http://doi.org/10.1590/1678-992X-2021-0076

SCIENTIA AGRICOLA

ISSN 1678-992X

Research Article

Optimization of oxalic acid production by fungi for biotechnological solubilization of rock phosphate

Jeniffer Kelly Cortes Amaro[1], Laura Vieira Xavier[1], Michelle Miranda Araújo de Carvalho Ribeiro[2], Bruno Sérgio Vieira[1], Gilberto de Oliveira Mendes[1]*

[1]Universidade Federal de Uberlândia/Instituto de Ciências Agrárias – Lab. de Microbiologia e Fitopatologia, Rod. LMG-746, km 1, Bloco 1B-MC – 38500-000 – Monte Carmelo, MG – Brasil.

[2]Universidade Federal de Uberlândia/Instituto de Química, Av. João Naves de Ávila, 2121 – 13400-970 – Uberlândia, MG – Brasil.

*Corresponding author <gilbertomendes@ufu.br>

Edited by: Tiago Osório Ferreira

Received March 23, 2021

Accepted February 10, 2022

ABSTRACT: This study describes a biotechnological strategy for producing and applying oxalic acid to solubilize phosphorus (P) from rock phosphate (RP). We evaluated six fungal species (*Aspergillus niger* FS1, *Penicillium islandicum* FS41, *Pleurotus ostreatus* PO1, *Rhizoctonia solani* Rhiz555, *Sclerotium rolfsii* Sr25, and *Sclerotinia sclerotiorum* Scl134) and three culture media (potato dextrose broth, Tsao and Strasser media) to maximize oxalic acid production. Among the fungal isolates tested and culture media, *S. rolfsii* Sr25 and Tsao medium showed efficient oxalic acid production. Tsao medium was optimized following a response surface methodology after initial screening of factors affecting RP solubilization. The optimized concentrations were 1 g L^{-1} $NaNO_3$, 100 g L^{-1} glucose, 2 g L^{-1} KH_2PO_4, 4.5 g L^{-1} yeast extract, and 25 mg L^{-1} $MgSO_4 \cdot 7H_2O$ used for 20 days of incubation. Under these conditions, 71 mmol L^{-1} oxalic acid was obtained, representing a three-fold increase over production under non-optimized conditions (20 mmol L^{-1}). Under optimized conditions, oxalic acid produced by *S. rolfsii* Sr25 reacted with low-solubility RP and solubilized 100 % of the P contained in ore. Thus, using *S. rolfsii* Sr25 to produce oxalic acid seems a promising biotechnological alternative for P solubilization from RP.

Keywords: phosphorus, microorganism, oxalate, fertilizer

Introduction

Adequate phosphorus (P) supply is vital at all stages of plant development (Grant et al., 2001). However, P forms that are readily absorbable by plants are deficient in most soils due to P fixation on soil particles. This is aggravated in tropical soils, where phosphate is chemically adsorbed to aluminum (Al) and iron (Fe) oxides (Fontes and Weed, 1996). One of the predominant methods to overcome this deficiency is the use of soluble phosphate fertilizers. On tropical soils, these fertilizers are typically applied at dosages higher than the requirement of crops as a large part of the applied phosphate is adsorbed by soil components (Roy et al., 2016).

The primary P source for the production of fertilizers is rock phosphate (RP), a non-renewable resource that is costly and has nevertheless been increasingly exploited (Cordell et al., 2009). New technologies to reduce RP processing costs may substantially affect fertilizer production and reduce costs for farmers by facilitating the use of low-quality RP reserves, which are currently neglected due to technical difficulties and lack of profitability for its exploration (Mendes et al., 2020, 2021b).

Solubilization of RP by microorganisms is a promising method to produce phosphate fertilizers using renewable carbon sources and low-cost substrates (Mendes et al., 2020, 2021b). In these biotechnological systems, cultivation conditions can be modified to increase the synthesis of organic acids by microorganisms, which can be used for the solubilization of phosphate minerals. Among microbial organic acids, oxalic acid shows high acidity to RP solubilization (pK_{a1} 1.25, pK_{a2} 3.81) (Lide, 2004) with the potential for complexing Ca^{2+} in the apatite mineral that forms the rock (Kpomblekou-A and Tabatabai, 1994; Mendes et al., 2021a, b).

Several fungi produce oxalic acid, by including ectomycorrhizal fungi, wood-degrading basidiomycetes, saprophytes, and phytopathogens (Dutton and Evans, 1996). Thus, oxalic acid-producing fungi show great biotechnological potential for phosphate solubilization. This research evaluated a high-performance biotechnological method of producing oxalic acid using fungi for RP solubilization.

Materials and Methods

Microorganisms

Isolates of *Rhizoctonia solani* (Rhiz555), *Sclerotium rolfsii* (Sr25), and *Sclerotinia sclerotiorum* (Scl134) were obtained from the Embrapa (Brazil) collection. *Aspergillus niger* (FS1) and *Penicillium islandicum* (FS41) were obtained from the collection of the Laboratory of Microbial Ecology, Federal University of Viçosa. *Pleurotus ostreatus* (PO1) was obtained from the collection of the Laboratory of Microbiology and Phytopathology, Federal University of Uberlândia. These fungal species have been reported as good oxalic acid producers (Durman et al., 2005; Mendes et al., 2014; Punja and Jenkins, 1984; Strasser et al., 1994; Tsao, 1963; Yang et al., 1993). The isolates were kept at 28 °C on Petri dishes with a culture medium of potato dextrose agar (PDA).

