TEMPERATURE, pH AND CARBON SOURCE AFFECT DRASTICALLY INDOLE ACETIC ACID PRODUCTION OF PLANT GROWTH PROMOTING YEASTS

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Abstract – Rhizosphere and phylloplane yeasts are able to produce phytohormones, acting as potential plant growth promoters. The aim of this study was to analyze the production of indole acetic acid (IAA) by yeasts using variations in the culture conditions. The yeasts evaluated were *Rhodotorula mucilaginosa* and *Trichosporon asahii*, which were cultivated in a potato broth medium with 20 g/L of glucose or sucrose as the carbon source. The cultivation was carried out at 22°C, 25°C or 30°C, and the initial pH was adjusted to 3.0, 4.5 or 6.0. The cultures were analyzed using a colorimetric technique with Salkowski reagent. The cell count was determined in all of the conditions. The results for *T. asahii* showed that IAA production with sucrose was superior at pH 6.0, and that with glucose was superior at pH 4.5. These results indicate an association between the carbon source and medium pH. *Rh. mucilaginosa* (2F32) presented a high IAA production when compared with *T. asahii* (3S44 and 4C06) or even with other microorganisms referenced in the literature. The highest production (0.655 g/L) was obtained at pH 6.0 with glucose as the carbon source. Variations in the culture conditions did not affect the cell growth of the yeasts.
Keywords: *Rhodotorula mucilaginosa*, *Trichosporon asahii*, culture conditions, kinetic evaluations

INTRODUCTION

The study of natural processes using autoctonous microorganisms that are able to protect and assist in the development of plants is presented as an alternative for more sustainable agricultural production. However, the lack of knowledge about microorganism function in the rhizosphere continues to be a challenge for scientists (Tiedje et al., 1999; Fitter et al., 2005; Torsvik and Ovreas, 2002; Van der Heijden et al., 2008; Maron et al. 2011). One of the goals in soil microbe studies is to stimulate microorganisms via agroecosystem management or by employing bio-products with beneficial species that are able to support plant production.

The challenge in this area is the expansion of knowledge regarding the behavior of microorganisms in the rhizosphere and the discovery of how they can assist the plant production process, contribute to the balance of the ecosystem and minimize the use of toxic products in the environment, such as fungicides and fertilizers responsible for soil, ground water, river and lake pollution.

The rhizospheric group of microorganisms known as

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Plant Growth Promoters (PGP) have the ability to produce compounds capable of stimulating plant growth via a variety of mechanisms, such as the production of phytohormones, biological control of phytopathogens and plant resistance induction, as well as the solubilization of phosphate and available minerals in the soil solution, promoting better nutrition for plants. Various studies in the literature have reported that rhizospheric microorganisms, especially rhizobacteria, show excellent results in promoting plant growth (Ahemad and Kibret, 2014; Lugtenberg and Kamilova, 2009; Myresiotis et al., 2014; Prashar et al., 2014). Another extensively studied group is the endophytic microorganisms, similar to the rhizobacteria, with the potential for use in various other economic crops (Brader et al., 2014; Hallmann et al., 1997; Rosenblueth and Martinez Romero, 2006; Shen et al., 2014).

Yeasts are unicellular fungi that can also be found in the rhizosphere of plants, but in smaller numbers compared to bacteria and filamentous fungi; however, little information is known about their function in this ecosystem. A wide variety of yeasts present characteristics that promote plant growth (Cloete et al., 2009; Limtong et al., 2014; Nutaratat et al., 2014) via the control of plant pathogens (El-Tarabily, 2004; El-Tarabily and Sivasithamparam, 2006; Korres et al., 2011; Rosa et al., 2010), the production of phytohormones (Nassar et al., 2005), the solubilization of phosphate (Hesham and Mohamed, 2011; Falih and Wainwright, 1995; Mirabal Alonso et al., 2008; Mundra et al., 2011), the oxidation and mineralization of nitrogen and sulfur compounds (Falih and Wainwright, 1995), the production of siderophores (Sansone et al., 2005) and the stimulation of root colonization by mycorrhizal fungi (Mirabal Alonso et al., 2008; Vassileva et al., 2000).

