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## EFFECT OF SUCROSE ON THE PHYSIOLOGY AND TERRESTRIC ACID PRODUCTION OF *PENICILLIUM AURANTIOGRISEUM*

### SUMMARY

*Penicillium aurantiogriseum* (*P. aurantiogriseum*) is a post-harvest pathogen that causes significant losses in agricultural production during storage. It plays an important role in food and feed spoilage, and it contaminates agricultural products with mycotoxins that are potentially harmful to human and animal health. *P. aurantiogriseum* is one of the most toxic species in the genus *Penicillium*, and it is often isolated from foods, vegetables, fruits and permafrost sediments from the Arctic and Antarctic. It has also been isolated from the marine environment. Thus, it is resistant to several types of stress related to nutrients and growing conditions. This study aimed to determine the effect of sucrose on the physiology of *P. aurantiogriseum* in order to control its growth and toxigenesis. Mycotoxin production was determined by TLC technique. Our results show a close relationship between the physiological state of *P. aurantiogriseum* and the secretion of mycotoxins under carbon stress conditions. The physiological state of the pathogen reveals a correlation between increased sucrose concentration and the intensity of aging signs. Aging signs begin to disappear at a sucrose concentration of 400 g/l, which allows the normal characteristics of *P. aurantiogriseum* to reappear. It is suggested that this transformation is meant to avoid the action of sucrose. Terrestrial acid production was recorded at the time of appearance of aging signs. Terrestrial acid is always maintained, even after returning to a normal physiological state, but its production was diminished. The growth of *P. aurantiogriseum* can be controlled by modifying the sucrose concentration in growth medium. This allowed us to determine the critical concentration at which the pathogen suffered and thus reached the phase of decline earlier while mycotoxin production was minimal.

**Keywords:** *P. aurantiogriseum*, sucrose, physiology, aging signs, terrestrial acid.

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## INTRODUCTION

Mycotoxins, as toxic secondary metabolites of molds, have been detected in several human or livestock foods (Khaddor *et al.*, 2006). The ingestion of mycotoxins represents a real menace to human and animal health (Faid and Tantaoui-Elaraki, 1989). *P. aurantiogriseum* is a particular species of *Penicillium* genus. It is ubiquitous in the terrestrial and marine environment (Yu *et al.*, 2010; Sonjak *et al.*, 2005). It is a post-harvest pathogen causes significant losses of agricultural production during storage (Khaddor *et al.*, 2006). This species is recognized as a prolific source of biologically active secondary metabolites. Mycotoxins of *P. aurantiogriseum* are of great importance given their largely variable effects between harmful and beneficial to human and animal health (Khaddor *et al.*, 2007). By this double effect, mycotoxins of *P. aurantiogriseum* could be used in pharmaceutical industry because of their therapeutic potential and also in agri-food industry which can minimize their unsafe effect by controlling growth factors. Previous studies had identified some mycotoxins such as penicillic acid, aurantiamine, and terrestric acid on *P. aurantiogriseum* (Khaddor *et al.*, 2007).

Terrestric acid is the least common toxin in *Penicillium* species (Peberdy, 1987). There are few studies of natural contamination with terrestric acid and its toxicity, and little is known about the factors influencing its production. The production of terrestric acid by *P. aurantiogriseum* was demonstrated in 1971 by Turner *et al.* (1971). The offending substance was found to be phytotoxic (Gausman, 1991) and cardiotoxic (Frisvad and Samson, 2004. ).

