Mutation breeding of *Aspergillus niger* by atmospheric room temperature plasma to enhance phosphorus solubilization ability

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Background. Phosphorus (P) is abundant in soils, including organic and inorganic forms. Nevertheless, most of P compounds cannot be absorbed and used by plants. *Aspergillus niger* v. Tiegh is a strain that can efficiently degrade P compounds in soils. **Methods.** In this study, *A. niger* xj strain was mutated using Atmospheric Room Temperature Plasma (ARTP) technology and the strains were screened by Mo-Sb Colorimetry with strong P-solubilizing abilities. **Results.** Compared with the *A. niger* xj strain, setting the treatment time of mutagenesis to 120 s, four positive mutant strains marked as xj 90–32, xj120–12, xj120–31, and xj180 – 22 had higher P-solubilizing rates by 50.3%, 57.5%, 55.9%, and 61.4%, respectively. Among them, the xj120–12 is a highly efficient P solubilizing and growth-promoting strain with good application prospects. The growth characteristics such as plant height, root length, and dry and fresh biomass of peanut (*Arachis hypogaea* L.) increased by 33.5%, 43.8%, 43.4%, and 33.6%, respectively. Besides available P, the chlorophyll and soluble protein contents also vary degrees of increase in the P-solubilizing mutant strains. **Conclusions.** The results showed that the ARTP mutagenesis technology can improve the P solubilization abilities of the *A. niger* mutant strains and make the biomass of peanut plants was enhanced of mutant strains.

1 **Mutation breeding of *Aspergillus niger* by atmospheric room**

2 **temperature plasma to enhance phosphorus solubilization ability**

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19 **ABSTRACT**

20   **Background.** Phosphorus (P) is abundant in soils, including organic and inorganic forms.

21 Nevertheless, most of P compounds cannot be absorbed and used by plants. *Aspergillus niger* v.

22 Tiegh is a strain that can efficiently degrade P compounds in soils.

23   **Methods.** In this study, *A. niger* xj strain was mutated using Atmospheric Room

24 Temperature Plasma (ARTP) technology and the strains were screened by Mo-Sb Colorimetry

25 with strong P-solubilizing abilities.

26   **Results.** Compared with the *A. niger* xj strain, setting the treatment time of mutagenesis to

27   120 s, four positive mutant strains marked as xj90–32, xj120–12, xj120–31, and xj180–22 had

28   higher P-solubilizing rates by 50.3%, 57.5%, 55.9%, and 61.4%, respectively. Among them, the

29   xj120–12 is a highly efficient P solubilizing and growth-promoting strain with good application

30   prospects. The growth characteristics such as plant height, root length, and dry and fresh biomass

31   of peanut (*Arachis hypogaea* L.) increased by 33.5%, 43.8%, 43.4%, and 33.6%, respectively.

32   Besides available P, the chlorophyll and soluble protein contents also vary degrees of increase in

33   the P-solubilizing mutant strains.

34   **Conclusions.** The results showed that the ARTP mutagenesis technology can improve the P

35   solubilization abilities of the *A. niger* mutant strains and make the biomass of peanut plants was

36   enhanced of mutant strains.

37   Keywords: Atmospheric Room Temperature Plasma; *Aspergillus niger*; Mutagenesis;

38   Phosphate-solubilizing ability.

39   **1. INTRODUCTION**

40   Phosphorus (P) is the second essential macronutrient for plant growth and development.

41   Although P only accounts for 0.2% of plant dry weight, low P availability can generate a strong

42   limit in plant growth and crop yield (Sharma et al., 2013). The natural reserve of soluble P

43   fractions is much lower than expected, which may just have a range of doses from 1.0 μg L^{-1} to

44   1.0 mg L^{-1} in soil solutions. This is formed due to mineral P compounds that are easily combined

45   with aluminum, calcium, iron, and magnesium together the tight fixation of in a form of soil

46   colloids (Kumar et al., 2001; Brady et al., 2002). Therefore, many crops are suffering the

47   problem of low P deficiency.

48   Phosphate-solubilizing microorganisms (PSM) play a great role in reducing P insufficiency

49   in soils because they can provide low-cost soluble P fractions in different production systems

50   (Khan et al., 2014). PSM have been shown to enhance the solubility of inorganic P compounds

51   and increase crop yields (Singh et al., 2011). The process of converting insoluble P into the

52   soluble forms by PSM involves a series of stages, such as acidification, chelation, and exchange

53 reactions (Narsian et al., 2000). The secretion of organic acids accounts for the major mechanism

54 to make P fractions soluble (Wahid et al., 2000). Organic acids of plant or microbial origin can

55 form organometallic complexes with Fe^{2+} and Al^{3+}, increasing P availability by the dissolution of

56 phosphate precipitates with these ions (Pavinato and Rosolem, 2008). Previous studies have

57 demonstrated that several mineral-solubilizing fungi could solubilize inorganic minerals into

58 available forms through the production of organic acids (e.g., citric, fumaric, gluconic, malic,

59 succinic, tartaric, and oxalic acids) (Acevedo et al., 2014; Wang et al., 2018; Khuna et al., 2021).

60 Specifically, the secretion of oxalic acid by strains under P-deficient soil conditions was one of

61 the most important P-solubilization mechanisms that plants have adapted to cope with P

62 deficiency (Song et al., 2008; Jmdn et al., 2021). Filamentous fungus *A. niger* is an outstanding

63 cell factory for organic acid production due to its high carbon conversion efficiency and

64 tolerance for low pH (Meyer et al., 2011; Yang et al., 2016). *A. niger* is a species widely used in

65 the food industry to produce various enzymes and metabolites, such as citric acid (Behera, 2020).

66 A gross estimation indicated that 80% of citric acids were fermented by *A. niger* all over the

67 world (Adeoye et al., 2015). *A. niger* ATCC 1015 exhibited great potential for malic acid

68 production (Xu et al., 2019). The *A. niger* WH-2 could biosynthesize L(+)-tartaric acid under

69 acidic conditions (Bao et al., 2020). The organic acids can chelate calcium, aluminum ferrous,

70 and magnesium, which can all further increase P availability (Mulik et al., 2020). The P-

71 solubilization activity of microorganisms is related to organic acid production. However, the

72 nature of the acid produced was also important, dependent on the carbon source supplied

73 (Vassileva, 1998; Srividya et al., 2009). Therefore, choosing the appropriate carbon resource or

74 other conditions to affect the nature of acid produced is important. *A. niger* could also promote

75 plant growth by enhancing P uptake. For example, a study revealed that *A. niger* could promote

76 the growth and nutrient uptake in groundnut (*Arachis hypogaea* L.) (Jitendra et al., 2011).

77 Besides, *A. niger* also promoted growth in soybean (*Glycine max L.*) (Elazouni, 2008). Although

78 the P-solubilizing ability of *A. niger* is well known, reports on improving the P-solubilizing

79 ability of strain and promoting plant growth by microbial mutation breeding technique

80 formulation are limited.

81   Microbial mutation breeding is an important technique, which has been broadly employed

82 for microbial improvement, especially in the fields of biotechnology, biomanufacturing, food

83 fermentation, and environmental protection (Brock 1976). Mutagenesis tools, including chemical

84 mutagens, physical or biological approaches, are used to increase mutation rate and accelerate

85 the subsequent evolutionary process to obtain mutant strains with desirable phenotypes (Huang

86 et al., 2021). Among them, Atmospheric Room Temperature Plasma (ARTP) mutagenesis

87 technology caused more serious DNA damage and a higher mutation rate among all the

88 mutagenesis methods (Zhang et al., 2015). ARTP is a novel type of atmospheric pressure non-

89 equilibrium discharge plasma source, which relies on a uniform distribution of highly

90 concentrated neutral active particles to alter microbial genetic traits and directly modify the

91 molecular structure at a nucleotide level (Zong et al., 2012). It has wide applications and high

92 efficiency of mutation diversity and offers good safety and ease-of-use (Zhang et al., 2014).