One of most important mechanisms applied by PGP is phytohormone production. The auxin group is a class of plant hormones known to stimulate responses in plants both quickly and in the long term. Indole acetic acid is a type of auxin produced by many groups of microorganisms, including bacteria (Ahmed and Hasnain, 2010; Tsavkelova et al., 2007), filamentous fungi (Manici et al., 2014; Hasan, 2002; Sirrenberg et al., 2007), and yeasts (Nassar et al., 2005).

The aim of this study was to evaluate the production of indole acetic acid by the yeasts isolated from the rhizosphere and phylloplane of maize and sugar cane and to evaluate the influence of temperature, pH and carbon source on production.

MATERIALS AND METHODS

Microorganisms

The yeasts evaluated in this study were isolated from sugarcane and maize in São Paulo State, Brazil. The strains consisted of 2 epiphytic yeasts from sugar cane leaves (*Rhodotorula mucilaginosa* - 2F32) and stems (*Trichosporon asahii* - 4C66), plus one rhizospheric yeast isolated from maize (*Trichosporon asahii* - 3S44). The choice of these isolates was based on preliminary screening of strains able to produce indole acetic acid (data not published), employing colorimetric analysis, according to Gordon and Weber (1951), as mentioned following in this manuscript. The yeasts were maintained in a culture collection at the Laboratory of Agricultural and Molecular Microbiology (LAMAM), Federal University of São Carlos, Brazil. The culture was kept in YEPD medium and stored at 8ºC until the start of the tests.

Temperature, pH and Carbon Source conditions

Yeast inoculum was prepared by transferring one loop of the colony into 10 mL of YEPD broth, which was incubated in a shaker at 160 rpm, 30ºC for 3 days. One milliliter of a suspension of concentration 1×10^7 cells/ml from this culture was used as the inoculum. The yeast strains were cultivated in Erlenmeyer flasks (500 mL) containing 200 mL of potato broth (infusion from potatoes) supplemented with 0.54 g/L of tryptophan and 20 g/L of the carbon source, either glucose or sucrose. The medium pH was modified according to the treatment to 3.0, 4.5 or 6.0 with 0.1N HCl before sterilization. The culture flasks were incubated on an orbital shaker at 160 rpm for 4 days at 22, 25 or 30°C. The medium was used as a control. The samples were taken for analysis every 12 hours for a total of 96 hours. At each sample time, 10 mL of the culture suspension was taken and the amount of cells was determined with a Neubauer chamber. The cell number was used as an estimate of cell mass (*Rh. mucilaginosa* - 1×10^6 cells/mL = 0.85 g of dry biomass and *T. asahii* – 1×10^6 cells/mL = 2.1 g of dry biomass). Indole acetic acid production was determined by colorimetric method (Gordon and Weber, 1951), using the remainder of the sample, as described below.

Indole acetic acid (IAA)

The samples were centrifuged at 3000 rpm for 5 min, and 1 mL of culture supernatant was mixed with 1 mL of Salkowski reagent (12 g.L^{-1} FeCl$_3$ in 7.9 M H$_2$SO$_4$) (Gordon and Weber, 1951), followed by an incubation for 30 minutes to complete pink-red color development. The color intensity was determined as A_{530} using a spectrophotometer (Thermo Electron Corporation, USA). The IAA concentration of the sample was compared with a linear calibration curve obtained from a commercial IAA standard using the performance of three replicates.

Kinetic analysis

The growth curves were plotted for the experimental

conditions, and the kinetic parameters were calculated according to Ahmad and Holland (1995). The maximum specific growth rates were estimated from the slope of semi-log plots of biomass concentration *vs*. time, while productivity was estimated from the IAA profile curves.

Statistical analysis

All of the data reported in this study are the mean of the values from three replicates. Differences between the treatments were tested by Analysis of Variance (ANOVA), and the mean values between treatments were compared by Tukey Test at p=0.05. Statistical analysis of the data was performed using the statistical computer program STATISTICA 6.0 (Statsoft, Tulsa-OK, USA, 2003).

RESULTS

Rh. mucilaginosa (strain 2F32) had a significantly higher production of IAA compared to the strains of *T. asahii* (3S44 and 4C06) in all of the tested conditions (Figure 1). The 3S44 strain showed a higher production of IAA compared to the 4C06 strain, although they are the same species. Even with the differences in production, the factors evaluated (pH, carbon source and temperature) presented the same influences, with an increase or decrease in IAA production for both strains of *T. asahii*.