Culture media and cultivation conditions

The following culture media previously reported as stimulating fungal oxalic acid production were examined: a) potato dextrose broth with adequate conditions for oxalic acid production by different fungi, mainly of the genera *Rhizoctonia* (Yang et al., 1993) and *Sclerotinia* (Durman et al., 2005); b) Tsao medium (2 g $NaNO_3$; 1.5 g KH_2PO_4; 1 g $MgSO_4 \cdot 7H_2O$; 40 g glucose; 2 g yeast extract; 1 L distilled water), which elicits high production of oxalic acid by the fungus *Pleurotus ostreatus* (Tsao, 1963); c) Strasser medium (100 g sucrose; 1.5 g $NaNO_3$; 0.5 g KH_2PO_4; 0.025 g $MgSO_4 \cdot 7H_2O$; 0.025 g KCl; 1.6 g yeast extract; 1 L distilled water), as optimized for oxalic acid production by *A. niger* (Strasser et al., 1994). Tsao medium was modified for the optimization experiments as indicated in the respective sections. Culture media were placed in 125-mL Erlenmeyer flasks and autoclaved at 121 °C for 20 min.

Selection of fungi and culture media

The best fungus and culture medium combination for oxalic acid production was screened in 125-mL Erlenmeyer flasks containing 30 mL of PDB, Tsao, or Strasser media. Flasks were inoculated with three mycelial plugs (5 mm) of each fungus taken from the edges of colonies grown in PDA for 7 days at 28 °C. Inoculated flasks were incubated for 15 days in an orbital shaker at 240 rpm and 28 °C. Uninoculated flasks were used as controls.

The experiment was set up in a completely randomized design in a 6 × 3 factorial scheme with three replications. Data were subjected to the analysis of variance (ANOVA) and treatments were compared using the Fisher's least significant difference (LSD) test ($p < 0.05$).

Optimization of oxalic acid production by *S. rolfsii* Sr25

The combination of fungus (*S. rolfsii* Sr25) and culture medium (Tsao) that produced the highest amount of oxalic acid was selected for optimization. Initially, the effect of each component of the Tsao culture medium, as well as the effect of Mn addition (Pedersen et al., 2000; Ruijter et al., 1999), pH buffering (Strasser et al., 1994), and incubation time on oxalic acid production by *S. rolfsii* Sr25 were tested in a two-level fractional factorial experiment (2^{8-4}, 1/16 fraction, resolution 4) with two replications of the complete experiment, totaling 32 runs. Each factor was assessed at two levels, coded as −1 (lower level) and +1 (upper level) in the experimental design (Table 1).

The experiment was conducted using 125-mL Erlenmeyer flasks containing 30 mL of culture medium. To produce a pH-buffered medium, components were diluted in 0.1 mol L^{-1} Tris-HCl buffer, whereas the unbuffered medium was prepared using distilled water.

Table 1 – Screening of factors controlling the biosynthesis of oxalic acid by *Sclerotium rolfsii* Sr25.

Factor	Lower level (–1)	Upper level (+1)	Effect[a]
Glucose (g L^{-1})	10	100	3.893*
$NaNO_3$ (g L^{-1})	1	3	–2.021
KH_2PO_4 (g L^{-1})	0.5	2	5.291*
Yeast extract (g L^{-1})	1	3	6.208*
$MgSO_4.7H_2O$ (g L^{-1})	0.025	1	–3.754*
$MnCl_2.4H_2O$ (g L^{-1})	0	0.2	0.986
pH (7.0)	Unbuffered	Buffered	1.815
Incubation time (d)	10	20	10.268*

*Significant by the *t* test ($p < 0.05$). [a]Effect = $m_+ - m_-$ (m_+ and m_- represent the average oxalic acid produced for the upper and lower level of each factor, respectively).

In both cases, the initial pH was adjusted to 7.0. The medium was autoclaved (121 °C, 20 min) and inoculated with three mycelial plugs (5 mm) of *S. rolfsii* Sr25 taken from the edges of colonies grown in PDA for 7 days at 28 °C.

The experimental design and analysis of the effect of each variable on oxalic acid biosynthesis were performed using the Design of Experiments (DOE) procedure in Minitab 18 software. The main effect of each factor was calculated according to Eq. (1) (Almeida et al., 2014; Mendes et al., 2015):

$$E_m = m_+ - m_- \quad (1)$$

where: E_m is the main effect, m_+ is the average amount of oxalic acid produced at the upper level of a factor, and m_- is the average amount of oxalic acid produced at the lower factor level. The effect values calculated were subjected to the *t* test to evaluate whether they differed from zero at a 5 % probability level.

Factors that affected oxalic acid biosynthesis were selected for optimization by the response surface methodology (RSM). The factor levels in the design were calculated based on the factor increment with the highest product between the variation unit and the estimated regression coefficient obtained in the screening experiment (Table 2) (Box and Wilson, 1951). In this calculation, the central point (coded as 0) of a central composite design (CCD) (Myers et al., 2016) was defined as the highest or lowest factor level in the screening experiment when its effect on oxalic acid biosynthesis was positive or negative, respectively. The CCD was completed with two levels above the central point (coded as 1 and 2) and two levels below (coded as −1 and −2) (Table 3), which were determined according to the relative increments calculated (Table 2). Factors with no effect were added to the most economical conditions, that is, $NaNO_3$ at the lowest concentration (1 g L^{-1}) and initial pH 7 without buffering. The experiment was conducted using 125-mL Erlenmeyer flasks containing 30 mL culture medium, according to CCD combinations (Table 3). The media were inoculated and incubated as described for the screening experiment.

Table 2 – Calculation of factor levels for experimental design to fit response surface. The changes in factor levels were based on a 5-unit increment in time, which was the variable with the largest regression coefficient. Data used in the calculations were obtained in the screening experiment.

	Glucose	KH_2PO_4	Yeast extract	$MgSO_4.7H_2O$	Time
	g L^{-1}				d
Base level (0)[a]	55	1.25	2	0.5125	15
Unit	45	1.5	1	0.4875	5
Estimated coefficient (f_t)[b]	1.946	2.646	3.104	–1.877	5.134
Unit × f_t	87.57	3.97	3.10	–0.92	25.67
Increment (Δ) relative to increment in time[c]	17.1	0.77	0.60	–0.18	5

The calculations were done according to Box and Wilson (1951). [a]Related to the experiment shown in Table 1, representing the average value between the levels –1 and +1. [b]Change in oxalic acid biosynthesis per unit, obtained in the screening experiment. [c]Changes in factor levels were calculated based on the change in the level of the factor with the highest regression coefficient in the screening experiment, that is, incubation time.