93 ARTP mutagenic technology has been successful in obtaining strains with desirable

94 characteristics among bacteria, fungi, and microalgae (Zhang et al., 2019a; Zhang et al., 2019b).

95 Zong et al., (2012) used ARTP technology to generate mutations in *Streptomyces albulus,* and

96 the spores concentration was 10^6 CFU mL^{-1}, which the positive mutation rates can reach a level

97 as high as up to 26.0%. *S. albulus* A-29 exhibited the highest ε-Poly-L-lysine productivity, which

98 was four times higher than that of the wild strain in the same culture condition. Song et al., (2017)

99 revealed that, compared with the original strain, the lipid productivity of *Chlorella pyrenoidosa*

100 was nearly improved over 16% by ARTP. The above examples illustrate that ARTP, as a novel

101 mutagenesis tool, has a good application prospect.

102   This study aims to screen highly effective P-solubilizing strains of *A. niger* by ARTP

103 mutagenesis and the peanut biomass and physiological indexes of the positive mutant strains

104 were determined and investigated. This research provides a fundamental basis for the further

105 development of multifunctional fungal agent and their applications. In addition, it provides a

106 reference model and a method for the mutation breeding of other microorganisms.

107 **2. MATERIALS AND METHODS**

108 *2.1 Aspergillus niger strain and reagents*

109   The original strain *A. niger* strain (xj) was isolated and identified by the Institute of Fungi

110 Resource, Guizhou University, and stored at the China Center for Type Culture Collection

111 (CCTCC NO. M206021) (Li et al., 2007). Chemical reagents of this experiment were purchased

112 from Sinopharm Chemical Reagent Co., Ltd. (China).

113 *2.2 Culture media*

114   Potato dextrose agar (PDA) medium (g/L): the boiled potato extract 200, glucose 20, agar

115 20.

116   Pikovskaya's medium (g/L): glucose 10, sodium chloride 0.3, potassium chloride 0.3,

117 ammonium sulfate 0.5, magnesium sulfate heptahydrate 0.3, ferric sulfate heptahydrate 0.03,

118 manganese sulfate 0.03 and tricalcium phosphate 5.

119 *2.3 Mutation of Aspergillus niger xj*

120   The method of mutagenesis of strains referred to Zhang et al., (2019). Helium was used as

121 the working gas and the mutagenic treatment distance was 2 mm, the processing power was 120

122 W, the gas flow rate was 10 slm (standard liter per minute), and the treatment time was from 0 s

123 to 300 s with intervals of 30 s. The calculation of lethality rate was carried out following the

124 protocol of Luo et al., (2018).

125
$$\text{lethality rate} = \frac{U-T}{U} \times 100\%$$

126   Where U represents the number of unmutated colonies (0 s) and T represents the number of

127 colonies after the mutagenesis.

128 *2.4 Screening of positive mutant strains*

129   Sterilized pikovskaya's agar medium was poured into sterilized petri dish. After

130 solidification of the medium, a pinpoint inoculation of the single mutagenic colony of *A. niger*

131 strain was made on the plates aseptically and incubated for seven days at 28 °C. The intensity of

132 tricalcium phosphate solubilisation was measured as the diameter of the halozone formed around

133 the colonies (Maurya et al., 2006). The above method aims to primarily screened the

134 mutagenesis strains with P-solubilization effect. The primary screening strains were selected

135 with a better P dissolving ability for re-screening, and P content was determined by Mo-Sb

136 Colorimetry (Li et al., 2020). The genetic variability of the mutant strains induced by artificial

137 mutagenesis was low and easy to repair or mutate. Therefore, the mutant strains were

138 subcultured based on their genetic stability (Tao, 2004). In this study, the genetic stability of the

139 mutant strain was evaluated by 10 passages. Values were shown as mean ±standard deviation

140 (SD) from triplicately repeated experiments.

141 *2.5 Measurement of organic acids*

142   In this study, seven kinds of organic acids (oxalic acid, malic acid, citric acid, tartaric acid,

143 lactic acid, acetic acid, and succinic acid) were determined by a method of Yang et al., (2018).

144 The organic acid concentration as the independent variable x and the peak area as the dependent

145 variable y, were taken to draw the standard curve by the external standard method. The unknown

146 organic acids in culture filtrate were determined by comparing the retention times (RT) and

147 peaks areas of chromatograms with the standard organic acids (Bakri, 2019).

148 *2.6 Determination of indole-3-acetic acid (IAA) content*

149   IAA concentration (0, 10, 20, 30, 40, and 50 µg mL^{-1}) was taken separately, and a volume

150 of 4 mL Salkowski was added to the mixture to promote full reaction at 28 °C for 30 min. IAA

151 concentration was measured by UV-VIS Spectrophotometer (BIOMATE 3S, Thermo Fisher,

152 USA) at 530 nm. IAA concentration as the abscissa x and the absorbance as the ordinate y to

153 draw the IAA standard curve. *A. niger* spore suspension (10^8 CFU mL^{-1}) was inoculated and

154 incubated at 28 °C and centrifuged at 150 r/min for five days. After that, it was centrifuged again

155 at 109 g (Allegra X-30R, Beckman Coulter, USA) for 10 min, mixed with liquid supernatant (1

156 mL) and Salkowski (4 mL) at 28 °C for 30 min. Three technical replicates were done in the

157 experiment, the IAA content was calculated by comparing the IAA standard curve.

158 *2.7 Determination of phosphorus content*

159   The determination of P content was carried out by the method of Zhang (2008). However,

160 the absorbance of the sample was measured by UV spectrophotometer at 700 nm. The standard

161 curve of phosphorus was drawn with P content as the abscissa x and absorption value as the

162 ordinate y. The content of soluble P in the centrifugal supernatant was determined by Mo-Sb

163 Colorimetry.

164 *2.8 Determination of phosphorus removal kinetics curve of strains*

165   The spore suspension was inoculated into a liquid medium containing 5% calcium

166 phosphate. The culture solution was inoculated at 28 °C and 150 r/min for seven days. During

167 the culturing, the samples were taken at regular intervals of 24 h to be centrifuged at 109 g for 5

168 min at 4 °C. The concentration of spore suspension was set to 1×10^8 CFU/mL for xj90–32,

169 xj120–12, xj120–31, and xj180–22 mutated strains. The content of soluble P in the centrifugal

170 supernatant was determined and the value of pH was determined by pH meter (PHS-3C, Dapu

171 Instrument, Shanghai, Co., Ltd., China).

172 *2.9 Effect of different factors on the phosphorus-solubilizing ability of mutant strains*

173   Studies have shown that the carbon source, nitrogen source, pH, and other factors of the

174 culture medium would affect the P-solubilizing ability of P-solubilizing fungi (Patel et al., 2008).

175 Therefore, the mutagenic strains of *A. niger* need to investigate the influence of these factors on

176 the ability of solubilizing P. These factors included (carbon source and concentration, nitrogen

177 source and concentration, temperature, NaCl, initial pH). Effects of changes in carbon sources

178 and concentration, nitrogen sources and concentration, temperature, salinity, initial pH on P-

179 solubilizing abilities were evaluated using calcium phosphate medium. The type of carbon

180 sources included glucose, sucrose, maltose, lactose, and starch, and concentration were 0.5%,

181 1%, 5%, 10% and 15%. Baseline experimental conditions were: concentration of *A. niger* spore

182 suspension 1×10^8 CFU mL^{-1}, ammonium sulfate concentration 0.5 g L^{-1}, initial pH 7.5, NaCl 3

183 g L^{-1}, and incubated aerobically (150 rpm min^{-1}, 30 °C) for five days. Nitrogen sources were

184 derived from beef extract, sodium nitrate, ammonium sulfate, potassium nitrate, and carbamide,

185   and concentration were 0, 0.05%, 0.5%, 1%, 5% and 10%, NaCl concentrations were regulated

186   to 0, 0.5%, 1.5%, 2%, 3%, 4%, 5%, 6%, 8%, and 10%, initial pH was adjusted to 3, 5, 7, 9, and

187   11, and temperatures were changed to 20 °C, 25, 30 °C, 35 °C, 40 °C, and 50 °C. The effects of

188   all these factors on the P solubilization were separately determined. Other conditions are the

189   same as for the above determination of P content. Values are shown by mean ±SD from triplicate

190   experiments.