The pH of the medium showed a significant influence on the production of IAA by *T. asahii*, with superior results at an initial pH of 4.5 and a maximum output of approximately 0.03 g/L in the medium containing 2% of glucose (in terms of mass) as the carbon source. The medium at pH 3 did not allow for the production of IAA by the yeast, and at pH 6 the production was very low, not exceeding 0.0027 g/L. For *Rh. mucilaginosa* (2F32), the highest production of IAA (0.655 g/L) was obtained at pH 6 in the medium that contained glucose as the carbon source (Figure 1).

According to Figure 2, the presence of sucrose in the medium promoted a higher production of IAA compared to glucose (both at initial pH of 6.0), with a maximum production close to 0.07 g/L. For *Rh. mucilaginosa,* glucose provided the maximum amount produced at any given initial pH value (Figure 2 and Figure 3). Independent of the carbon source used for *Rh. mucilaginosa* culture, IAA production occurred during the stationary phase of cell growth (after 24 hours of incubation). For *T. asahii* strains, however, when sucrose was utilized in the cultivation, the production started during the log phase growth (after 12 hours of incubation) (Figures 1, 2, 3 and 4).

The incubation temperature had an influence on the start of IAA production by the two strains of *T. asahii*; the temperature of 25°C provides the production of IAA in less time (after 24 hours), while the culture at 22°C causes production delays, which initiated only after 72 hours of incubation. For *Rh. mucilaginosa,* a temperature of 30°C after 60 hours of cultivation, followed by 25°C after 24

hours were the culture conditions with the best production results (Figure 4).

The yeast cell growth was not significantly affected by the variation of the factors evaluated. For the yeast *Rh. mucilaginous* (2F32), the conditions that provided the best result of IAA production were the same for the better development of the lineage, with a μ_{max} (maximum specific growth rate) and IAA productivity of approximately 0.19 h^{-1} and 12 μg $mL^{-1}h^{-1}$, respectively, in the medium containing glucose at an initial pH of 6 and 25°C. For *T. asahii* (4C06), the μ_{max} was 0.119 h^{-1} with 2% glucose, at pH 6, 25°C; however, the maximum productivity (0.64 g mL^{-1} h^{-1}) was obtained at an initial of pH 4.5. For *T. asahii* (3S44), the μ_{max} (0.0722 h^{-1}) was observed in a medium with 2% glucose, at pH 6, 25°C, but the maximum productivity (2.49 g mL^{-1} h^{-1}) in the sucrose medium was observed with an initial pH of 6 at 30°C. The optimal cell growth conditions by the specific growth rates were the same for all three of the tested strains, i.e., at 25°C, in culture medium with glucose as the carbon source and an initial pH of 6 (Table 1).

DISCUSSION

The production of IAA by all of strains evaluated occurred during the stationary growth phase when glucose was utilized as the carbon source. This finding has already been observed in other microorganisms, such as bacteria (*Azospirillum* sp. and *Arthrobacter* sp. (Cacciari et al., 1989), *Streptomyces albidoflavus* (Narayana et al., 2009), *Rhizobium* sp. (Nalini and Rao, 2014)) and mold (*Aspergillus niger* (Bilkay et al., 2010)). The production of IAA during the stationary phase must be related to the decrease of carbon and yeast nutritional stress. The results for the *T. asahii* strains showed that sucrose as the carbon source yielded higher IAA production and that the production started during the log phase. This result could have occurred due to the yeast having suffered nutritional stress when using sucrose as a carbon source since the production of invertase is required for obtaining glucose by cells; the efficiency of this process was not determined in this study.

The maximum IAA productivity by *Rh mucilaginosa* (2F32) was over ten times higher than the maximum value obtained with the *T. asahii* strains, a finding that was observed in all of the experimental conditions.