Data of oxalic acid biosynthesis by *S. rolfsii* Sr25 were used to adjust a polynomial quadratic model with a multiple regression procedure using the least squares method. The largest model was as described in Eq. (2) (Almeida et al., 2014):

$$\hat{Y} = \beta_0 + \sum_{i=1}^{n}\beta_i x_i + \sum_{i=1}^{n}\beta_{ii}x_i^2 + \sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\beta_{ij}x_i x_j + \varepsilon_{ij} \qquad (2)$$

where: $\hat{Y}$ is the estimated concentration of oxalic acid produced by *S. rolfsii* Sr25, i and j assume a value between one and the total number of factors (n), β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the coefficient of the quadratic term, β_{ij} is the interaction coefficient, x_i and x_j are the factor levels, and ε_{ij} is the experimental error. An ANOVA of the adjusted coefficients and lack-of-fit tests was performed on the adjusted model ($p < 0.05$). Coefficients with a p-value greater than or equal to 0.1 were removed from the model. The experimental design and all calculations and analyses were executed in the DOE option of Minitab 18.

Table 3 – Oxalic acid biosynthesis by *Sclerotium rolfsii* Sr25 under different combinations of factor levels in a central composite design (CCD).

#	Glucose	KH_2PO_4	Yeast extract	$MgSO_4.7H_2O$	Incubation time	Oxalic acid observed	Oxalic acid estimated
	g L^{-1}				d	mmol L^{-1}	
1	80 (–1)[a]	1.25 (–1)	2.25 (–1)	12.5 (–1)	22.5 (1)	25.50	23.40
2	120 (1)	1.25	2.25	12.5	17.5 (–1)	35.92	39.32
3	80	2.75 (1)	2.25	12.5	17.5	29.36	25.99
4	120	2.75	2.25	12.5	22.5	50.03	46.82
5	80	1.25	3.75 (1)	12.5	17.5	28.78	35.57
6	120	1.25	3.75	12.5	22.5	60.48	56.41
7	80	2.75	3.75	12.5	22.5	-	43.08
8	120	2.75	3.75	12.5	17.5	68.85	59.00
9	80	1.25	2.25	37.5 (1)	17.5	38.88	41.90
10	120	1.25	2.25	37.5	22.5	34.34	31.72
11	80	2.75	2.25	37.5	22.5	40.76	49.41
12	120	2.75	2.25	37.5	17.5	33.62	34.31
13	80	1.25	3.75	37.5	22.5	-	58.99
14	120	1.25	3.75	37.5	17.5	43.16	43.89
15	80	2.75	3.75	37.5	17.5	61.74	61.58
16	120	2.75	3.75	37.5	22.5	53.75	51.40
17	60 (–2)	2 (0)	3 (0)	25 (0)	20 (0)	54.85	48.20
18	140 (2)	2	3	25	20	45.53	53.93
19	100 (0)	0.5 (–2)	3	25	20	31.38	28.25
20	100	3.5 (2)	3	25	20	34.09	38.34
21	100	2	1.5 (–2)	25	20	48.33	45.55
22	100	2	4.5 (2)	25	20	70.92	74.82
23	100	2	3	0 (–2)	20	37.50	45.61
24	100	2	3	50 (2)	20	58.58	56.52
25	100	2	3	25	15 (–2)	29.87	28.69
26	100	2	3	25	25 (2)	31.30	33.60
27	100	2	3	25	20	56.24	51.07
28	100	2	3	25	20	55.81	51.07
29	100	2	3	25	20	60.90	51.07
30	100	2	3	25	20	55.44	51.07
31	100	2	3	25	20	46.93	51.07
32	100	2	3	25	20	37.75	51.07

[a]Values in parentheses are the coded levels.

RP solubilization using fungal oxalic acid

The combination of factor levels that resulted in the highest production of oxalic acid (71 mmol L^{-1}) by *S. rolfsii* Sr25 in the response surface experiment was used in the RP solubilization experiment: 1 g L^{-1} $NaNO_3$, 100 g L^{-1} glucose, 2 g L^{-1} KH_2PO_4, 4.5 g L^{-1} yeast extract, and 25 mg L^{-1} $MgSO_4 \cdot 7H_2O$. The experiment was carried out using 125-mL conical flasks with 30 mL of sterile culture medium and with the pH adjusted to 7, but without buffering. Flasks were inoculated and incubated as described for the screening experiment.

Rock phosphate was added to the medium at a concentration of 10 g L^{-1} at the beginning of the incubation together with the fungus (one step) or 20 days after inoculation of the medium with the fungus (two steps). RP was sterilized together with the culture medium in the one-step system, while in the two-step system, RP was sterilized separately by autoclaving at 121 °C for 20 min. Uninoculated flasks containing a sterile mixture of RP and liquid medium were incubated as controls. The RP sample was obtained in the municipality of Pratápolis, Minas Gerais State, Brazil (20°47′58.2″ S, 46°50′39.9″ W, altitude 836 m). It contained 10.5 % P, with particles measuring < 63 µm. The experiment was conducted in five replications per treatment. Differences between treatments were tested using the ANOVA, followed by the Tukey's post hoc test ($p < 0.05$) using the Experimental Designs package in R version 3.6.3.

Analytical methods

At the end of the incubation period in the oxalic acid production experiments, all samples were vacuum-filtered through a 0.2-µm membrane. Fungal biomass retained in the filter was transferred to a crucible and dried to constant weight in an oven at 80 °C. The filtrate was analyzed to quantify the amount of produced oxalic acid and measure the pH. Oxalic acid was quantified using capillary electrophoresis. Electropherograms were produced using capillary electrophoresis equipment with two detectors of capacitively coupled contactless C^4D conductivity (Francisco and Lago, 2009; Silva and Lago, 1998), which were positioned near the capillary at 10 cm from each end. The fused silica capillary used in all experiments was 50 cm long and 50 µm i.d. × 375 µm o.d. The effective lengths were 10 and 40 cm for the first and second detectors, respectively. Before use, the capillary was washed using deionized water for 10 min, 0.1 mol L^{-1} NaOH for 10 min, deionized water for 10 min, and background electrolyte (running buffer) for 10 min.

