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Saccharomyces cerevisiae and Pichia pastoris Optimize NH_4NO_3 by 50% in Solanum lycopersicum Preventing N_2O Release

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Abstract

In agriculture, the unregulated application of NH_4NO_3 for healthy growth of Solanum lycopersicum is associated in the soil with the denitrification of free NO_3- (nitrate), with an increase in N_2O, a greenhouse gas, that contributes to global warming, loss of soil fertility. An ecological option to avoid denitrification that releases N_2O as other environmental problems is to inoculate S. lycopersicum seeds with endophytic yeasts that promote plant growth, such as Saccharomyces cerevisiae and Pichia pastoris, that increase the uptake of reduced doses of NH_4NO_3. In this sense, the purpose of this work was to analyze the response of S. lycopersicum to P. pastoris and S. cerevisiae at a dose of 50% NH_4NO_3. The experiment in a greenhouse, was made in agricultural soil, with a low level of nitrogen available, for the plant. Endophytic yeast were isolated flowers, from wild plants with beneficial activity, for domestic plants were used. To analyze the response of S. lycopersicum to P. pastoris and S. cerevisiae, a randomized trial was used: S. lycopersicum uninoculated with the yeasts: a) irrigated with water only, b) fed with 100% NH_4NO_3; 3 treatments of S. lycopersicum at 50% NH_4NO_3 inoculated with: c) S. cerevisiae, d) P. pastoris, e) S. cerevisiae + P. pastoris, and 6 repetitions: The following were considered as response variables: the germination of S. lycopersicum, at the seedling level: plant height (PH) and root length (LR), as well as aerial and radical fresh weight (AFW/RFW) and dry weight: aerial and radical (ADW/RDW). Numerical results were validated with ANOVA/Tukey $P<0.05$% The main results showed, a better positive response of S. lycoperiscum to P. pastoris at 50% NH_4NO_3; than with S. cerevisiae or the combination of both. Due to the ability of endophytic yeasts, to invade at the beginning of the formation of the root system, in germination, then in root development, that improved phenology and biomass at the seedling level. This positive effect of this yeasts on S. lycopersicum supports, that they converted organic compounds, from the roots into phytohormones, that maximally increased the uptake of NH_4NO_3 to 50%, which in the soil, prevents the generation of N_2O, the loss of fertility and contamination of surface or underground water, due to excess free nitrogen fertilizer.

Keywords: Soil, nitrogen fertilizer, endophytic yeasts, plant growth promoting microorganisms, global warming

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INTRODUCTION

Globally, the agricultural sector is recognized as the second largest, contributor to greenhouse gas emissions. It is estimated that by 2030 about 30-50% of N_2O, in the atmosphere will be due, to the indiscriminate use of nitrogen fertilizers, such as NH_4NO_3 (Malhi et al., 2021; Panchasara et al., 2021), [1,2] since between 60 and 90%, of the dose applied to promote and maintain, the growth of economically, important vegetable crops such as *Solanum lycoperiscum*, is not assimilated by the scarce root system, and therefore, the remainder causes in the soil the rapid degradation of organic matter, and as a consequence causes the generation of N_2O, a gas that contributes to global warming and with a global warming potential 298 times, greater than CO_2 (Kopittke et al., 2019; Hernández-Terrón et al., 2021).[3,4] To prevent these problems a sustainable option is to apply, in *S. lycoperiscum* seeds with *Saccharomyces cerevisiae* and *Pichia pastoris* genus, and species of plant growth promoting endophytic yeasts, that are competitive and effective in optimizing NH_4NO_3 to 50% to avoid N_2O generation and simultaneously maintain soil fertility (Wu et al., 2019; Panchasara et al., 2021;Salvador et al, 2022), [2,6,8] since some endophytic yeasts live inside the roots; there they can utilize organic metabolites in phytohormones, which accelerate germination in seeds, and uptake of reduced dose of NH_4NO_3 optimization (Türkanoğlu et al., 2019; Mukherjee et al., 2020; Poveda et al., 2021). The aim of this research was, to analyze effect of *S. cerevisiae* and *P. pastoris* on optimization of NH_4NO_3 at 50 % on healthy growth of *S.lycopersicum.*