This study is not the first to show IAA production results for the yeast species *Rh. mucilaginosa* and *T. asahii*. Xin et al. (2009) obtained endophytic strains of *Rh. mucilaginosa* from *Populus* trees and evaluated their ability to produce IAA. These authors evaluated two strains and observed significant differences in their IAA production, such that PTD2 reached 18 mg of IAA/mg of dry cell mass and PTD3 produced less than 10 mg of IAA/mg of dry cell mass. In this study, differences were observed in IAA
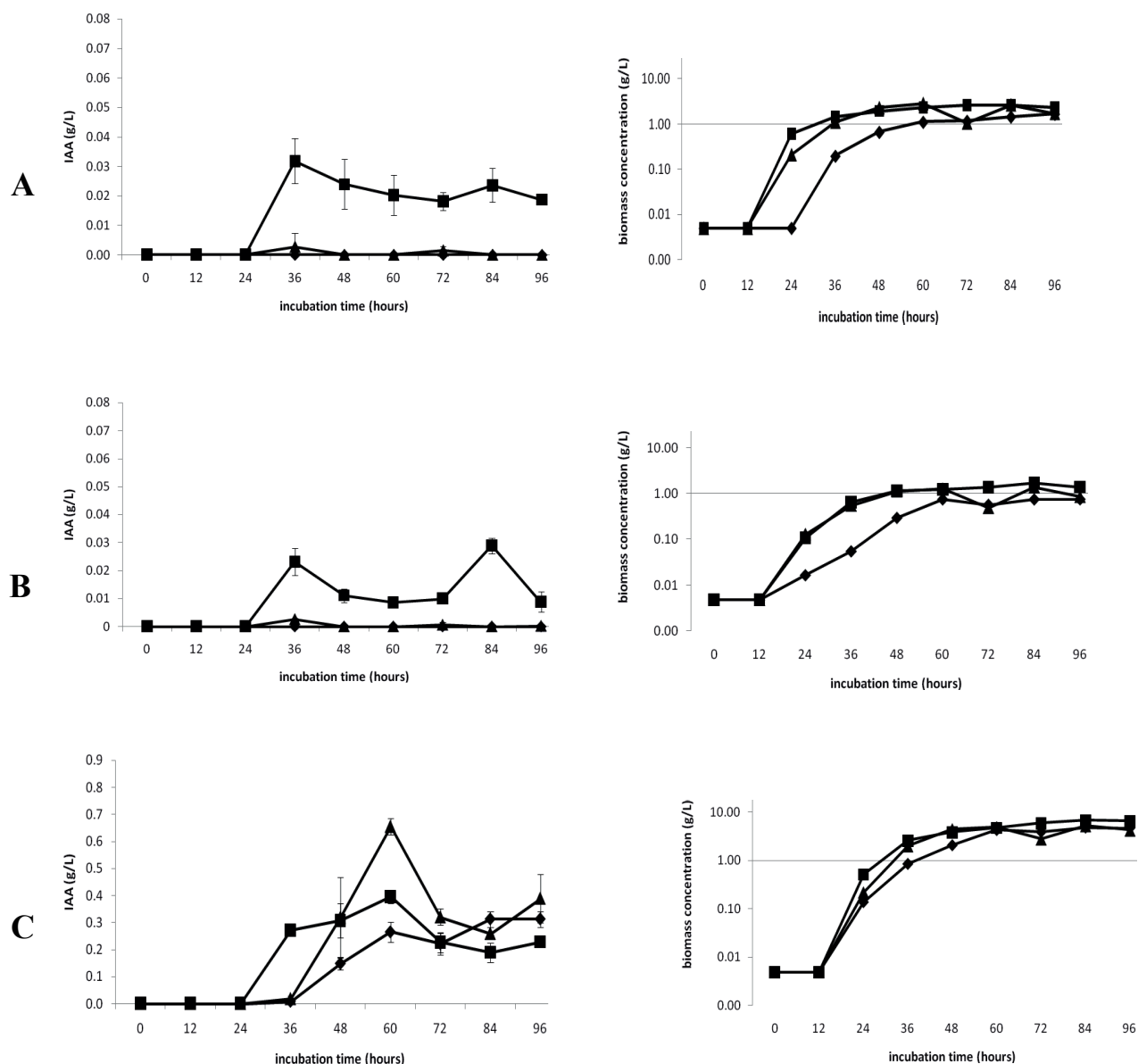
Figure 1. IAA production and cell biomass concentration of *T. asahii* (A – strain 3S44 and B – strain 4C06) and *Rh. mucilaginosa* (C – strain 2F32) at different medium pH ($\blacklozenge$- pH 3.0; $\blacksquare$ - pH 4.5; $\blacktriangle$ - pH 6.0). Cultures were incubated in a shaker (160 rpm) at 25ºC, with 2% glucose as the carbon source. Data was the means of three replicates. Bars at each point indicate ± SE.