At the end of the 30 day of the RP solubilization experiment, all samples were filtered using quantitative filter paper (25 µm pore size) for the pH and P analysis. Soluble P was determined by molecular absorption spectrophotometry using the ascorbic acid method (Braga and Defelipo, 1974).

Results

Selection of fungi and culture media for oxalic acid production

The highest oxalic acid production (20 mmol L^{-1}) was achieved by *S. rolfsii* Sr25 in Tsao medium (Figure 1A). Similar oxalic acid concentrations were produced by *A. niger* FS1 and *S. rolfsii* Sr25 in PDB medium. In general, fungi that produced more oxalic acid induced more significant acidification of the medium (Figure 1B). The Strasser medium stimulated biomass production; however, without increasing oxalic acid production (Figure 1C).

Optimization of oxalic acid production by *S. rolfsii* Sr25

Only five evaluated factors affected oxalic acid production by *S. rolfsii* Sr25 (Table 1). Higher dosages of glucose, KH_2PO_4, and yeast extract and longer incubation time increased oxalic acid biosynthesis, whereas a higher dosage of $MgSO_4 \cdot 7H_2O$ decreased the production (Figure 2). The other factors (pH, $NaNO_3$, and $MnCl_2 \cdot 4H_2O$) did not influence oxalic acid production.

Three-fold higher concentrations of oxalic acid (an increase from 20 to 71 mmol L^{-1}) were produced using RSM to optimize the levels of factors affecting oxalic acid biosynthesis (Table 3). Contour plots show the optimal ranges of each factor (Figure 3). The highest oxalic acid production was observed at approximately 2.0 g L^{-1} KH_2PO_4 and after approximately 20 days of incubation. The optimal concentration of yeast extract ranged from 4.0 to 4.5 g L^{-1} and $MgSO_4 \cdot 7H_2O$ was required at concentrations below 25 mg L^{-1}.

RP Solubilization using fungal oxalic acid

No difference between the one-step and two-step RP solubilization processes was observed ($p > 0.05$), with a mean concentration of 1,832 mg L^{-1} soluble P (Figure 4). On average, 1,395 mg P L^{-1} was solubilized by *S. rolfsii* Sr25 by subtracting the soluble P added to the medium as KH_2PO_4. The amount of RP added yielded 1,050 mg L^{-1} P, indicating that *S. rolfsii* Sr25 solubilized 100 % of the P present in RP.

The pH values of the culture medium differed ($p < 0.05$) between treatments at the end of the incubation period, with values of 2.4, 3.4, and 5.3 for the one-step, two-step, and control treatments, respectively.

Discussion

Solubilization of 100 % of the P in low-solubility RP was achieved using oxalic acid produced by *S. rolfsii* Sr25. An initial screening showed that oxalic acid production by *S. rolfsii* Sr25 was affected by the concentrations of glucose, KH_2PO_4, yeast extract, and

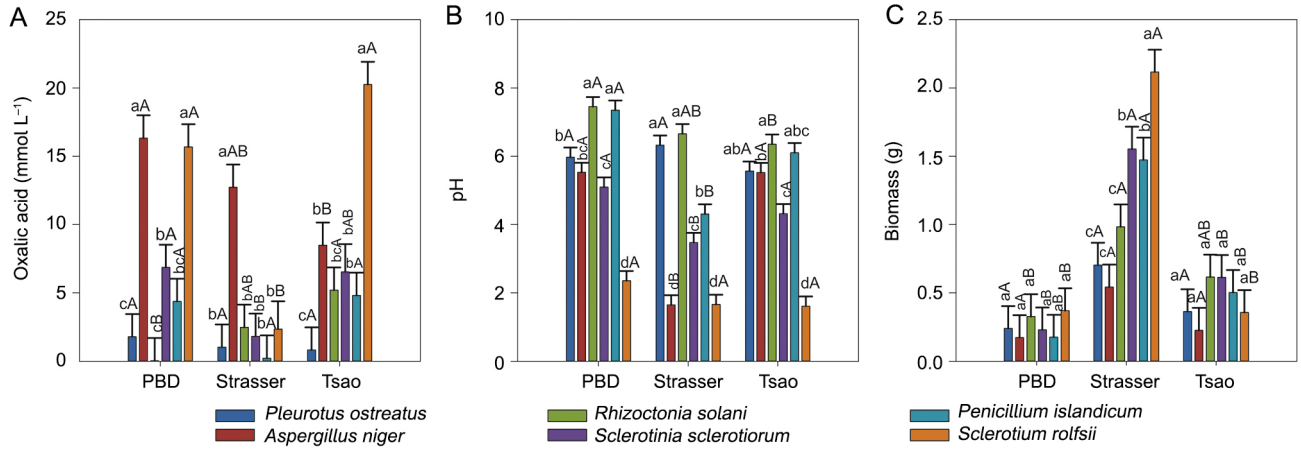

Figure 1– Oxalic acid biosynthesis (A), the pH of the culture media (B), and fungal biomass (C) produced by fungi in different culture media. Lowercase letters compare different fungi within each culture medium, while uppercase letters compare culture media for each fungus (Fisher's LSD test, $p < 0.05$).