MATERIALS AND METHODS

A trial of inoculating seeds of *S. lycopersicum* with endophytic yeast was made in a glass house under the following environmental conditions: temperature of 23.2°C, luminosity of 450 µmol m-2 s^{-1} and relative humidity of 67%. For this test, soil collected from a site located at 19° 37' 10" of north latitude 101° 16' 41.999" west longitude, with an altitude of 2013 meters above sea level with a temperate climate in an agricultural land called "Uruapilla" belonging to the municipality of Morelia, on the Morelia-Pátzcuaro highway, Michoacan, Mexico, which the following properties with a pH of 6.64 slightly acidic, an organic matter concentration of 4.57% indicated as a moderate amount of organic carbon; the level of available nitrogen was poor, less than 0.10%, while one of texture: clay 22.16%, silt 37.28% and sand 40.56% in soil, classified as loam-clayey-sandy (Nardi et al., 2018). To homogenize the particle size of the soil, it was sieved through a 0.4mm diameter mesh. While to avoid interference from agricultural diseases and pests, it was solarized for 48 h. The isolation of plant growth promoting endophytic yeasts (PGPEY) was from the root, stem and leaf of *Amarylis belladonna* (summer lily), *Clivia miniata* (clivia), *Spanthodea campanulata* (african tulip). For this, plant tissues were disinfected with a 3% sodium hypochlorite solution/3 min, washed 6 times with sterile water, then disinfected with 75% ethanol/3 min and washed 6 times with sterile water. The plant organs were cut into 5 cm pieces, macerated with a 0.85% saline solution *Roma*^{MR} 0.01% detergent (SSD), from there 1.0 mL was taken in yeast malt agar (YMA) (g/L): 3.0 yeast extract, 3.0 malt extract, 10.0 sucrose, 5.0 peptone of casein, 18.0 of bacteriological agar, adjusted to pH 5.5 and 10 mL of ciprofloxacin at 50 mg/mL to eliminate any bacteria, then incubated at 30°C/36 h. The microorganisms that grown in YMA based in microscopy and colonial morphology characteristics. The biochemical identification of the endophyticyeasts was carried out according to Ling et al., 2020; including morphological and physiological characteristics in: Sabouraud Agar or SA (g/L): glucose 25, peptone 10, extract yeast 1.0, bacteriological agar 18, pH of 5.5 to 6; Malt Yeast Extract Agar or MYEA (g/L): malt extract, 3.0; yeastextract, 3.0; peptone, 5.0; glucose, 10.0; 18.0 agar, pH 5.0; Malt Yeast Broth or MYB (g/L): malt extract, 3.0; yeast extract, 3.0; peptone, 5.0; glucose, 10.0, pH at 5.0; Wickerham-1 (g/L): glucose 10, yeast extract, 4.5; peptone, 7.5 and pH 6.5; and Wickerham-2 (g/L): KH_2PO_4, 0.15; $MgSO_4H_2O$, 0.5; NaCI, 0.1; CaCI, 0.1; dextrose, 10.0; NH_4CI, 0.5, pH 6.5, 1 mL of ciprofloxacin was added to each culture medium at a concentration of 50 mg/mL. (Ling et a., 2020; Fernández-San Millan et al., 2020). Subsequently, all the culture media were inoculated with 500 and 1000 µL, then incubated until growth was observed at 27°C and performed the following biochemical tests: catalase, fermentation of glucose and sucrose, Simmons citrate. Three collection strain from the Environmental Microbiology Laboratory were used as a reference: *P. pastoris* and *S. cerevisiae*, were grown on MYEA, *S. cerevisiae* and *P. pastoris* strains in yeast extract and casein broth with the following chemical composition (g/L): sucrose, 10; casein peptone, 5; yeast extract, 5; and pH of 6; incubated at 30°C/24h/150 rpm (Mukherjee et al., 2020; Poveda et al., 2021; Salvador et al 2022) [8,11,12]. Subsequently, seeds of *S. lycopersicum* were disinfected with sodium hypochlorite (Clorox®) 0.5%/2.0 min and washed 3 times, followed by 60% alcohol/3min, finally washed 3 times sterile deionized water. Then, for every 10 seeds, they were inoculated with 1.0 mL of a suspension of *S. cerevisiae* and *P. pastoris* individually and mixed in a 1:1 ratio (v/v) with a density equivalent to 1.5×108 CFU/mL obtained by viable plate count (VPC) on yeast extract and casein agar with pH adjusted to 7.0. The seeds inoculated with *S. cerevisiae* and *P. pastoris* were left in agitation at 150 rpm for 30 min, then sown in 1.0 kg of previously sieved and solarized soil in the upper part of Leonard jars (Figure 1) and in the lower part water or 50% mineral solution was added according to the experimental design shown in Table 1. The volume to ensure field capacity at 80% was 16 mL, which was applied every third day until the end of the experiment, that was carried out under the following conditions: average temperature of 23.2°C. The following were considered as response variables: the germination of *S. lycopersicum*, and then at seedling level: plant height (PH) and root length (LR), as well as aerial and radical fresh weight (AFW/RFW) and dry weight: aerial and radical (ADW/RDW). Experimental data will be validated with the statistical program ANOVA/Tukey HSD P<0.5% with Statgraphics Centurion (Fernández-San Millan et al., 2020).