**Table 1.** Maximum specific growth rate (μ_{max}) for yeast strains in the culture conditions evaluated.

Yeast strain	μ_{max} (h^{-1})	culture conditions		
		Temperature	pH	Carbon source
Rh. mucilaginosa (2F32)	0.19	25°C	6.0	glucose
T. asahii (4C06)	0.119	25°C	6.0	glucose
T. asahii (3S44)	0.072	25°C	6.0	glucose
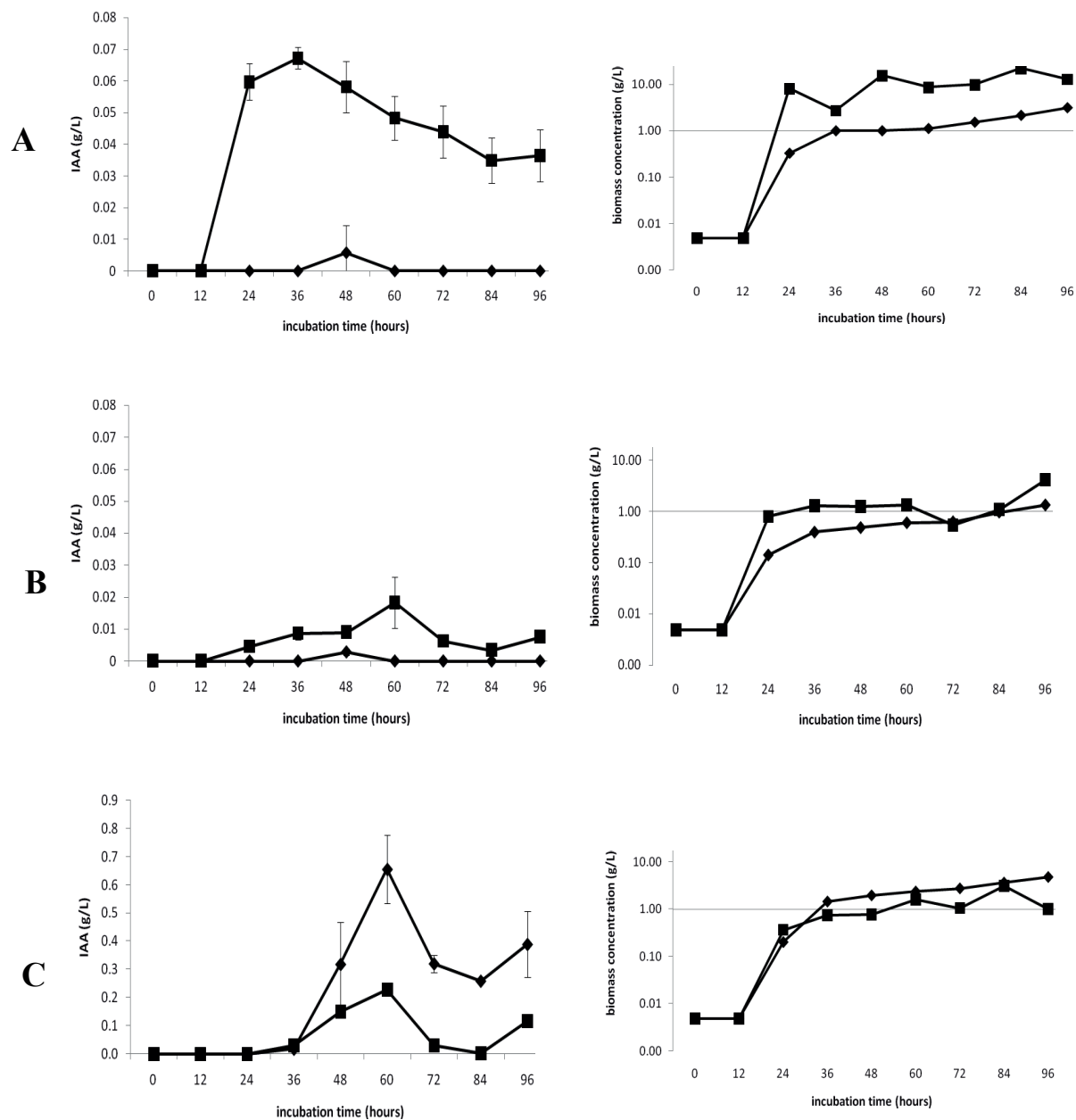
Figure 2. IAA production and cell biomass concentration of *T. asahii* (A – strain 3S44 and B – strain 4C06) and *Rh. mucilaginosa* (C – strain 2F32) with different carbon sources (♦ - 2% glucose; ■ – 2% sucrose). Cultures were incubated in a shaker (160 rpm) at pH 6.0, 25°C. Data was the means of three replicates. Bars at each point indicate ± SE.