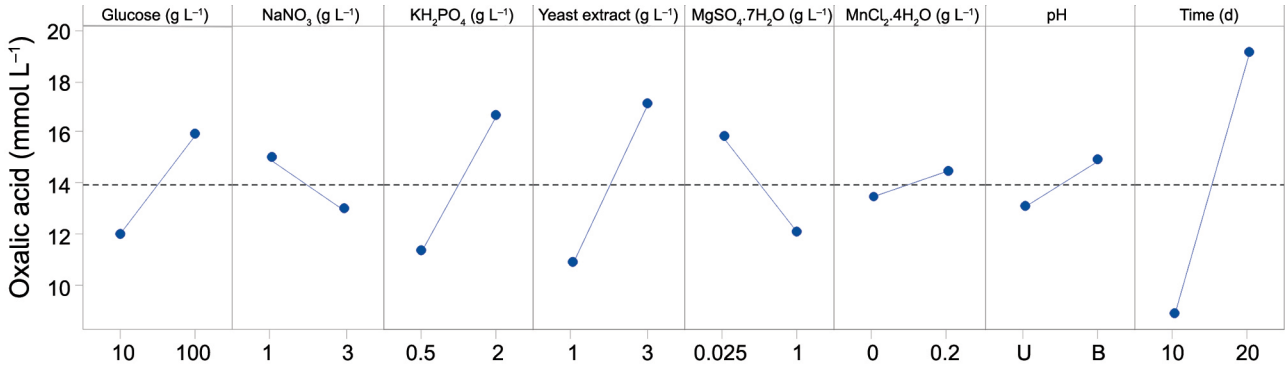

Figure 2 – Oxalic acid biosynthesis by *Sclerotium rolfsii* Sr25 as affected by the concentration of glucose, $NaNO_3$, KH_2PO_4, yeast extract, $MgSO_4.7H_2O$, and $MnCl_2.4H_2O$ by the pH buffering (U = unbuffered, B = buffered) and incubation time. The dashed line represents the mean biosynthesis of oxalic acid.

$MgSO_4 \cdot 7H_2O$ and by incubation time. The use of RSM tripled oxalic acid production by *S. rolfsii* Sr25 from 20 to 71 mmol L^{-1}. The highest oxalic acid biosynthesis was achieved using 1 g L^{-1} $NaNO_3$; 100 g L^{-1} glucose; 2 g L^{-1} KH_2PO_4; 4.5 g L^{-1} yeast extract; 25 mg L^{-1} $MgSO_4 \cdot 7H_2O$, and incubation for 20 days.

Oxalic acid has excelent potential to solubilize RP of different origins and reactivities (Mendes et al., 2020). Moreover, oxalic acid was more efficient than sulfuric acid, the traditional reagent used for RP solubilization to produce fertilizers, releasing more P per mmol acid (Mendes et al., 2020). The reaction between oxalic acid and apatite reached complete P extraction at stoichiometric proportions of reagents. In addition, the reaction was fast, and most P was solubilized in the first hours, releasing up to 75 % of P after 1 h and 88 % after 24 h (Mendes et al., 2021b). These results support our findings, as we observed that 100 % of the RP were solubilized using oxalic acid produced by *S. rolfsii* Sr25. Medium acidification is one of the mechanisms of apatite solubilization in RP, as well as the capacity of oxalate to form a sparingly-soluble complex with calcium (CaC_2O_4) (Kpomblekou-A and Tabatabai, 1994; Mendes et al., 2020, 2021a, b). These features show the efficiency of oxalic acid in P solubilization, as evidenced by our results. Moreover, none of the organic acids commonly associated with phosphate solubilization (citric, gluconic, and malic acids) were detected in the strain of *S. rolfsii* Sr25 used in our study (data not shown), which suggests that oxalic acid was the primary fungal metabolite involved in RP solubilization. The amount of soluble P measured in the solubilization experiment was higher than the amounts of P added in the form of KH_2PO_4 and RP (Figure 4). Possibly, this excess of P originates from the mineralization of organic P in yeast extract in the culture medium (Hidayat et al., 2006; Thompson et al., 2017).

Under optimized conditions, oxalic acid production by *S. rolfsii* Sr25 was tripled, reaching a concentration of 71 mmol L^{-1} and the crucial optimization factors were concentrations of KH_2PO_4, glucose, yeast extract, and $MgSO_4 \cdot 7H_2O$ and incubation time. $NaNO_3$ induces expression of the *oah* gene, which encodes oxaloacetate