Sucrose is the most used carbon source in growth media for development and production of *Penicillium* mycotoxins. Except for sucrose, the substitution between carbon sources in a growth medium does not present a large difference in colony production (Bode *et al.*, 2002). This production is also influenced by the addition of nitrate. The addition of complex accessory factors such as yeast extract to the medium increases the rate of growth while having little effect on the colony (Smith *et al.*, 1981; Pitt, 1973). Glycerol facilitates the consistent development of *Penicillium* colonies (Pitt, 1973) and it is an excellent carbon source for mycotoxin production (Mulè *et al.*, 2004). Hocking and Pitt (1979) recommend the use of glycerol to adjust water activity with the least harmful effects on fungal growth. Several studies have used G25N (25% glycerol nitrate) as an identification and purification medium, but not for the production of mycotoxins (Park *et al.*, 2014; Zhao *et al.*, 2014). Khaddor *et al.* (2007) showed that penicillic acid and aurantiamine are produced by *P. aurantiogriseum* in CYA (Samson and Gams, 1984) and terrestric acid in YES liquid (Samson and Gams, 1984). Therefore thus, we suggest that the basic medium that will be used for the growth and toxigenesis of *P. aurantiogriseum* is the G25N (Pitt, 1973) medium added at different sucrose concentrations (to get the G25N\* medium).

The present work is devoted to study the effects of sucrose on the physiology and terrestric acid production of *P. aurantiogriseum*. Thus, it may help to control the growth conditions of *P. aurantiogriseum* in order to improve

the production of its mycotoxins with therapeutic interest or restrict its growth to minimize the harmful effects of this species.

## MATERIAL AND METHODS

### *Fungal Strains*

The strain of *P. aurantiogriseum* is part of the collection of the Environmental and Food Biotechnology Research Team (EFBRT) used in previous studies (Bouhoudan et al., 2018; Khaddor et al., 2007; Maouni et al., 2002). The stored strain is placed in the MEA (malt extract agar) and incubated at 25°C for 7 days. After incubation, the spores were suspended in 0.1 % of tween 80. The density of suspension was adjusted to 10<sup>7</sup> spores /ml.

### *Growth medium*

*P. aurantiogriseum* was inoculated on G25N\* medium (at different concentrations of sucrose from 0 g/l to 700 g/l). The dishes are incubated for 10 days at 25°C.

### Determination of mycelial dry weight and colony diameter

Physiological studies are based on morphology, texture, color, growth rings, growth status, mycelial weight, and colony diameter of *P. aurantiogriseum* colonies, as well as the aspect of its hyphae.

Mycelia were harvested by filtration using Buchner funnel. Then they were washed thoroughly with distilled water and the excess of water was removed by plotting with filter papers. The mycelia were dried at 80°C until constant weight obtained which is a dry weight (Zain et al., 2009). Radial growth was estimated by measuring the diameter of each colony with a ruler (Zain et al., 2009). All the experiments were performed in triplicate.

### *Mycotoxins extraction*

The toxigenesis study was made according to the method reported by Bouhoudan et al. (2018). The colonies grown in G25N\* medium were added with 25 ml of chloroform and agitated during 2 minutes. The chloroform phase recovered was filtered on anhydrous sodium sulfate. The filtrate was concentrated to the rotavapor and then evaporated to dryness in nitrogen current. Thin Layer Chromatography (TLC) highlights mycotoxins in concentrated filtrates thus obtained. The thin layer chromatography technique (TLC) adopted in this study is described by Mills et al. (1995). The TLC plates used are 60 Kieselguhr F254. Mycotoxins standards used by the reference of migration forehead (Rf) were patulin (P), citrinin (C), ochratoxin A (OTA), penicillic acid (PA), and griseofulvin (Gi). Ten ml each of ethanol extract and of standard solutions (1 mg/ml) were spotted on TLC plates. Elution systems used are toluene-ethyl acetate - formic acid (5/4/1, v/v/v) and chloroform - acetone - 2-propanol (85/15/20, v/v/v). The plates were examined in daylight and by ultraviolet 365 and 254 nm after spraying the spots by ANIS (p-anisaldehyde solution) and 8 min heating to 120°C. The ratio (Rf), color and fluorescence intensity of the extracts were compared with different reference concentrations of P, C, Gi, PA and OTA

(Cunniff, 1995). Fluorescence intensity was expressed by a variable number of "+" signs (Hameed *et al.*, 2012).

#### *Statistical study*

Statistical analysis of the obtained results was performed by the test "Duncan's multiple range" at the threshold of 5% [Stat Soft]. For each medium, nine tests were performed. The averages obtained in the nine trials ( $n = 9$ ) were compared by analysis of variance (ANOVA) with the Duncan's Multiple Range test at the 5% threshold. This test is then used to define more precisely if the factor (carbon source) has seen a really significant effect on the response (mycotoxin production and lipase and fungal growth).