Table 2. IAA yield (Y_{IAA}) from a given carbon source, for yeast strains in the culture conditions evaluated.

| Yeast strain	Y_{IAA} (µg/ml/h)	culture conditions			
		Temperature	pH	Carbon source	Time (hours)
Rh. mucilaginosa (2F32)	12	25°C	6.0	glucose	60
T. asahii (4C06)	0.64	25°C	4.5	glucose	36
T. asahii (3S44)	2.49	30°C	6.0	sucrose	24
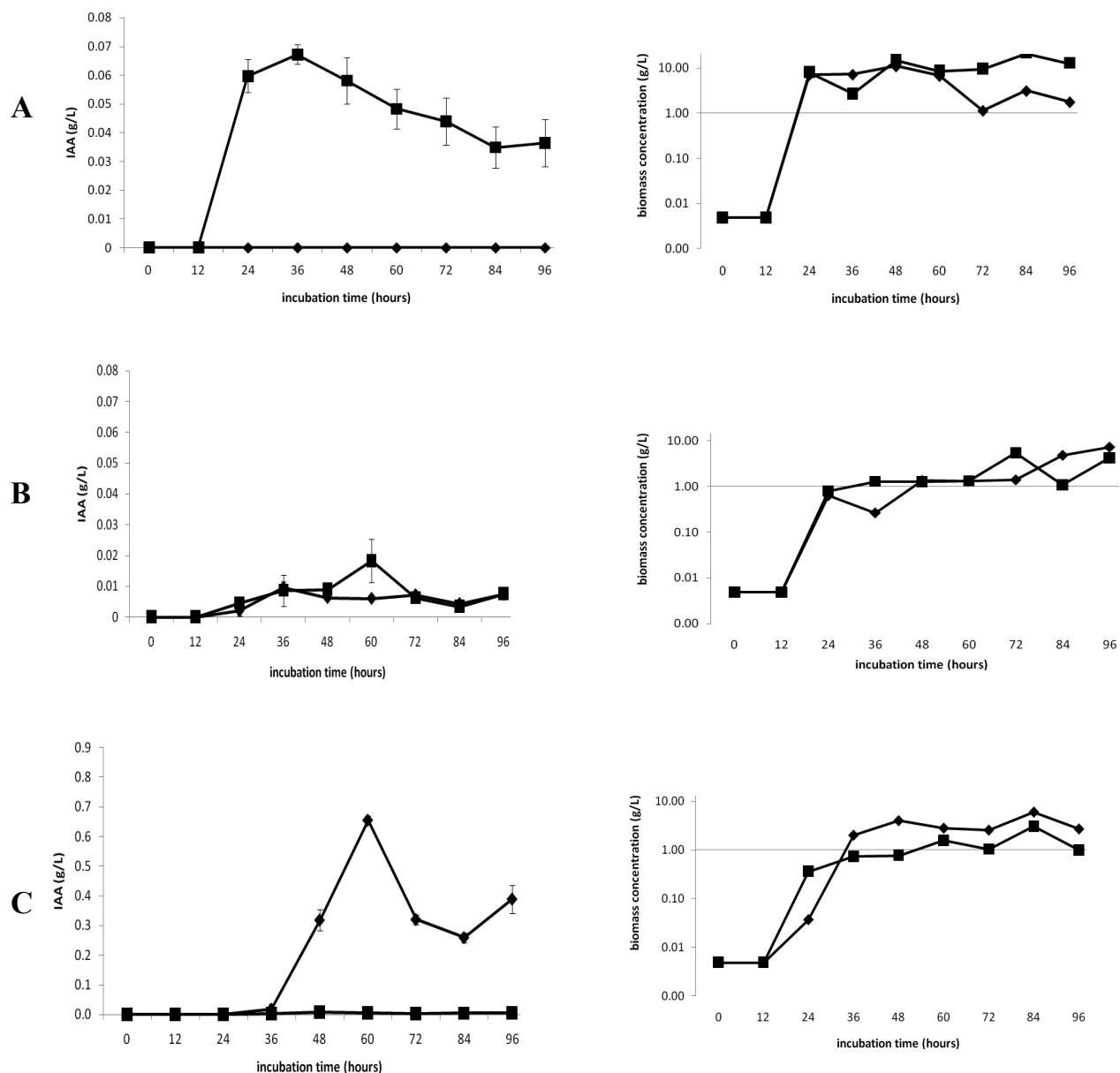
Figure 3. IAA production and cell biomass concentration of *T. asahii* (A – strain 3S44 and B – strain 4C06) and *Rh. mucilaginosa* (C – strain 2F32) at different pH (♦ - pH 4.5; ■ – pH 6.0) with 2% sucrose as the carbon source. Cultures were incubated in a shaker (160 rpm) at 25ºC, pH 6.0. Data was the means of three replicates. Bars at each point indicate ± SE.