191   *2.10 Collection of peanut samples and measurement of growth characteristics*

192   Peanut seeds were firstly disinfected with hydrogen peroxide, rinsed with sterile water, and

193   subjected to 28 °C for 24 h to accelerate the germination. Subsequently, seeds were placed in a

194   test pot containing 300 g of soil, which was collected from the campus of Guizhou University

195   with the geographical coordinates of 106 66 E' and 26 44 N'. The larger particles and debris

196   were filtered, which were mixed with fine sand in a 1:2 (V:V) ratio. Besides, a certain amount of

197   insoluble calcium phosphate was added to each pot of soil at the ratio of 1:1000 (W:W). The *A.*

198   *niger* positive mutant strains (10^6 CFU g^{-1}) were inoculated with the peanut that was grown for

199   three days, and the blank controls were prepared (inoculating the same amount of sterile water

200   for CK control). When peanuts were growing up 30 days and was collected. Then, sterile water

201   was used to clean the soil at the root of the peanut, and the filter paper was used to suck water.

202   the growth indicators (plant height, root length, and dry and fresh biomass), chlorophyll content,

203   and protein content were measured. the plant height and root length were measured by a ruler.

204   Fresh biomass was measured with an analytical balance (BS110S, Sartorius Tianping, Co., Ltd.,

205   Beijing, China). Besides, the peanut was put into a sterilization carton and sterilized at 105 °C

206   for 20 min and dried at 80 °C to constant weight. The dry biomass of the peanut was measured

207   by analytical balance. Values shown are mean ±SD from triplicate experiments.

208   *2.11 Determination of physiological indexes (chlorophyll and total protein content)*

209   Around 100 grams of peanut leaves were collected, washed, and dried. They were next cut

210   into slices in 1-mm width and placed in a 10 mL test tube. Leaves were fully soaked in 2 mL of

211   dimethyl sulfoxide (DMSO) at 65 °C until they turned white or transparent. The extracted liquid

212 was cooled to room temperature, and the extracted liquid was mixed fully with 80% acetone (8

213 mL) (Qiu et al., 2016). Chlorophyll concentration was determined using UV-VIS

214 Spectrophotometer at 663.6 nm, and 646.6 nm and the calculation formula referred to Porra et al.,

215 (1989).

216   The standard protein curve of bovine serum protein was made by a method of Bradford (1976).

217 Around 0.5 g of fresh peanut leaves was taken, washed, and wipe-dried. The dried leaves were

218 placed in a mortar and about 5 mL of phosphate buffer saline (PBS) was added to the mortar.

219 Leaves were grounded to extract the protein, which was thoroughly mixed with Coomassie

220 brilliant blue G-250 (0.1 mg mL^{-1}) and stand for 5 min. The absorbance value was measured at

221 595 nm by UV-VIS Spectrophotometer and the protein content in the sample was calculated. The

222 formula is as follows:

223
$$\text{The protein content of sample (mg/g)} = \frac{C \times V_T V_1}{FW \times 1000}$$

224   Where C represents the protein content calculated by the standard protein curve, unit (μg);

225 V_T represents the total volume of the extract, unit (mL); V_1 represents the added sample amount

226 when determination, unit (mL); FW represents the fresh weight of the sample, unit (g).

227 *2.12 Effect of the Aspergillus niger spore suspension on available phosphorus content in the soil*

228   Soil samples were air-dried and filtered using a 1 mm sieve. Around 2 grams of dried samples

229 were transferred to a 100 mL conical flask filled with 20 mL of 0.50 mol L^{-1} sodium bicarbonate

230 solution. Then, samples were put at 28 °C and 150 r/min for 30 min. After, the samples were

231 filtered and centrifuged at 109 g for 5 min. 1 mL of supernatant was used to determine the

232 available P content by Mo-Sb Colorimetry. The test was performed in triplicate.

233 *2.13 Statistical analysis*

234   One-way analysis of variance (ANOVA) was performed for data analysis using IBM SPSS

235 (ver. 23.0, SPSS Inc., USA). The differences among the groups were evaluated by Duncan's test,

236 and *p*-value <0.05 was considered significant.

237 **3. RESULTS**

238   *3.1 Investigation of the ARTP mutagenesis time*

239   The lethality rate of the strain increased with time. The positive mutation rate showed a trend

240   of increase first and then a decrease. When the treatment time reached 120 s, the lethality rate

241   was 37%, and the positive mutation rate was 25.5%. The treatment time was 240 s, the lethality

242   rate was 85%, whereas the positive mutation rate was only 6.38%. Therefore, the treatment time

243   with the highest positive mutation rate was considered as the best condition for the mutagenesis

244   of the xj strain, and the treatment time was 120 s (Fig. 1).

245   *3.2 Screening of mutagenic strains*

246   Certain strains among a total of 670 mutant xj strains obtained by ARTP mutagenesis were

247   efficient in solubilizing the inorganic phosphorus (calcium phosphate) in the medium. The 141

248   and 24 mutant strains were acquired by preliminary and secondary screening, respectively. The

249   standard curve of P content was prepared and analyzed by linear regression, resulting in the

250   following correlation model: $y = 0.0103x+0.0069$, $r = 0.994$, which showed a good linear

251   relationship and met the sample testing requirements. The analysis results revealed that the xj90–

252   32, xj120–12, xj120–31, and xj180–22 strains did not show differences in P-solubilizing abilities

253   within ten generations, indicating that the four strains were good genetic stabilities and the P-

254   solubilizing abilities increased by 50.3%, 57.5%, 55.9%, and 61.4%, respectively (Table 1).

255   *3.3 Determination of organic acid contents*

256   HPLC was used to analyze the organic acids in the medium containing calcium phosphate

257   and revealed the P-solubilization mechanism of the xj strain. In this experiment, the standard

258   curves of seven organic acids were prepared and analyzed by linear regression, resulting in the

259   following coefficients: oxalic acid $(y = 0.0987x+0.2843$, $r = 0.9944)$, malic acid

260   $(y = 0.0228x+0.118$, $r = 0.9995)$, citric acid $(y = 0.0101x+0.2628$, $r = 0.9984)$, tartaric acid

261   $(y = 0.0146x+0.7278$, $r = 0.9946)$, lactic acid $(y = 0.0057x+0.2372$, $r = 0.9987)$, acetic acid

262   $(y = 0.0055x+0.6848$, $r = 0.9951)$ and succinic acid $(y = 0.0055x+0.4042$, $r = 0.9983)$. All

263   above-mentioned curves revealed close linear relationships, which met the requirements of

264   samples testing. We observed differences in the kinds and contents of organic acids between the

265   xj strain and the four positive mutant strains (Table 2). To be more specific, the xj strain and

266   xj90–32, xj120–12, xj120–31, and xj180–22 strains secreted oxalic acid with contents reaching

267   the peaks of 1363.7 mg L^{-1}, 1426.6 mg L^{-1}, 1402.4 mg L^{-1}, 1329.4 mg L^{-1}, and 1394.2 mg L^{-1},

268   respectively and also secreted citric acid, the contents reached the maximum of 140.2 mg L^{-1},

269   151.4 mg L^{-1}, 156.3 mg L^{-1}, 145.6 mg L^{-1}, and 172.9 mg L^{-1}, respectively. In addition, the

270   xj120–12 strain could secret succinic acid and the highest content was 543.5 mg L^{-1}.

271   *3.4 Determination of IAA content*

272   The standard curve of IAA was prepared and analyzed by linear correlation, resulting in the

273   following coefficient: $y = 0.0138x+0.0138$, and $r = 0.9972$. The curve displayed a good linear

274   relationship, which met the requirements of sample testing. As shown in Fig. 2 the xj and four

275   mutated strains could produce IAA in a medium containing 0.5 g L^{-1} tryptophan. The positive

276   mutant strain xj120–12 exhibited a significant difference from xj strains ($P<0.05$). The highest

277   IAA content was 10.5%, which was higher than that in xj strain. However, strains of xj90–32,

278   xj120–31, and xj180–22 were not significantly different from xj strains ($P>0.05$).

