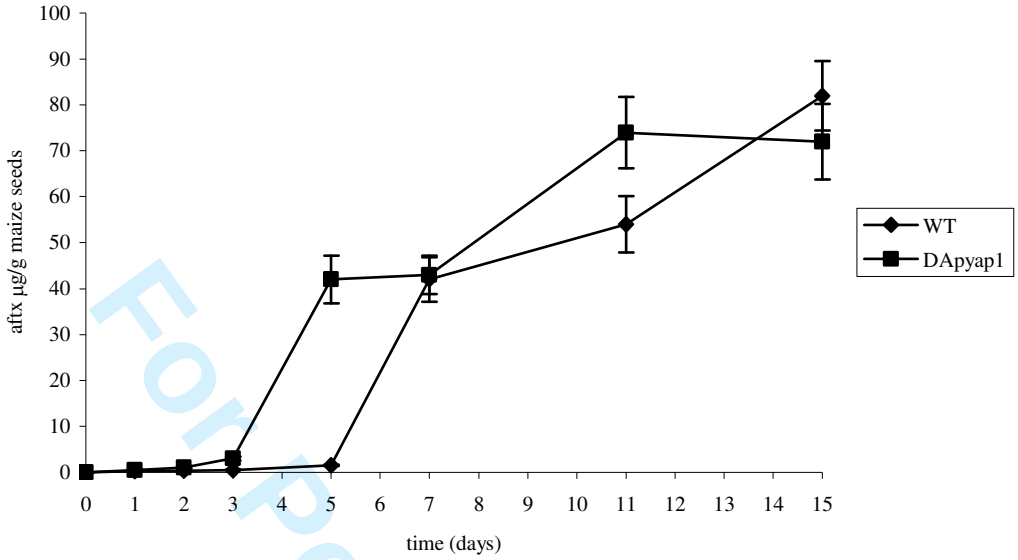




**Figure 4. Reverberi et al.**

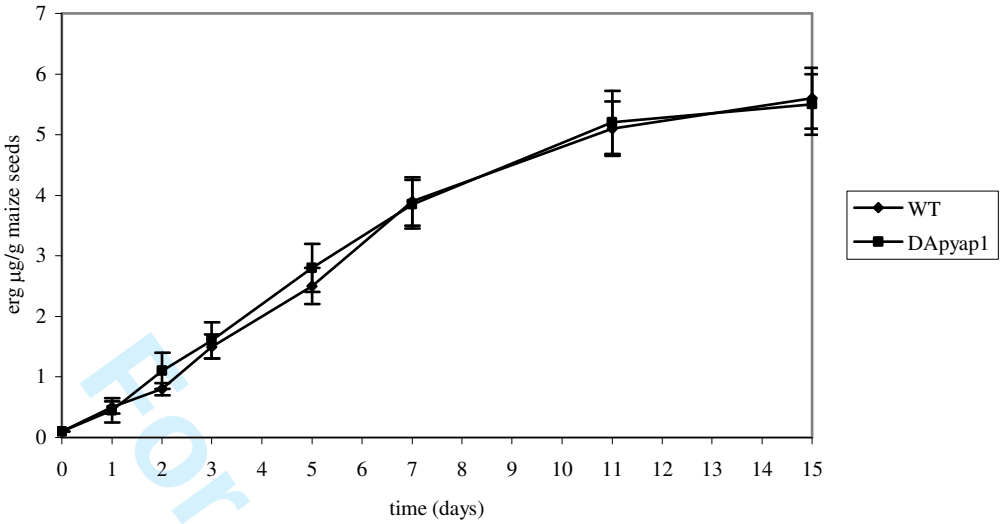




**Figure 5. Reverberi et al.**