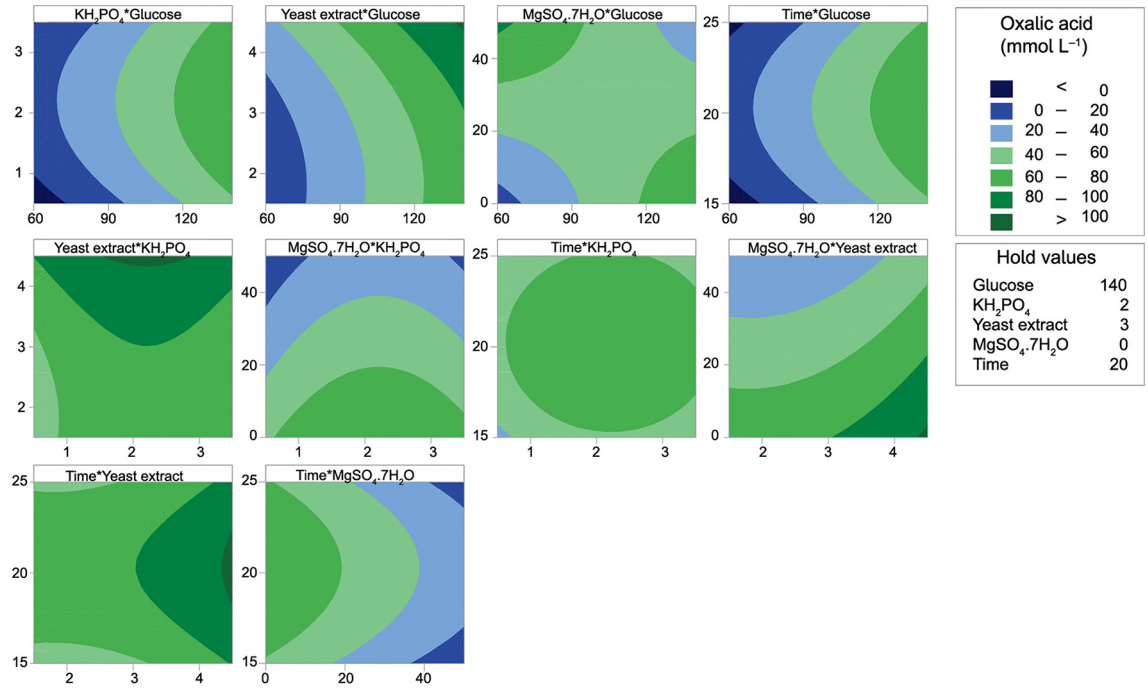

Figure 3 – Oxalic acid produced by *Sclerotium rolfsii* Sr25 in response to different combinations of glucose, yeast extract, KH_2PO_4, $MgSO_4.7H_2O$ and incubation time. In each panel, the variables not presented were fixed as indication in the box. Calculated with the fitted regression equation: $\hat{Y} = -398.7 + 0.847x_1 + 34.96x_2 - 14.6x_3 + 3.320x_4 + 32.36x_5 - 7.90x_2^2 + 4.05x_3^2 - 0.797x_5^2 - 0.03102x_1x_4$ ($\hat{Y}$ = oxalic acid produced, x_1 = glucose, x_2 = KH_2PO_4, x_3 = yeast extract, x_4 = $MgSO_4.7H_2O$, x_5 = incubation time).

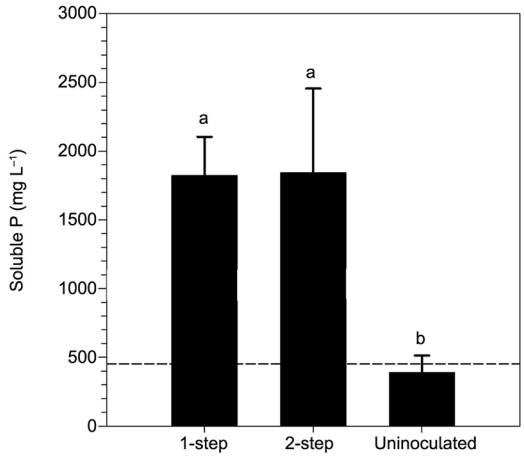

Figure 4 – Soluble P in culture medium after incubation of rock phosphate (RP) with oxalic acid produced by *Sclerotium rolfsii* Sr25. In the one-step process, RP was added at the beginning of the incubation period, while in the 2-step process, RP was added on the 20th day of incubation. The dashed line represents the dose of soluble P added as a component (KH_2PO_4) of the culture medium for oxalic acid biosynthesis. Error bars represent standard deviation (n = 5).

hydrolase (Pedersen et al., 2000). However, in our study, an increase in $NaNO_3$ concentrations above 1 g L^{-1} did not result in higher oxalic acid production by *S. rolfsii* Sr25. Thus, $NaNO_3$ concentrations lower than in Tsao medium appear to supply enough N. Oxalic acid production increased when more significant amounts of P were used, similar to reports for *A. niger*, where the accumulation of oxalic acid occurred in the presence of 2.5 g L^{-1} KH_2PO_4 (Kubicek et al., 1988). In our study, only a small amount of Mg was necessary, or may not have been required, for oxalic acid biosynthesis by *S. rolfsii* Sr25. Production of organic acids by *A. niger* is stimulated in the presence of C excess (Papagianni, 2007; Strasser et al., 1994). Our results demonstrate a similar mechanism in *S. rolfsii* Sr25. At higher dosages of glucose and yeast extract than in the Tsao medium (2.5- and 2.25-fold increase, respectively), the biosynthesis of oxalic acid tripled.

In general, optimal oxalic acid production by fungi occurs between 10-15 days of incubation (Amadioha, 1993; Tsao, 1963). In our study, the optimal duration of *S. rolfsii* Sr25 incubation for oxalic acid production was approximately 20 days. Moreover, the culture medium containing the fungus assumed gel consistency after 20 days due to scleroglucan production (Castillo et al., 2015; Fariña et al., 1998; Survase et al., 2007), which is an undesirable characteristic regarding the subsequent application of oxalic acid-rich medium for RP solubilization due to the difficulty of homogenization.

Manganese stimulates oxaloacetase activity in *A. niger* (Pedersen et al., 2000; Ruijter et al., 1999); however, in our study, Mn addition did not affect oxalic acid production by *S. rolfsii* Sr25. Furthermore, contrary to observations on *A. niger* (Strasser et al., 1994), the pH

buffering close to neutral values did not increase oxalic acid production by *S. rolfsii* Sr25. The optimum pH range for oxalate production was proposed at 5 to 8 (Ruijter et al., 1999), with the highest oxalate accumulation at approximately pH 6 (Kubicek et al., 1988). No corresponding effect was observed in our study. This discrepancy may be because oxalic acid is a product of the glyoxylate pathway of the tricarboxylic cycle in *S. rolfsii* and glyoxylate dehydrogenase is responsible for oxalic acid production (Maxwell and Bateman, 1968), whereas in *A. niger* oxalic acid is produced by oxaloacetase (Kobayashi et al., 2014; Kubicek et al., 1988).

Our findings indicate that oxalic acid may be an effective biotechnological option for solubilizing P from RP, potentially replacing sulfuric acid (Mendes et al., 2020). The oxalic acid concentration required to attain 100 % RP solubilization (71 mmol L^{-1}) was achieved by *S. rolfsii* Sr25 in a culture medium using renewable carbohydrates, such as glucose and yeast extract. Although *S. rolfsii* Sr25 is phytopathogenic and can cause considerable losses to agricultural production, its use in a controlled production system may help explore its physiology for the benefit of agriculture. This study provides insights to support oxalic acid production by *S. rolfsii* Sr25 in a two-stage bioprocessing scheme aiming at producing P fertilizer. In the first stage, oxalic acid was produced by the fungus using renewable organic substrates as energy and carbon sources. In the second stage, the medium containing oxalic reacted with RP to generate a product with soluble phosphate that could be applied as a liquid fertilizer (Mendes et al., 2017) or dried in a subsequent step to produce solid fertilizer formulations (Gilmour, 2013). In this bioprocessing scheme, fungal biomass could be removed by filtration after the first stage to prevent fungal propagules from being disseminated with the fertilizer.