production between the strains of *T. asahii*, such that strain 3S44 (isolated from the rhizosphere) showed better results compared with strain 4C06 (isolated from the stem). Limtong and Koowadjanakul (2012) isolated yeasts from the phylloplane of several plant species and noted that not all of the strains within the same species had the ability to produce IAA. Variations in IAA biosynthesis between strains within the same species have been reported by other investigators (Nutaratat et al., 2014; Ruanpanun et al., 2010; Sun et al., 2014; Tsvkelova et al., 2006).

Results suggests that temperature, pH and carbon source did not influence the cell growth for strains, indicating microbial stability in the different conditions. This adaptability is a very important feature for microorganisms with potential use in the scaling up of biotechnological processes, whether they are industrial or agricultural. For *Rh. mucilaginosa* (2F32), the results indicate that the optimum growth conditions also result in high IAA productivity. For the strains of *T. asahii*, the conditions for higher productivity were not the same as those for the maximum growth rate, in which one of the variables was changed, such as pH (for 4C06 strain), sugar source or temperature (for 3S44 strain). Sun et al. (2014) evaluated temperature and pH variations on IAA production by
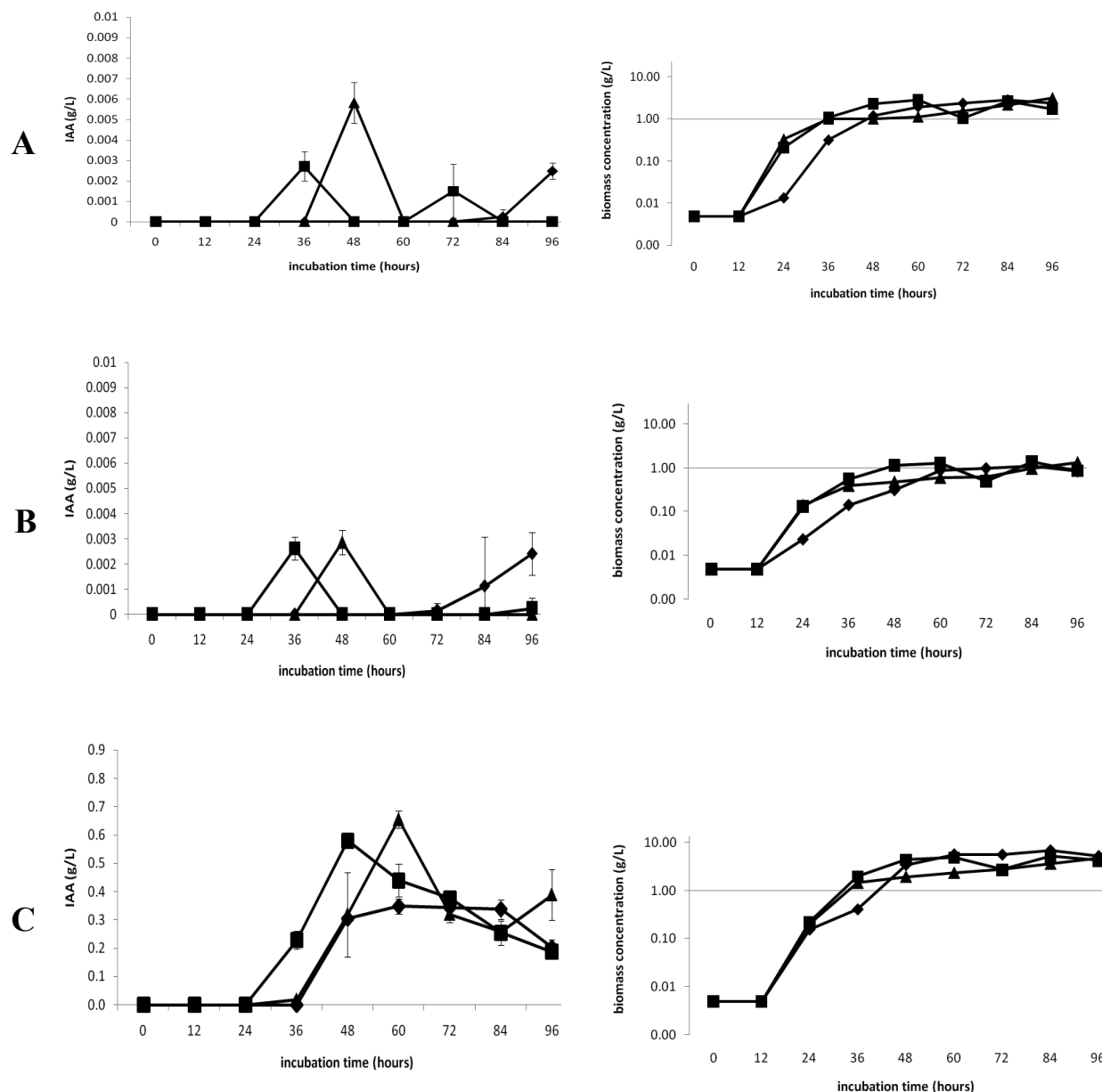
Figure 4. IAA production and biomass concentration of *T. asahii* (A – strain 3S44 and B – strain 4C06) and *Rh. mucilaginosa* (C – strain 2F32) at different temperatures (♦- 22°C; ■ - 25°C; ▲- 30°C). Cultures were incubated in a shaker (160 rpm) at pH 6.0, with glucose as the carbon source. Data was the means of three replicates. Bars at each point indicate ± SE.