## RESULTS

### *Macromorphological characteristics of P. aurantiogriseum*

Our results revealed that the mycelia weight and the diameter of *P. aurantiogriseum* colonies were increased with increasing concentration of sucrose (Figure 1 and Table 1).

Table 1: Average colony diameters, dry weight and AT intensity of *P. aurantiogriseum* as a function of sucrose concentration of G25N medium.

Sucrose concentration (g/l)	Mycelial Dry weight (g/100 ml) (95% IC)	Colony Diameter (cm) (95% IC)	Intensity of Terrestrial Acid
0	0.68 d	1.98 d	–
30	4.00 ab	40.00 ab	+
50	4.48 a	41.00 ab	+
100	4.88 a	45.00 b	+
200	6.05 c	50.00 e	++
300	6.96 c	55.00 c	+++
400	8.72 e	36.37 a	++++
500	4.03 ab	57.70 c	++
700	3.19 b	62.77 f	++
(-) No (+) low, (++) medium, (++) strong (+++) very strong			

On the same column, 2 results followed by the same letter do not differ significantly at the 5% threshold. For each concentration of YES, nine tests were performed. The averages obtained in the nine trials ( $n = 9$ ) were compared by analysis of variance (ANOVA) with the Duncan's Multiple Range test at the 5% threshold.

At different sucrose concentrations ranging from 0 to 700 g/l, signs of aging are observed at the macroscopic level. *P. aurantiogriseum* colonies change color in a centrifugal direction. They are whiter from the center to the periphery. The colony diameter increases with the concentration of sucrose. The rough shape extends centrifugally over the entire colony. We also noted a change of relief that results in the elevation of the colony central area. The aging degree is proportional to the sucrose concentration used (Figure 1).

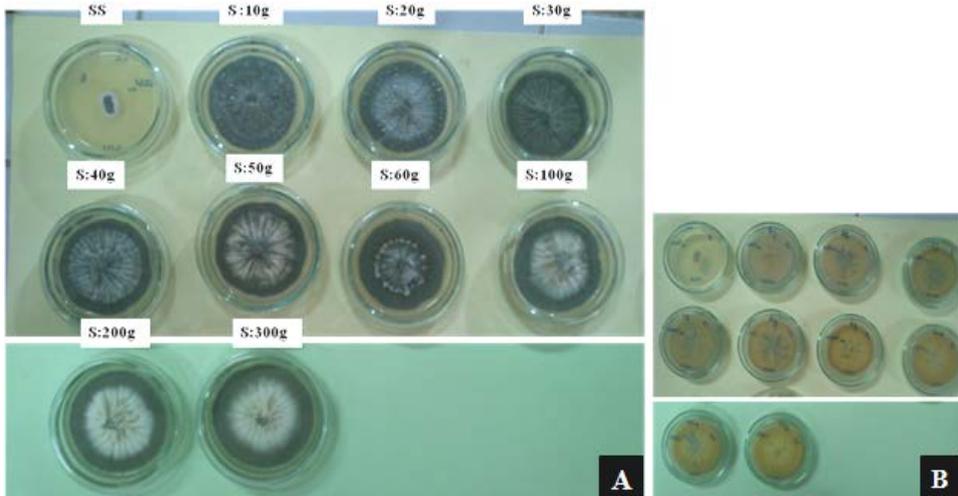


Figure 1: Physiological modification of *P. aurantiigriseum* in the medium containing different sucrose concentrations (g/l); A: front view of the colony, B:reverse view of the colony; S: sucrose; SS: no sucrose