Currently, industrial production of oxalic acid largely relies on the oxidation of carbohydrates using sulfuric and nitric acids (Riemenschneider and Tanifuji, 2011). Furthermore, industrial RP solubilization for fertilizer production is carried out with sulfuric acid, produced from elemental sulfur mainly from the oil industry (Gilmour, 2013). Thus, the biotechnological scheme proposed here for RP solubilization using oxalic acid of microbial origin could eliminate the need for sulfuric acid in both industrial processes and represent a renewable and ecological alternative for producing phosphate fertilizers.

Acknowledgments

We are thankful to the Center for Research on Electroanalysis (NuPE, Institute of Chemistry, Federal University of Uberlândia) for performing the oxalic acid analyses. This work was supported by the Minas Gerais State Agency for Research and Development (FAPEMIG) [grant number APQ-01842-17], the Coordination for the Improvement of Higher Level Personnel (CAPES) [grant number 001], and the Foundation for University Support (FAU).

Authors' Contributions

Conceptualization: Mendes, G.O.; Vieira, B.S.; Amaro, J.K.C. **Data acquisition:** Amaro, J.K.C.; Xavier, L.V. **Data analysis:** Amaro, J.K.C.; Ribeiro, M.M.A.C.; Mendes, G.O. **Design of methodology:** Mendes, G.O.; Amaro, J.K.C.; Ribeiro, M.M.A.C. **Writing and editing:** Amaro, J.K.C; Mendes, G.O.; Vieira, B.S.

References

Almeida, M.N.; Guimarães, V.M.; Falkoski, D.L.; Paes, G.B.T.; Ribeiro Jr., J.I.; Visser, E.M.; Alfenas, R.F.; Pereira, O.L.; Rezende, S.T. 2014. Optimization of endoglucanase and xylanase activities from *Fusarium verticillioides* for simultaneous saccharification and fermentation of sugarcane bagasse. Applied Biochemistry and Biotechnology 172: 1332-1346. https://doi.org/10.1007/s12010-013-0572-9

Amadioha, A.C. 1993. A synergism between oxalic acid and polygalacturonases in the depolymerization of potato tuber tissue. World Journal of Microbiology and Biotechnology 9: 599-600. https://doi.org/10.1007/BF00386304

Box, G.E.P.; Wilson, K.B. 1951. On the experimental attainment of optimum conditions. Journal of the Royal Statistical Society. Series B - Methodological 13: 1-45. https://doi.org/10.1111/j.2517-6161.1951.tb00067.x

Braga, J.M.; Defelipo, B.V. 1974. Spectrophotometric determination of phosphorus in soil extracts and plant material = Determinação espectrofotométrica de fósforo em extratos de solo e material vegetal. Revista Ceres 21: 73-85 (in Portuguese).

Castillo, N.A.; Valdez, A.L.; Fariña, J.I. 2015. Microbial production of scleroglucan and downstream processing. Frontiers in Microbiology 6: e1106. https://doi.org/10.3389/fmicb.2015.01106

Cordell, D.; Drangert, J.-O.; White, S. 2009. The story of phosphorus: global food security and food for thought. Global Environmental Change 19: 292-305. https://doi.org/10.1016/j.gloenvcha.2008.10.009

Durman, S.B.; Menendez, A.B.; Godeas, A.M. 2005. Variation in oxalic acid production and mycelial compatibility within field populations of *Sclerotinia sclerotiorum*. Soil Biology and Biochemistry 37: 2180-2184. https://doi.org/10.1016/j.soilbio.2005.03.017

Dutton, M.V.; Evans, C.S. 1996. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology 42: 881-895. https://doi.org/10.1139/m96-114

Fariña, J.I.; Siñeriz, F.; Molina, O.E.; Perotti, N.I. 1998. High scleroglucan production by *Sclerotium rolfsii*: influence of medium composition. Biotechnology Letters 20: 825-831. https://doi.org/10.1023/A:1005351123156

Fontes, M.P.F.; Weed, S.B. 1996. Phosphate adsorption by clays from Brazilian Oxisols: relationships with specific surface area and mineralogy. Geoderma 72: 37-51. https://doi.org/10.1016/0016-7061(96)00010-9

Francisco, K.J.M.; Lago, C.L. 2009. A compact and high-resolution version of a capacitively coupled contactless conductivity detector. Electrophoresis 30: 3458-3464. https://doi.org/10.1002/elps.200900080

Gilmour, R. 2013. Phosphoric Acid: Purification, Uses, Technology, and Economics. CRC Press, Boca Raton, FL, USA. https://doi.org/10.1201/b16187

Grant, C.A.; Flaten, D.N.; Tomasiewicz, D.J.; Sheppard, S.C. 2001. The importance of early season phosphorus nutrition. Canadian Journal of Plant Science 81: 211-224. https://doi.org/10.4141/P00-093

Hidayat, B.J.; Eriksen, N.T.; Wiebe, M.G. 2006. Acid phosphatase production by *Aspergillus niger* N402A in continuous flow culture. FEMS Microbiology Letters 254: 324-331. https://doi.org/10.1111/j.1574-6968.2005.00045.x

Kobayashi, K.; Hattori, T.; Honda, Y.; Kirimura, K. 2014. Oxalic acid production by citric acid-producing *Aspergillus niger* overexpressing the oxaloacetate hydrolase gene oahA. Journal of Industrial Microbiology and Biotechnology 41: 749-756. https://doi.org/10.1007/s10295-014-1419-2

Kpomblekou-A, K.; Tabatabai, M.A. 1994. Effect of organic acids on release of phosphorus from phosphate rocks. Soil Science 158: 442-453. https://doi.org/10.1097/00010694-199415860-00006

Kubicek, C.P.; Schreferl-Kunar, G.; Wöhrer, W.; Röhr, M. 1988. Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. Applied and Environmental Microbiology 54: 633-637. https://doi.org/10.1128/aem.54.3.633-637.1988

Lide, D.R. 2004. CRC Handbook of Chemistry and Physics. 8ed. CRC Press, Boca Raton, FL, USA.