279   *3.5 Determination of kinetic curve of phosphorus solution*

280   According to Fig. 3A, all strains started releasing soluble P into the culture medium on day

281   first. On the fifth day, the soluble P content of xj, xj90–32, xj120–12, xj120–31, and xj180–22

282   strains reached the maximum values of 554.3 μg mL^{-1}, 744.4 μg mL^{-1}, 787.6 μg mL^{-1}, 769.9 μg

283   mL^{-1}, and 810.9 μg mL^{-1}, respectively. Afterward, the P content began decreasing at 6 and 7

284   days affecting by time. Simultaneously, the pH value of five strains sharply decreased during the

285   first two days and remained at a lower pH value from 2 to 7 days (Fig. 3B). During the

286   dissolution of calcium phosphate, the soluble P content of five strains presented a relatively

287   negative relationship with pH.

288

289   *3.6 Effect of carbon sources on the phosphorus-solubilization ability of strain*

290   The effects of different carbon sources (monosaccharides, disaccharides, and

291   polysaccharides) on the ability of *A. niger* to dissolve calcium P were investigated. Effects of

different carbon sources on the P content were significantly different in order, as follows: glucose > sucrose > maltose > starch > lactose (Fig. 4A). When glucose was used as the sole carbohydrate component, P content was the highest ($P<0.05$). Compared with the xj strain, the P-solubilizing rates of four positive mutant strains marked as xj90–32, xj120–12, xj120–31, xj180–22 increased by 41.2%, 49.2%, 45.8%, and 52.4%, respectively. In addition, glucose concentration had a significant effect on the P-solubilizing ability of the strains. In the range of 5~15%, the P-solubilizing increased with the increase of glucose concentration. Compared to the xj strain, the P-solubilizing rates of four positive mutant strains improved 40.1%, 30.9%, 31.2%, and 40.1%, respectively (Fig. 4B).

3.7 Effect of nitrogen sources on the phosphorus-solubilization ability of strain

Nitrogen sources had different influences on the P removal ability of the strains. When ammonium sulfate was used as the nitrogen component, the P content was the highest ($P<0.05$). In contrast to the xj strain, the xj90–32, xj120–12, xj120–31, and xj180–22 strains had higher P-solubilizing rates boosted by 40.5%, 49.1%, 46.6%, and 53.1%, respectively (Fig. 5A). Besides, when ammonium sulfate concentration was 0.5%, the P-solubilizing quantities of the mutant strains enhanced 34.1%, 41%, 36.6%, and 62.2%, respectively (Fig. 5B).

3.8 Effect of NaCl, temperature and pH on the phosphorus-solubilization ability of strain

The effects of different NaCl concentrations on the ability of strains to dissolve calcium P were determined. The P-solubilizing abilities of strains were the highest at lower NaCl concentration 1% (Fig. 6A). Compared to xj strain, the P-solubilizing quantities of strains (xj90–32, xj120–12, xj120–31, and xj180–22) increased by 40%, 47.1%, 43.4%, and 49.2%, respectively. The effect of temperature ranging from 20 to 50 °C on the ability of *A. niger* to hydrolyze calcium phosphate was determined. The P-solubilizing ability was the highest at the temperature 35 °C. Compared to the xj strain, P-solubilization quantities of strains (xj90–32, xj120–12, xj120–31, and xj180–22) increased by 27.4%, 36.9%, 30.2%, and 32.2%, respectively (Fig. 6B). To assess the pH adaptability of *A. niger* strains during P solubilization, the effect of pH (ranging from 3 to 11) on the ability of five strains to dissolve calcium P was determined.

319   The P-solubilizing abilities of strains had the highest at pH 7.0. Compared to the xj strain, the P-

320   solubilization quantities of strains (xj90–32, xj120–12, xj120–31, and xj180–22) enhanced 40%,

321   44.3%, 43.5%, and 50%, respectively (Fig. 6C).

322   *3.9 Effect of the Aspergillus niger spore suspension on the biomass of peanut plant*

323   As it is shown in Fig. 7, the positive mutant strains showed different effects on the biomass

324   of *peanut.* The xj120–12, xj90–32, and xj180–22 strains significantly promoted the growth of

325   height of peanut ($P<0.05$), which the biomass increased 68.7%, 55%, 47%, respectively higher

326   than the CK and enhanced 33.5%, 22%, 16%, respectively, more than the xj strain (Fig 7A). As

327   it is shown in Fig. 7B, the xj120–12, xj120–31, xj180–22, and xj90–32 strains obviously

328   improved the growth of root length of peanut ($P<0.05$). Their biomass improved 132.7%, 103%,

329   100%, 90.9%, respectively, higher than the CK. They enhanced by 43.8%, 26%, 24%, and 18%,

330   respectively, over than the xj strain. As it is shown in Fig. 7C, compared to the CK, the xj120–12,

331   xj180–22, and xj120–31 strains evidently increased the fresh biomass of 67.7%, 61%, and 40%,

332   respectively. Simultaneously they improved 43.4%, 38%, and 20%, respectively higher than the

333   xj strain. In addition, compared to the CK, the xj120–12 strain markedly boosted the dry biomass

334   of 117.2%. It improved 33.6% than the xj strain (Fig 7D).

335   *3.10 Effect of mutant positive strain on physiological indexes*

336   The strains influenced the physiological indexes of peanuts, including chlorophyll and

337   soluble protein content (Fig 8). The xj180–22 and xj120–12 strains improved the chlorophyll

338   content compared with the CK ($P<0.05$) by 49.5% and 27.9%, respectively. The enhancements

339   were 36% and 16%, respectively, higher than the xj strain (Fig. 8A). In addition, the positive

340   mutant strains increased soluble protein content than the CK ($P<0.05$). The soluble protein

341   content of xj180–22, xj120–31, xj120–12, and xj90–32 strains increased 12%, 10.7%, 9.2%, and

342   6%, respectively, than the CK. However, compared with the xj strain, the soluble protein

343   contents were not significantly different in all the positive mutant strains ($P>0.05$) (Fig. 8B).

344   *3.11 Effect of mutant strain on the available phosphorus content in the soil*

345   The available P content in soil for the CK was 6.88 mg kg^{-1}, which was considered low.

346   The inoculation with the xj spore suspension increased the P content to 12.85 mg kg^{-1}. The

347   xj120–12, xj120–31, xj90–32 and xj180-22 increased by 169.4%, 127.6%, 121.3% and 98.3%,

348   respectively, higher than CK ($P<0.05$). In addition, the xj120–12, xj120–31 and xj90–32 boosted

349   44.3%, 21.9%, and 18.5%, respectively higher than the xj strain ($P<0.05$) (Fig. 9). In general,

350   inoculation with the mutant strains xj120–12 could improve the P content in soils and promote

351   peanut growth.

352   **4. DISCUSSION**

353   With the establishment of ARTP mutation and using primary screening, re-screening and

354   subcultured 10 passages, we eventually obtained four representative mutants: xj90–32, xj120–12,

355   xj120–31, and xj180–22. The positive mutation rate was 25.5%, when the optimal treatment time

356   was 120 s. The good genetically stable of four strains were obtained, and the P-solubilization

357   abilities increased more than 50%. Due to the occurrence of positive mutations is related to the

358   mutagenic parameters and characteristics of the strain (Zhang et al., 2019). The positive mutation

359   rate of strains were the highest in the study when the fatality rate was 37%.

360   With the decrease in the pH value, P content increased by contrast. This indicated that pH

361   could be related to the P-solubilization ability of the mutant strain. Several reports have revealed

362   that pH decreased due to the release of organic acids (Nath et al., 2012; Padamavathi, 2015a;

363   Whitelaw, 2000). Oxalic acid and critic acid were detected in four positive mutant strains and the

364   xj strain. In addition, succinic acids were also secreted by xj120–12. The nature of organic acid

365   produced is a specific characteristic of each fungal isolate but may vary under different

366   cultivation conditions. Arguably, the mutagenesis treatment affected the kinds of organic acid,

367   resulting in differences in metabolic capabilities and affecting the P-solubilization ability of

368   mutant strains.