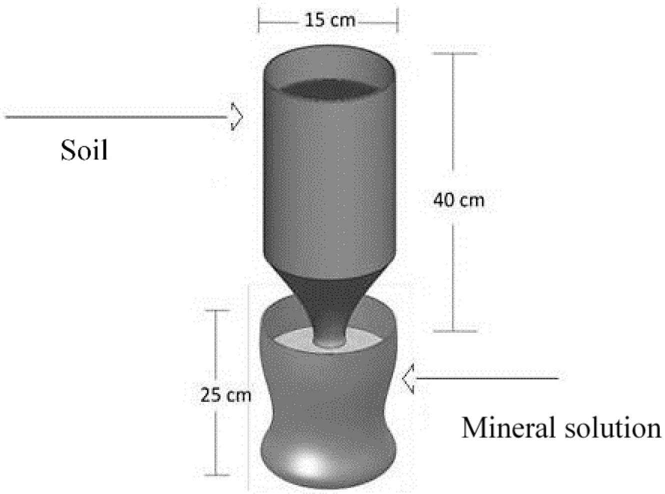

Figure 1. Design diagram of Leonard jar.

Table 1. Experimental design to analyze the effect of *Saccharomyces cerevisiae* and *Pichia pastoris* on *Solanum lycopersicum* at 50% NH_4NO_3

Solanum lycopersicum*	***Saccharomyces cerevisiae***	***Pichia pastoris***	**Water**	**NH_4NO_3 (%)**
Absolute control	-	-	+	-
Relative control	-	-	-	100
Treatment 1	+	-	-	50
Treatment 2	-	+	-	50
Treatment 3	+	+	-	50

**Number of replicates (n) = 6; added (+); not added (-).*

RESULTS AND DISCUSSION

Table 2 shows the positive effect of *S. cerevisiae* on *S. lycopersicum* t 50% NH_4NO_3, where 98.6% germination was registered, a numerical value with statistical difference compared to the numerical values registered in the germination of *S. lycopersicum* with *S. cerevisiae* and *P. pastoris* at 50% NH_4NO_3, the same way 83.3% germination of *S. lycopersicum* uninoculated, fed at 100% NH_4NO_3 regard as a relative control. The better per cent on germination of *S. lycopersicum* seeds with *S. cerevisiae* and *P. pastoris* supports both due to are able to live inside of vegetal tissues could to convert organic compounds derived from endosperm degradation into phytohormones that ended embryonic dormancy to accelerate germination percentage of *S. lycopersicum* seeds (Türkanoğlu et al., 2019; Ling et al., 2020; Poveda et al., 2021) [9–15]. This fact supports that endophytic yeast are an option for sustainable agriculture to improve uptake nitrogen fertilizer as well as other plant growth promoting bacteria (Ling et al., 2020; Mukherjee et al 2020) [11,13]. In Figure 2 is shown the positive effects of *S. cerevisiae* and *P. pastoris* on *S. lycopersicum* based in phytohormons as well as auxin and gibberellins related with germination and first step of growth of the primordium of young plants of *S. lycopersicum* compared to the same plant uninoculated irrigated only with water or absolute control (AC) and with *S. lycopersicum* uninoculated fed with at 100% of NH_4NO_3 (Nardi et al., 2018; Zhou et al., 2018; Fernandez-San Millan et al., 2020) [14-16].

Table 2. Effect of *Saccharomyces cerevisiae* and *Pichia pastoris* on germination of *Solanum lycopersicum* at 50% NH_4NO_3.

Treatment/*Solanum lycopersicum****	**Germination percentage (%)**
Absolute control (AC) Irrigated with water	75.5^{d**}
Relative control (RC) Fed at 100% NH_4NO_3	83.3^{c}
Treatment 1 (T1) *Saccharomyces cerevisiae* + 50% NH_4NO_3	98.6^{a}
Treatment 2 (T2) *Pichia pastoris* + 50% NH_4NO_3	91.5^{b}
Treatment 3 (T3) *S. cerevisiae* and *P. pastoris* + 50% NH_4NO_3	92.3^{b}

**n =6; **different letters indicate statistical difference according to ANOVA/Tukey (P<0.05).*

Figure 2. Effect of Saccharomyces cerevisiae and Pichia pastoris on germination of Solanum lycopersicum seeds with 50% NH_4NO_3.