yeasts isolated from a carnivorous plant (*Drosera indica*) and noted that the results were different for each strain evaluated. The authors reported that the yeasts were unable to produce IAA under alkaline conditions, and the majority of the strains presented high production values at 28°C compared to 37°C and 16°C. Yeast strains have different responses to environmental conditions, and these variations affect the production of phytohormones.

According to studies in the literature, IAA production is directly related to morphological changes in the yeast cells (production of pseudo-hyphae/stranded), which in turn are related to a "search" for nutrients (in the case of nutritional stress conditions) or host tissue invasion (for pathogenic yeasts) (Antonini and Sudbery, 2004; Prusty et al., 2004; Rao et al., 2010). In this study, however, IAA was produced under optimal or very close to ideal growing conditions. Furthermore, no cell morphological changes were observed during periods of higher IAA production, indicating that in this case there was no direct relationship between this production and the filamentation in the evaluated yeast strains.

CONCLUSIONS

Temperature, pH and carbon source can influence the IAA production of yeast strains of *Rhodotorula mucilaginosa* and *Trichosporon asahii*; however, these factors did not significantly influence cell growth. IAA production, when glucose was used as the carbon source, started after the initial stationary growth phase of yeast development. For *Rh. mucilaginosa*, the maximum production was obtained in the most appropriate conditions for yeast development. No relationship between the IAA production and morphological changes in the yeast cells was observed.

Our future goals include testing these strains in plant experiments to observe whether they are able to promote plant growth *in vivo*, and improving the use of these beneficial yeasts as an agricultural tool for more sustainable field production.

ACKNOWLEDGEMENTS

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