369   P-solubilization is a complex phenomenon that depends on many factors such as the

370   culture's nutritional, physiological, and growth conditions (Reyes et al., 1999). In this

371   experiment, the kinetics curve showed an optimal culture time of 5 days, which was taken as the

372   optimal culture time for the influencing factors. Glucose was used as a carbon source, and the P-

373   solubilization abilities were obviously increased. Glucose is a monosaccharide with a simple

374   structure and facilitates the acid production for the strain (Wang et al., 2015). Using ammonium

375   sulfate as nitrogen sources also caused high P-solubilization efficiency. Similar results have been

376   reported in previous studies that nitrogen in the ammonium form is necessary for P solubilization

377   in organic acid production through NH^+_4/H^+exchange mechanisms (Omar. 1998). The P-

378   solubilizing abilities of the four mutant strains were the highest at 1% NaCl concentration,

379   afterward, decreased with the NaCl concentration increase. These results were similar to the of

380   Srividya et al., (2009). The reason is that too much chloride ions may chelate or neutralize proton

381   ions or acid produced in the media then, decrease the P-solubilizing ability. Except for carbon

382   source, nitrogen source type and concentration and NaCl concentration, the formation process,

383   and growth of *A. niger* mycelium will be affected by temperature and pH value. In this study, the

384   P-solubilizing ability started decreasing with temperature and pH value over 35 °C and 7,

385   suggesting that microbial metabolism and biological activity affected the P-solubilizing ability of

386   the strain. In summary, choosing the composition of the medium and conditions would increase

387   the strains' P-solubilization ability; thus, improving the utilization rate of P in the soil (Liu et al.,

388   2015).

389   The four positive mutant strains could increase in different degrees on height, root length,

390   and dry and fresh biomass of peanut. The reason may be that the strains secreted IAA. According

391   to reports, IAA is an endogenous plant growth hormone, promoting seed germination and thus

392   plant growth (Wu et al., 2014). The xj180–22 and xj120–12 strains could improve the

393   chlorophyll content. The cause is that chlorophyll is the most important pigment in plant

394   photosynthesis, and its content is positively correlated with leaf color, which can roughly reflect

395   the nutritional status of plants and affect the photosynthetic rate of plants (Zhang et al., 2008).

396   The soluble protein as a kind of nutrient substance could directly affect the respiration of plants

397   and catalyze all kinds of chemical reactions (Berry. 1982). In this study, the four mutant strains

398 improved the soluble protein content compared with the CK. Besides, the xj120–12 strain

399 improved the P content in the soil.

400 **5. CONCLUSIONS**

401   To sum up, the xj120–12 can be used as the dominant fungi with the potential of P

402 solubilization and growth promotion and provided the basis for the production of P solubilization

403 fungal fertilizer.

404

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560

Figure 1

The lethality rate and positive mutation rate of *Aspergillus niger* strain were determined by atmospheric toom temperature plasm mutagenesis.
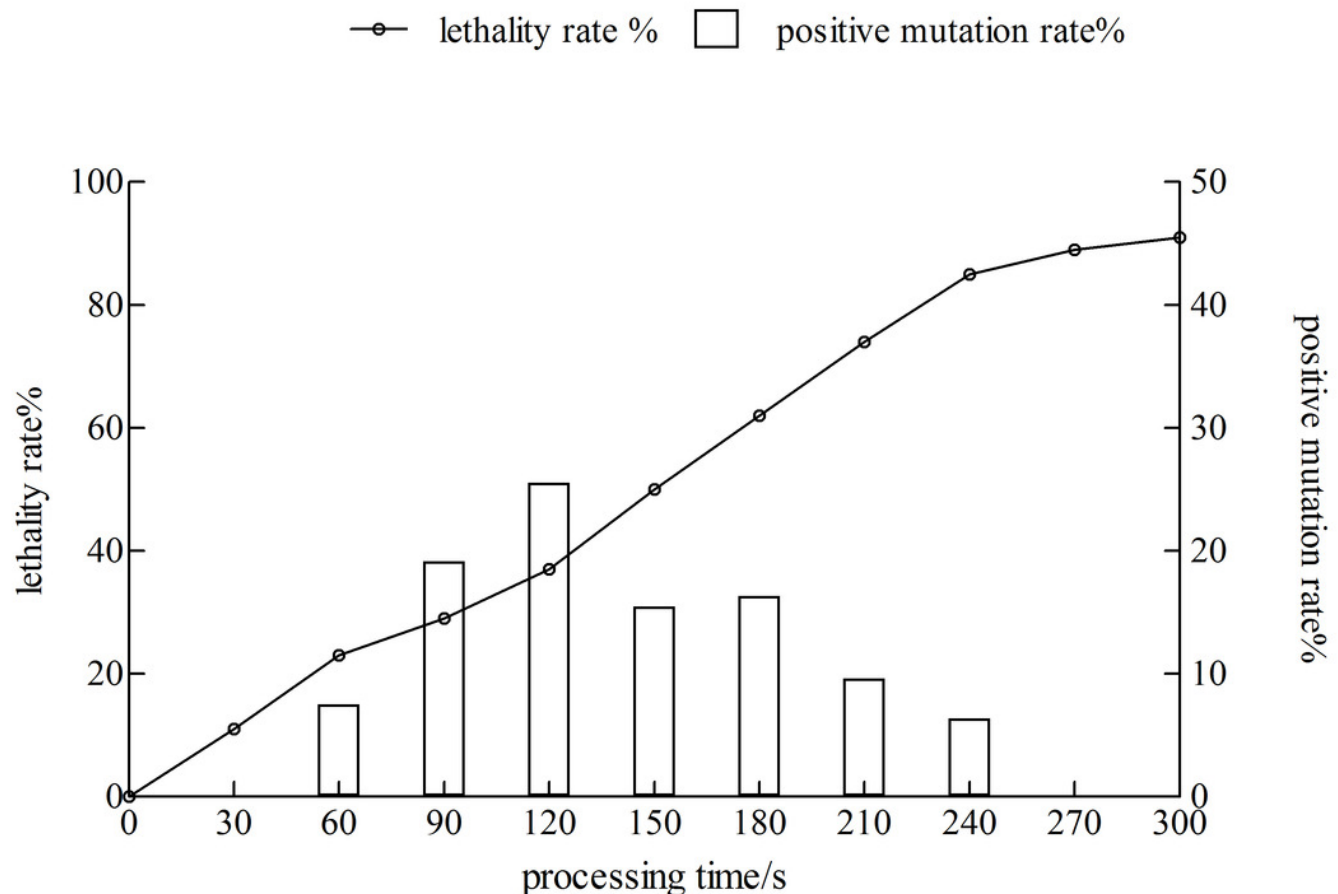
Figure 2

The concentrations of IAA produced by *Aspergillus niger* xj and mutant strains.