Absolute control (AC) = *S. lycopersicum* uninoculated irrigated with water; Relative control (RC) = *S. lycopersicum* uninoculated fed at 100% NH_4NO_3; T1= *S. lycopersicum* plus *S. cerevisiae* at 50% NH_4NO_3; T2= *S. lycopersicum* plus *P. pastoris* at 50% NH_4NO_3; T3= *S. lycopersicum* plus *S. cerevisiae* and *P. pastoris* at 50% NH_4NO_3.

Table 3. Phenology and biomass of *Solanum lycopersicum* with *Saccharomyces cerevisiae* and *Pichia pastoris* at 50% NH_4NO_3 at seedling level.

Treatment/*Solanum lycopersicum****	**Plant height (cm)**	**Root length (cm)**	**Fresh weight (g)**		**dry weight ((g)**	
			Aerial	***Radical***	***Aerial***	***Radical***
Irrigated with water Absolute control (AC)	14.18^{d**}	8.45^{d}	1.09^{d}	0.26^{c}	0.12^{d}	0.02^{d}
Fed at100% NH_4NO_3 Relative control (RC)	17.75^{c}	11.28^{c}	1.89^{c}	0.37^{b}	0.20^{c}	0.04^{b}
Saccharomyces cerevisiae + 50% NH_4NO_3 Treatment 1 (T1)	20.53^{b}	13.90^{b}	2.19^{b}	0.39^{b}	0.22^{c}	0.04^{b}
Pichia pastoris + 50% NH_4NO_3 Treatment 2 (T2)	24.93^{a}	16.89^{a}	3.59^{a}	0.51^{a}	0.46^{a}	0.05^{a}

S. cerevisiae and *P. pastoris* + 50% NH_4NO_3 Treatment 3 (T3)	21.76^b	14.71^b	2.78^b	0.40^b	0.31^b	0.04^b

**n =6; **different letters indicate statistical difference according to ANOVA/Tukey (P<0.05).*

Table 3 shows the phenology at the seedling level, of *S. lycopersicum* with *P. pastoris* at 50% NH_4NO_3, there 24.93 cm of PH and 16.89 cm of RL were registered, both statistically different values, compared to the numerical data, registered in *S. lycopersicum* with *S. cerevisiae* and *P. pastoris*, at 50% NH_4NO_3, as well as the 17.75 cm of PH, and 11.28 cm of RL of *S. lycopersicum*, uninoculated, fed at 100% or relative control (RC). Regarding fresh and dry biomass, *S. lycopersicum* with *S. cerevisiae*, at 50% NH_4NO_3, were registered: 3.5912 g AFW, 0.1591 g RFW, 0.4658 g ADW and 0.0591 g RDW. These numerical values, have statistical difference, compared to these registered, in *S. lycopersicum* uninoculated with endophytic yeasts, fed with 100% NH_4NO_3 or RC. The phenology and biomass data registered, for *S. lycopersicum* inoculated singly, and in mixture with *S. cerevisiae* and *P. pastoris*, indicate that both yeasts, endophytically colonized the root, and stem primordium, by transforming organic compounds, from photosynthesis into phytohormones necessary, to induce abundant formation of the root system, sufficient to optimize 50% NH_4NO_3 (Fernandez-San et al., 2020). In that sense, the Figure 3, shows the response *S. lycopersicum* to *P. pastoris* and 50% NH_4NO_3, registered an increase in stem diameter, and root system area, compared to *S. lycopersicum* uninoculated, with *S. cerevisiae* and *P. pastoris* fed with 100% NH_4NO_3, referred as a RC. It was clear, that *S. lycopersicum* has better responded, in terms of phenology to *P. pastoris*, according on the treatment 2 (T2,) due to better transformation of the plant organic compound, into phytohormons, from the root metabolism, to enhance its healthy growth (Ling et a., 2020; Fernández-San Millan et al., 2020; Poveda et al., 2021),[12-14] compared to, the same respond of *S. lycopersicum*, to *S. cerevisiae* on treatment 1 (T1), where less phytohormons, were release to improve its growth at 50% NH_4NO_3, including when both yeast were applied, in *S. lycopersicum* on treatment 3 (T3), in that sense an evident, competition in the colonization of the radical system, for organic compounds from the root metabolism, that caused the positive effect to be, not similar on the phenology of *S. lycopersicum*, to when only *P. pastoris* was applied (Sarabia et al., 2018; Wu et al., 2019).[5,6] However except in the case of *S. lycopersicum*, with *S. cerevisiae* and *P. pastoris*, fed at 50% NH_4NO_3, in the soil. While *S. lycopersicum* fed by 100% NH_4NO_3, uninoculated with endophytic yeasts, allows the emission of N_2O, since NH_4NO_3, is not efficiently uptake, by the roots of *S. lycopersicum*, it facilitates that the nitrogen fertilizer, to be released as N_2O, by the via denitrification, to promote global warming besides other environmental problems (Nardi et al., 2018; Kopittke et al., 2019; Wu et al., 2019 Mali et al., 2021). [3-7]Reason why it is important to do: reduction of the dose of NH_4NO_3, and simultaneously inoculate the seeds, of *S. lycopersicum* with these beneficial endophytic yeasts, that ensure that there is no free nitrogen fertilizer, to avoid the generation of N_2O, as well as global warming.