Maxwell, D.P.; Bateman, D.F. 1968. Oxalic acid biosynthesis by *Sclerotium rolfsii*. Phytopathology 58: 1635-1642.

Mendes, G.O.; Freitas, A.L.M.; Pereira, O.L.; Silva, I.R.; Vassilev, N.B.; Costa, M.D. 2014. Mechanisms of phosphate solubilization by fungal isolates when exposed to different P sources. Annals of Microbiology 64: 239-249. https://doi.org/10.1007/s13213-013-0656-3

Mendes, G.O.; Silva, N.M.R.M.; Anastácio, T.C.; Vassilev, N.B.; Ribeiro, J.I.; Silva, I.R.; Costa, M.D. 2015. Optimization of *Aspergillus niger* rock phosphate solubilization in solid-state fermentation and use of the resulting product as a P fertilizer. Microbial Biotechnology 8: 930-939. https://doi.org/10.1111/1751-7915.12289

Mendes, G.O.; Galvez, A.; Vassileva, M.; Vassilev, N. 2017. Fermentation liquid containing microbially solubilized P significantly improved plant growth and P uptake in both soil and soilless experiments. Applied Soil Ecology 117-118: 208-211. https://doi.org/10.1016/j.apsoil.2017.05.008

Mendes, G.O.; Murta, H.M.; Valadares, R.V.; Silveira, W.B.; Silva, I.R.; Costa, M.D. 2020. Oxalic acid is more efficient than sulfuric acid for rock phosphate solubilization. Minerals Engineering 155: e106458. https://doi.org/10.1016/j.mineng.2020.106458

Mendes, G.O.; Bahri-Esfahani, J.; Csetenyi, L.; Hillier, S.; George, T.S.; Gadd, G.M. 2021a. Chemical and physical mechanisms of fungal bioweathering of rock phosphate. Geomicrobiology Journal 38: 384-394. https://doi.org/10.1080/01490451.2020.1863525

Mendes, G.O.; Dyer, T.; Csetenyi, L.; Gadd, G.M. 2021b. Rock phosphate solubilization by abiotic and fungal-produced oxalic acid: reaction parameters and bioleaching potential. Microbial Biotechnology 1-14. https://doi.org/10.1111/1751-7915.13792

Myers, R.H.; Montgomery, D.C.; Anderson-Cook, C.M. 2016. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. 4ed. John Wiley, Hoboken, NJ, USA.

Papagianni, M. 2007. Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. Biotechnology Advances 25: 244-263. https://doi.org/10.1016/j.biotechadv.2007.01.002

Pedersen, H.; Hjort, C.; Nielsen, J. 2000. Cloning and characterization of oah, the gene encoding oxaloacetate hydrolase in *Aspergillus niger*. Molecular and General Genetics 263: 281-286. https://doi.org/10.1007/s004380051169

Punja, Z.K.; Jenkins, S.F. 1984. Influence of medium composition on mycelial growth and oxalic acid production in *Sclerotium rolfsii*. Mycologia 76: 947. https://doi.org/10.1080/00275514.1984.12023935

Riemenschneider, W.; Tanifuji, M. 2011. Oxalic acid. p. 529-541. In: Ullmann's Encyclopedia of Industrial Chemistry. Wiley-VCH, Weinheim, Germany.

Roy, E.D.; Richards, P.D.; Martinelli, L.A.; Coletta, L.D.; Lins, S.R.M.; Vazquez, F.F.; Willig, E.; Spera, S.A.; VanWey, L.K.; Porder, S. 2016. The phosphorus cost of agricultural intensification in the tropics. Nature Plants 2: e16043. https://doi.org/10.1038/nplants.2016.43

Ruijter, G.J.G.; van de Vondervoort, P.J.I.; Visser, J. 1999. Oxalic acid production by *Aspergillus niger*: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology 145: 2569-2576. https://doi.org/10.1099/00221287-145-9-2569

Silva, J.A.F.; Lago, C.L. 1998. An oscillometric detector for capillary electrophoresis. Analytical Chemistry 70: 4339-4343. https://doi.org/10.1021/ac980185g

Strasser, H.; Burgstaller, W.; Schinner, F. 1994. High-yield production of oxalic acid for metal leaching processes by *Aspergillus niger*. FEMS Microbiology Letters 119: 365-370. https://doi.org/10.1111/j.1574-6968.1994.tb06914.x

Survase, S.A.; Saudagar, P.S.; Bajaj, I.B.; Singhal, R.S. 2007. Scleroglucan: fermentative production, downstream processing and applications. Food Technology and Biotechnology 45: 107-118.

Thompson, K.A.; Summers, R.S.; Cook, S.M. 2017. Development and experimental validation of the composition and treatability of a new synthetic bathroom greywater (SynGrey). Environmental Science: Water Research and Technology 3: 1120-1131. https://doi.org/10.1039/C7EW00304H

Tsao, G.T. 1963. Production of oxalic acid by a wood-rotting fungus. Applied Microbiology 11: 249-254. https://doi.org/10.1128/am.11.3.249-254.1963

Yang, J.; Tewari, J.P.; Verma, P.R. 1993. Calcium oxalate crystal formation in *Rhizoctonia solani* AG 2-1 culture and infected crucifer tissue: relationship between host calcium and resistance. Mycological Research 97: 1516-1522. https://doi.org/10.1016/S0953-7562(09)80227-X