The vertical bars indicate standard deviation of biological replicate and different lowercase letters indicate significant differences (*P* < 0.05).
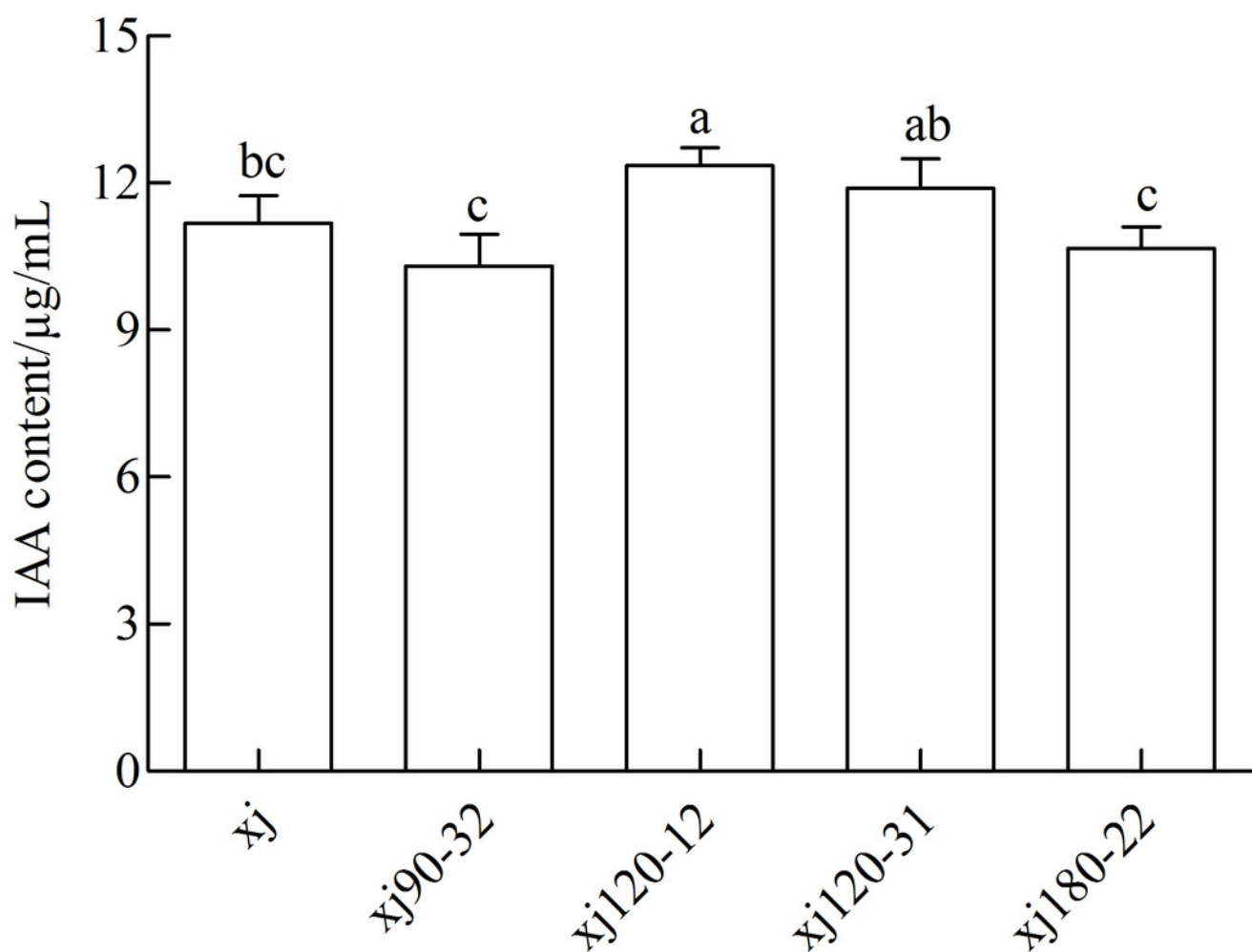
Figure 3

The change of soluble P (A) and pH (B) during incubation by seven days for *Aspergillus niger* xj and mutant strains.

Data are expressed as the average of three experiments. Error bars represent mean±SD.
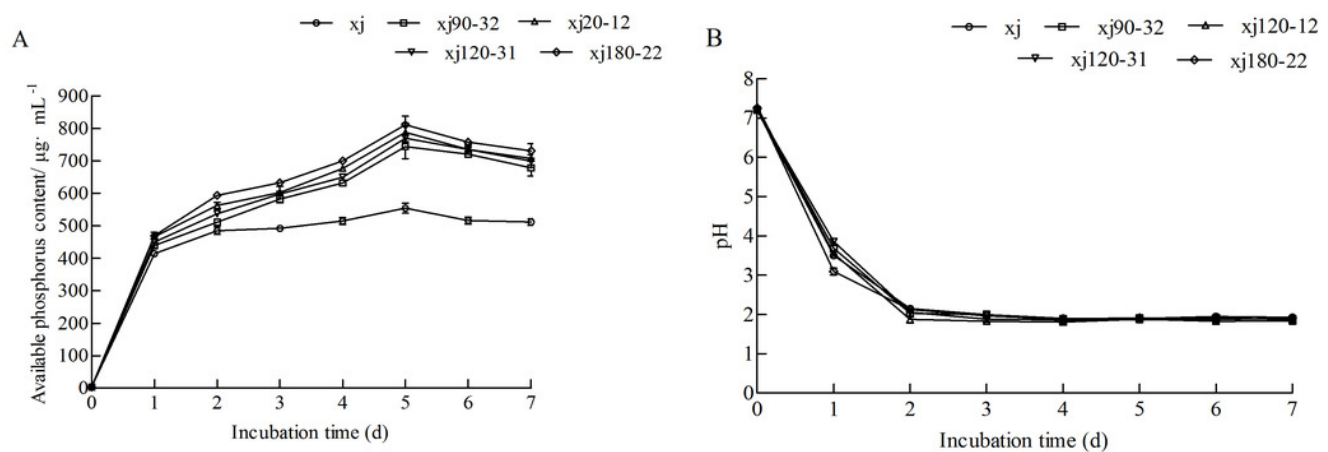
Figure 4

The effects of carbon sources (A) and glucose concentration (B) on the P-dissolving capability of *Aspergillus niger* xj and mutant strains.

Different lowercase letters indicate significant differences, and the lowercase letters used for the significance comparisons apply only to comparisons within the same strain ($P < 0.05$).
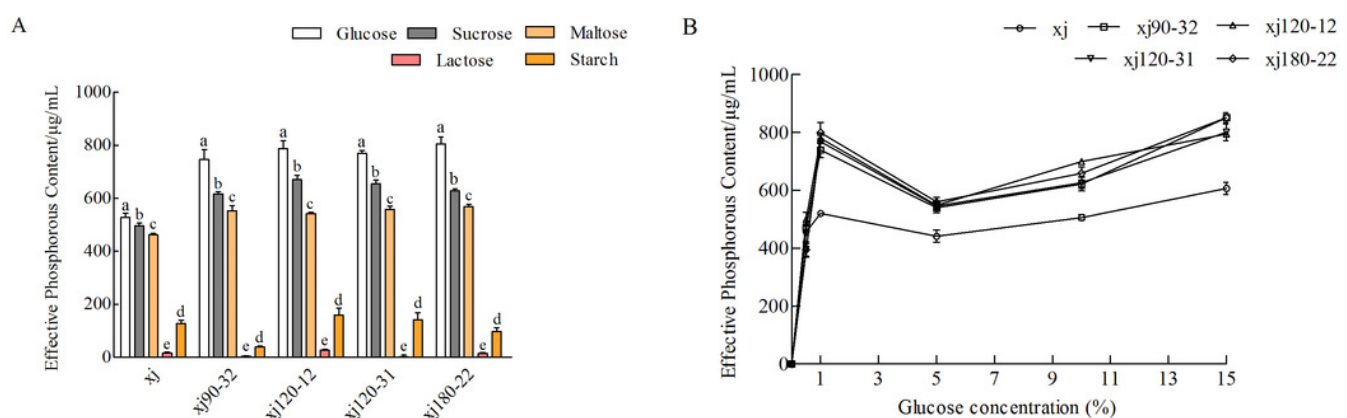
Figure 5

The effect of nitrogen sources (A) and ammonium sulfate concentration (B) on the P-dissolving capability of *Aspergillus niger* xj and mutant strains.