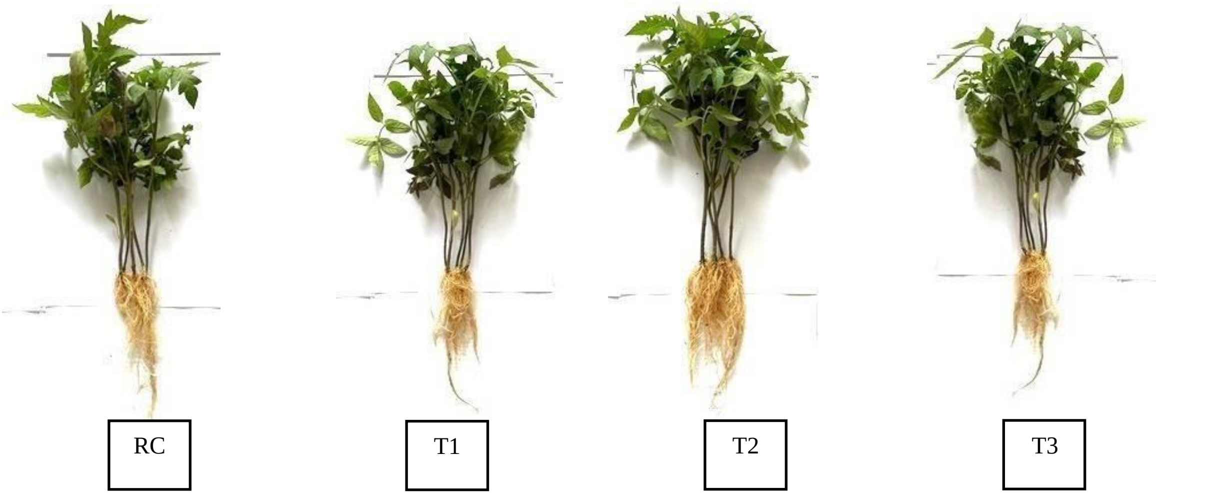

Figure 3. Response of *Solanum lycopersicum* to *Saccharomyces cerevisiae* and *Pichia pastoris* at

50% NH_4NO_3

Relative control (RC)= *S. lycopersicum* uninoculated fed at 100% NH_4NO_3; T1= *S. lycopersicum* with *S. cerevisiae* at 50% NH_4NO_3;

T2= *S. lycopersicum* with *P. pastoris* at 50% NH_4NO_3. T3= *S. lycopersicum* with *S. cerevisiae* and *P. pastoris* at 50% NH_4NO_3.

CONCLUSION

According to the results, *S. cerevisiae* and *P. pastoris* are useful for optimizing by up to 50% NH_4NO_3, preventing the loss of fertility of agricultural soils, as well as the contamination of surface and groundwater, additionally stopping the generation of N_2O, a greenhouse gas and global warming, caused by excess nitrogen fertilizers, to advance in the direction of sustainable agriculture, and not harm nature and recover the condition of quality of human life.

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Author contributions: Experimentation JLIC, PLTL, conceptualization JMSY methodology, JLIC and PLTL software, JLIC and JMSY; validation, JLIC and JMSY; results JLIC, DAPG and JMSY.; investigation, JLIC and JMSY; resources JMSY a writing—original draft preparation JMSY writing—review and editing JMSY; visualization DAPG and JMSY; supervision JMSY. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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