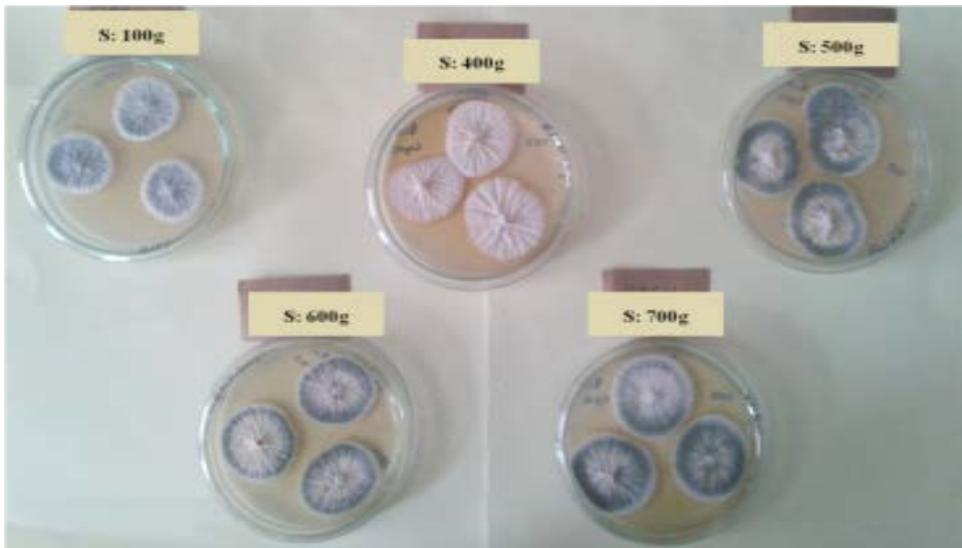


Figure 2: Phenomenon of escape to the high sucrose concentration in *P. aurantiigriseum*. At the concentration of 400 g/l, the strain begins to resume its normal characters and the aging signs disappear. S: sucrose

The use of increasing sucrose concentrations allowed us to observe signs of suffering and aging reflected by the physiological activity of the strain. This results in morphological changes on the colonies (Table 1).

There was a critical concentration (400g/l) for which the strain responded aggressively to the concentration and this appeared at the macroscopic level

where the colony has a whitish and very rigorous appearance. The aging signs begin to disappear from the sucrose concentration of 400 g/l, revealing the normal characters of *P. aurantiogriseum*. We have considered this transformation as an escape phenomenon to the action of sucrose (Figure 2).

#### *Micromorphological characteristics of P. aurantiogriseum*

At the microscopic level, *P. aurantiogriseum* showed an identical appearance on all sucrose concentrations added to G25N\* medium. We distinguished two areas: a central zone, which contains older cells and a peripheral zone, which represents the young cells.

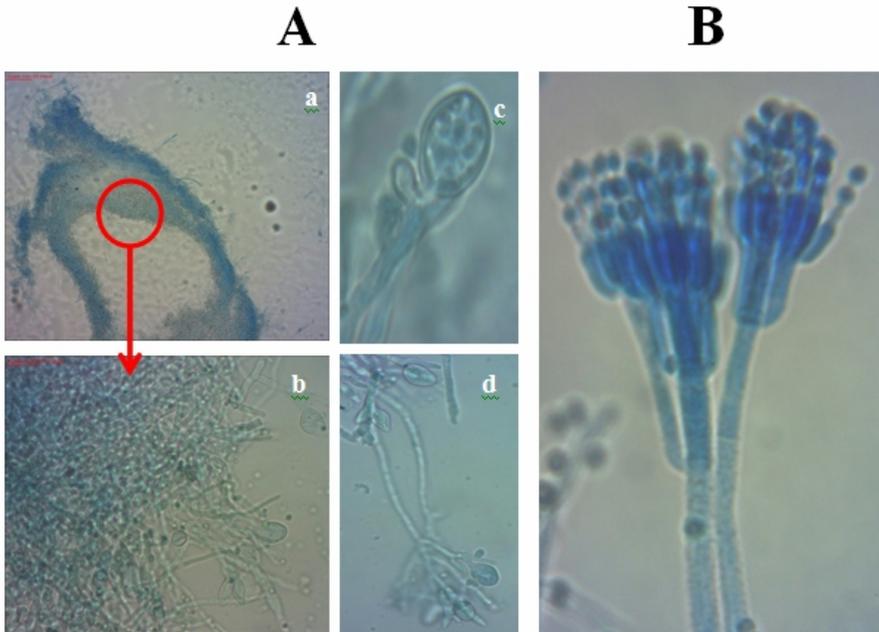


Figure 3: Thallus appearance in the same colony of *P. aurantiogriseum* at the concentration of 100 g/l of sucrose:

A: center of the colony; the cells lose branched hyphae and show an abnormal appearance: (a) with a resolution x10 (b) with a resolution x 40. The thallus shows conidiophores without phialides and metulae clearly differentiable: (c) with a resolution x100, (d) with a resolution x40.

B: periphery of the colony; the strain retains its asexual reproduction type with the presence of conidiophores as well as spores.

During carbon stress, the colonies of *P. aurantiogriseum* showed a dispersed morphology. In the colony center, where sucrose began to decline, we observed empty hyphae compartments emerged and the diameter of growing hyphae decreased significantly (Figure 3A-b). Throughout the prolonged decrease, the fraction of the empty hyphae compartments increased, but the exoskeleton of the cell wall appears to have remained intact (Figure 3A-a). We also observed asexual reproductive structures morphologically paralyzed which

resembled to low-density conidiophores without clearly distinguishable phialides and metulae (Figure 3A-c and 3A-d). At the peripheral zone, the mycelia appear normal with a penicil containing phialides and metules (Figure 3B). All hyphae have the same extension ratio and the same diameter; extension zones have the same shape and size. In differentiated mycelia, there is a hierarchy such that parental hyphae extend faster, have larger extension areas, and are wider than the branches they support.

#### *Production of terrestrial acid*

Our results revealed that the mycotoxins profile of *P. aurantiogriseum* was greatly affected by the sucrose concentrations of G25N\* growth medium (Table 1). Toxigenesis study allowed us to detect a significant production of terrestrial acid at the time of the appearance of aging signs. The aging signs are more important when the concentration of terrestrial acid produced is high.

## DISCUSSION

#### *Morphological response*

Sugars act not only as nutrients, but also as important regulators of gene expression. The influence of sucrose concentrations on *Penicillium* growth has been extensively studied (Cunniff, 1995). Thus, the identified phenotypic responses are likely caused by changes in fungal growth rate (Hameed et al., 2012; Zain et al., 2011; Gasch et al., 2000). Our results revealed that the mycelium weight, the colony diameter, and the terrestrial acid production of *P. aurantiogriseum* were significantly affected by the increase in sucrose concentrations added to the G25N\*medium. We have observed that the high sucrose concentration in the medium induces a kind of trauma in the strain. This was reflected first by a change at the morphological level and then at the behavioral level. The changes in *P. aurantiogriseum* growth parameters, possibly induced by sucrose, could be related to the significant decrease in the total sugar content of the cell walls observed after the stationary phase. However, the oldest cells in the center did not find oxygen for survival and so began to increase. The shape of the colony became rougher because of the intense growth of cells and the high concentration of sucrose gave an aged appearance. According to Sinclair (2002), the accumulation of sugar resulted in over-expression of free radicals in mitochondria, led to a mitochondrial dysfunction and consequently accelerated cell aging.

#### *Physiological response*

In the presence of high sucrose concentration in the medium, all the metabolism of *P. aurantiogriseum* developed as rapidly as possible by extension of the hyphal end. During exponential growth, all mycelial hyphae of biomass contribute to growth. However, as the hypha spreads, the nutrients must diffuse through the hyphae and the mycelia in the center become progressively limited in nutrients so that exponential growth is limited to the periphery. According to Zain et al. (2009), filamentous fungi respond to carbon deficiency with very specific

responses, including fungal cell wall degradation (autolysis) and the onset of asexual spore formation. Similar results reported morphological data from *Aspergillus oryzae*. Pollack *et al.* (2008) indicate a clear transition between thick and thin compartments in response to carbon starvation. This was also observed in this study suggesting, as well, that hyphal diameters can be used to distinguish aged and young cells formed during growth. On the other hand, the microscopic analysis of the thallus showed us a change in the reproduction type at the same colony (unpublished results). The area showing aging signs (center of the colony) shows sexual reproduction with presence of ascospores while moving away from the center to the periphery. The reproduction remains asexual; this can be explained by the degree of sucrose resistance in function of age. The modification of reproduction type in older cells is probably due to the production of mycotoxins secreted during the stationary phase. Li *et al.* (2008) report that some mycotoxins have easily observable effects on morphological differentiation and can induce sexual sporulation.