Different lowercase letters mean significant differences, and the lowercase letters used for the significance comparisons apply only to comparisons within the same strain ($P < 0.05$).
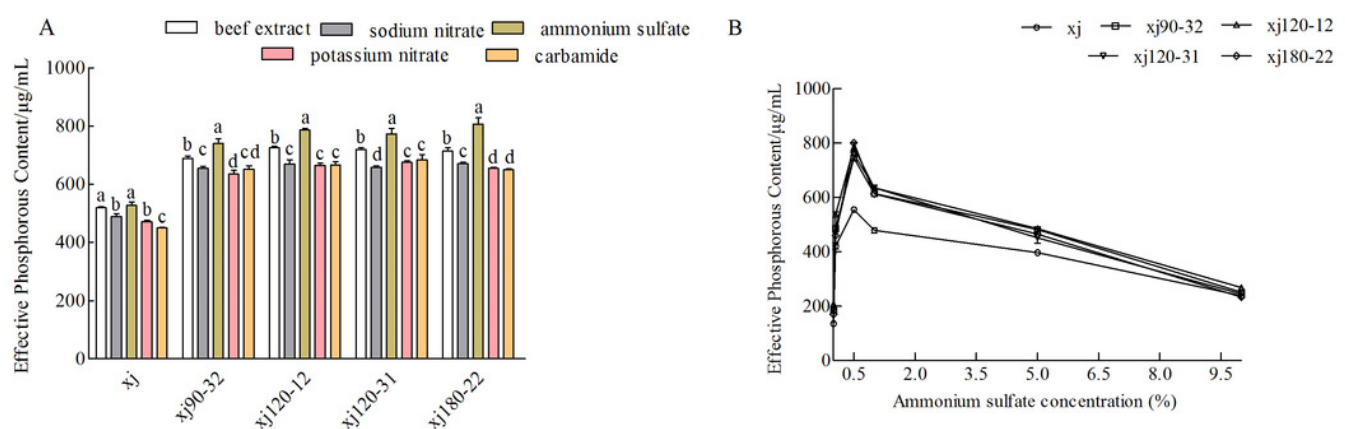
Figure 6

The effect of NaCl concentration (A), temperature (B), and pH value (C) on the P-dissolving capacity of *Aspergillus niger* xj and mutant strains.

Data are expressed as the average of three experiments. Error bars represent mean±SD.
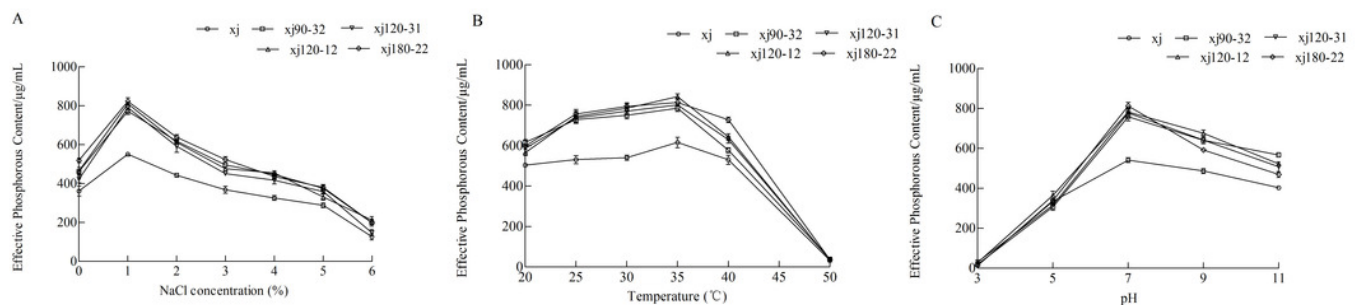
Figure 7

The effects of *Aspergillus niger* xj and mutant strains on plant height (A), root length (B), fresh biomass (C)and dry biomass (D) of peanut grown in pot experiment with soil sand mixture by 30 days.

CK represented blank group.

A

B

C

D
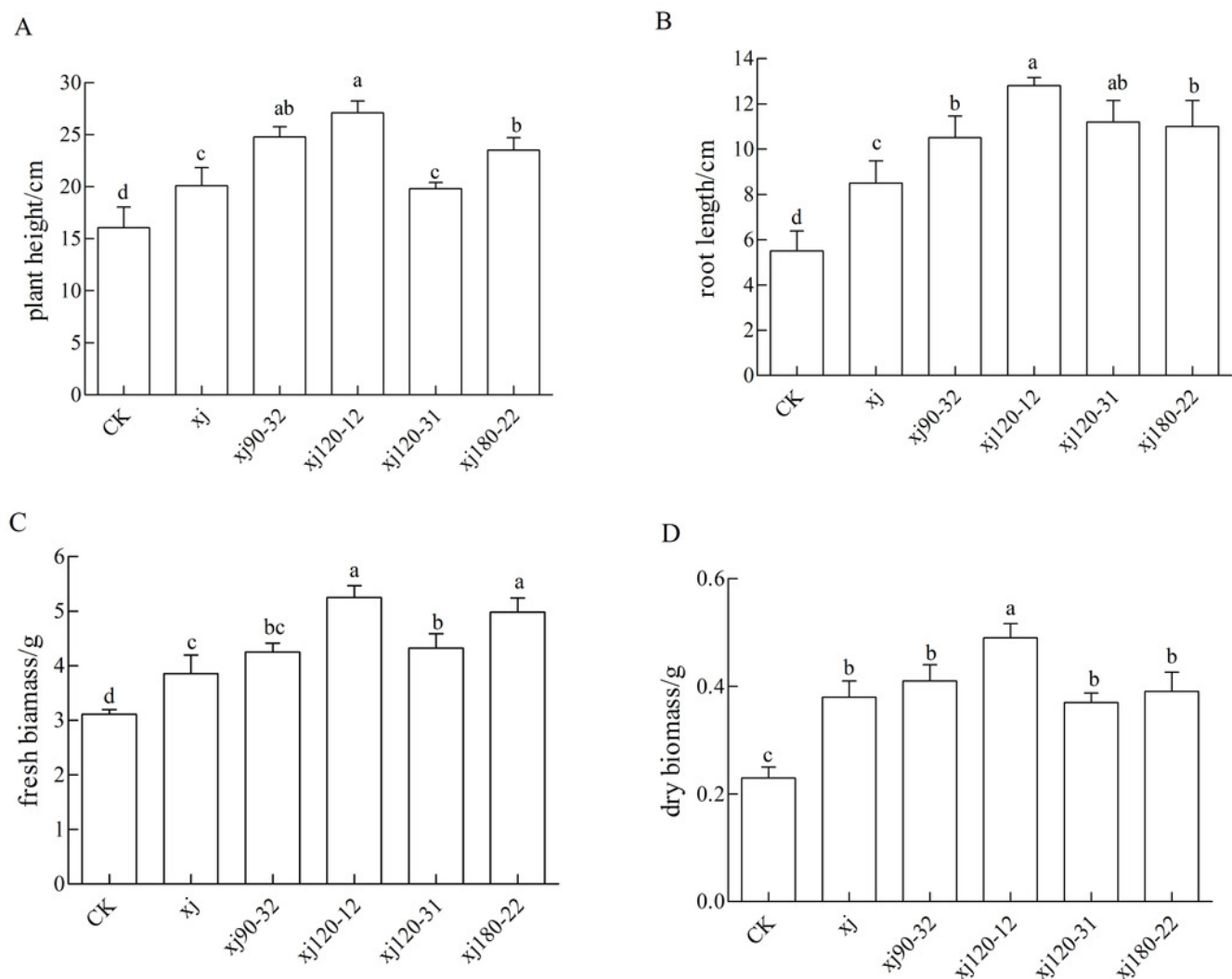
Figure 8

The effects of *Aspergillus niger* xj and mutant strains on physiological indexes (chlorophyll content and soluble protein content).