#### *Metabolic response*

In this work, the study of toxigenesis has shown that the production of terrestric acid is proportional to the intensity of the aging signs. Indeed, the high sucrose concentration in the medium caused a kind of cellular stress and therefore led to overproduction of terrestric acid in the cell during the stationary phase. This means that the terrestric acid probably caused the early cells aging. These results are in agreement with Chander (1981) who reported that the high concentration of the carbon source causes the production of mycotoxins in molds. In addition, Rouvier (2002) and Meisner (1974) showed that terrestric acid is produced in the Krebs cycle, a process that occurs in mitochondria, which enhance its involvement in the respiratory cell metabolism. Consequently, the intensity of cell growth in the central zone of *P. aurantiogriseum* colony caused respiratory problems related to mitochondrial dysfunction (Coppola and Ghibelli, 2000; Sámi *et al.*, 2001). This dysfunction, reported by Moore and Truelove (1970) and Meisner and Chan (1974) leads to early cell aging

#### *Escape phenomenon at high concentration of sucrose*

This phenomenon appeared in the concentrations from 500 to 600 g/l of sucrose. Our strain was normal in appearance with good biomass and mycelium production and moderate production of terrestric acid. We consider this finding as escape phenomenon at the high sucrose concentration. According to other studies (Nilsson and Bjurman, 1998; Robin *et al.*, 2001; Park and Gander, 1998; El-Kady *et al.*, 1995) molds are well known for their ability to adapt to high osmolarity environments through the polyols intracellular accumulation. *Penicillium* species accumulate glycerol as major osmoregulation substance (Blomberg and Adler, 1992; Harris, 1981; Hocking, 1986). Our analysis suggests that the presence of glycerol in the medium (G25N\*) creates some cell permeability. Thus allows the oxydo-reductase genes to hinder protein activity and consequently maintaining the cells during the growth phase.

On the other hand, we suggest that between 10g /l and 400g /l of sucrose, *P. aurantiogriseum* was forced to adapt to the sucrose stress. It used its panoply of intracellular proteins to maintain growth and survival, which was remarkable in colony diameter and terrestrial acid production. At certain levels, the difficulty of nutrients absorption by the colony center cells and mycotoxins production have led to a premature senescence which has been reflected in the relief degree and rigorously in the colony morphology.

However, at a concentration of 600 g/l of sucrose, *P. aurantiogriseum* behaved as being in osmotic shock. This leads it to call, in addition to its protein heritage, its genetic heritage by a signaling cascade in order to respond to the stress. This reaction allows the strain to maintain its growth in a normal state. This explains its normal morphology and the minimal production of terrestrial acid compared to other concentrations.

### CONCLUSIONS

This study is a continuation of a previous study (Bouhoudan et al., 2018) showing the effect of different carbon sources on the production of secondary metabolites in *P. aurantiogriseum*. Other stress-related studies are running on 3 different strains of *Penicillium* by analyzing their metabolic profile with the HPLC-MS method.

In conclusion, this study demonstrated that cell autolysis, morphological changes, and terrestrial acid production by *P. aurantiogriseum* are influenced by the sucrose concentration in the medium. Indeed, terrestrial acid production was faster and more important by increasing the sucrose concentration in the medium. Therefore, the carbon stress can prove to be an effective procedure to reduce the life duration of *P. aurantiogriseum* and control the terrestrial acid production or to accelerate the aging process that occurs during sucrose stress. The possibility of recovering large quantities of terrestrial acid is a key advantage that could later allow studying this mycotoxin well.

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