CK represented blank group. Different lowercase letters indicate significant differences ($P <$ 0.05).
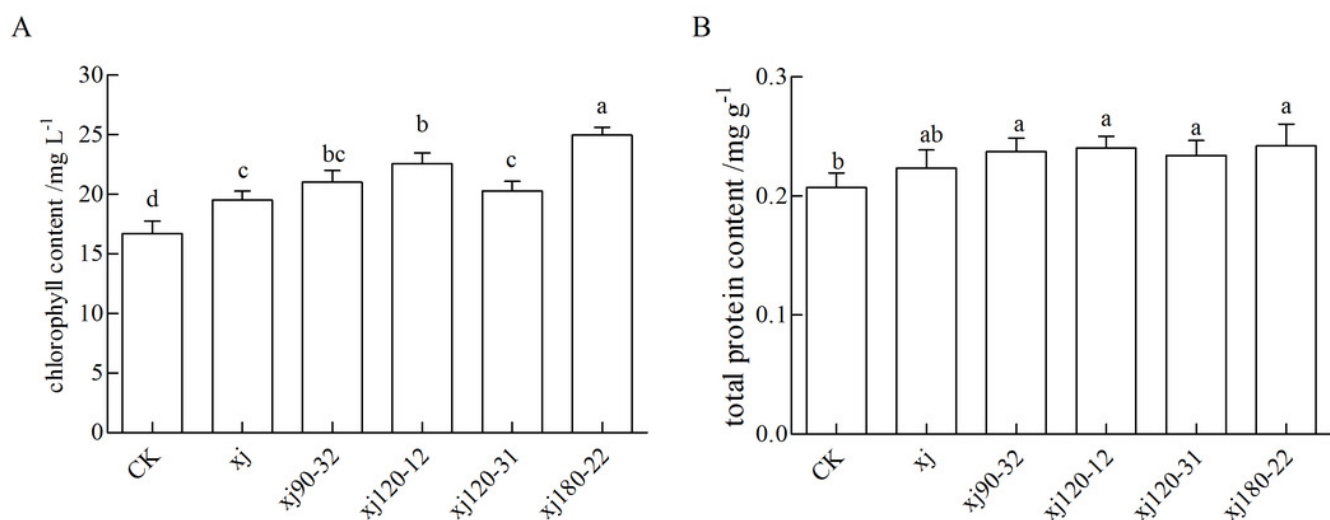
Figure 9

Effects of *Aspergillus niger* xj and mutant strains on the content of plant available phosphorus in the soil after cultivation of peanut in pot experiment with soil sand mixture by 30 days.

CK represented blank group.
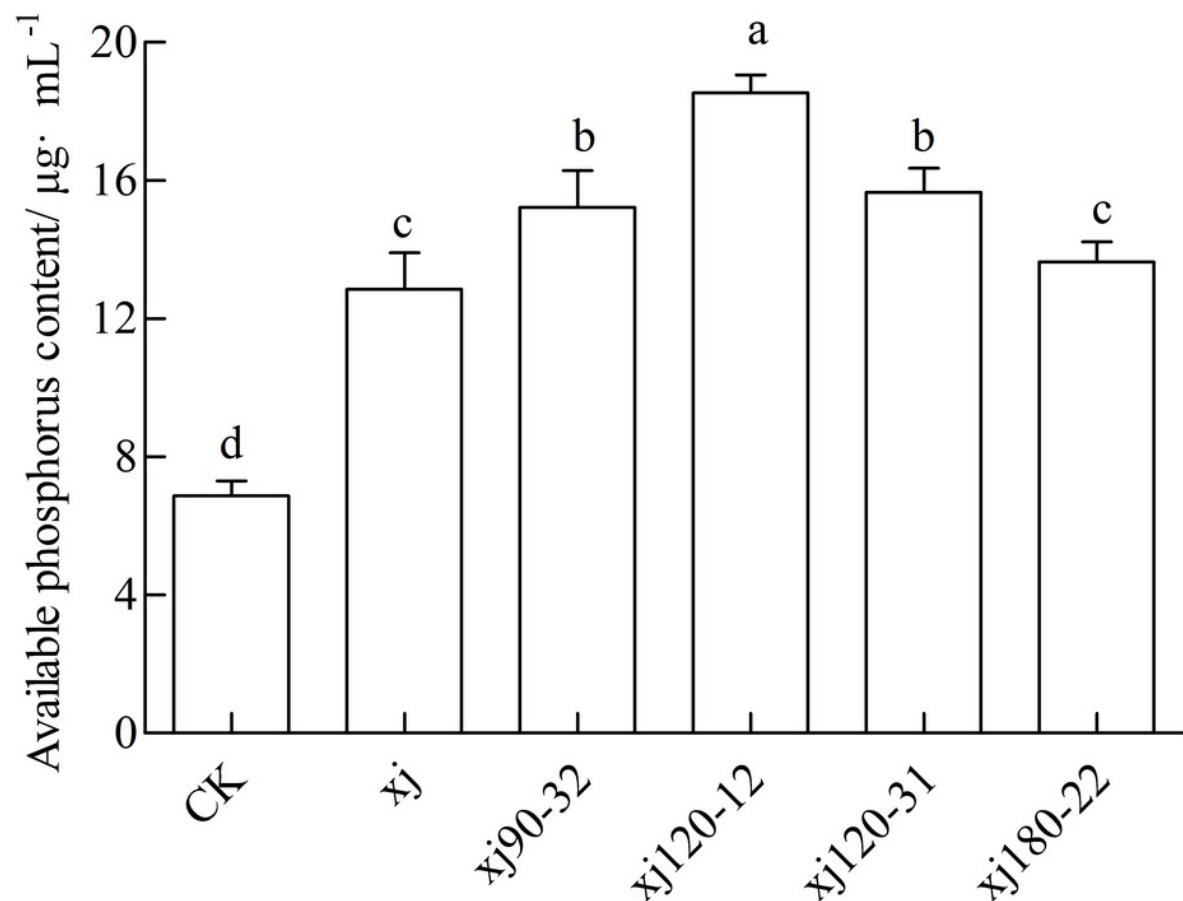
Table 1. The results of genetic stability testing of mutant strains of *Aspergillus niger.*

Note: $P < 0.05$ means a significant difference among means using Duncan's test.

Fungi strains	Phosphate-solubilizing quantity (µg/mL) (generation)										p-values	
	one	two	three	four	five	six	seven	eight	nine	ten	mean value	
xj	508.3 ±16.3	512.9 ±9.4	518.3 ±24.2	520.2 ±31.4	502.4 ±19.8	498.3 ±30.1	490.1 ±18.3	491.5 ±22.6	480.7 ±20.5	497.1 ±18.8	502.0	>0.05
xj90-32	776.2 ±25.8	775.1 ±17.3	770.0 ±23.2	761.2 ±3.2	736.6 ±20.7	750.5 ±38.5	746.0 ±8.2	745.7 ±19.3	753.9 ±29.6	748.6 ±38.2	754.4	>0.05
xj120-12	792.1 ±19.8	781.6 ±24.5	800.5 ±25.1	787.2 ±25.01	786.4 ±28.7	777.8 ±10.3	776.1 ±17.2	795.5 ±33.2	808.1 ±27.8	801.4 ±20.8	790.6	>0.05
xj120-31	776.8 ±12.4	788.1 ±28.8	774.8 ±30.3	779.9 ±19.5	776.5 ±30.2	781.3 ±21.9	782.3 ±34.35	781.15 ±21.3	789.8 ±32.2	791.9 ±17.1	782.8	>0.05
xj180-22	810.1 ±29.2	828.0 ±61	823.8 ±69.6	819.9 ±1.62	832.8 ±6.5	822.1 ±24.1	818.1 ±24.1	816.8 ±23.4	812.5 ±24.1	807.1 ±37.4	819.1	>0.05

Table 2. Contents of organic acids secreted by the *Aspergillus niger* strain within five days.

		organic acid content(mg·L^{-1})						
Fungi strains	time	oxalicacid	malic acid	citric acid	tartaric acid	lactic acid	acetic acid	succinic acid
xj	1d	730.5	-	110.1	-	-	-	-
	2d	942.3	-	112.2	-	-	-	-
	3d	1363.7	-	140.2	-	-	-	-
	4d	1239.2	-	134.2	-	-	-	-
	5d	1216.3	-	135.8	-	-	-	-
xj90-32	1d	843.5	-	142.0	-	-	-	-
	2d	1002.4	-	148.4	-	-	-	-
	3d	1424.6	-	151.4	-	-	-	-
	4d	1339.2	-	141.1	-	-	-	-
	5d	1304.1	-	138.5	-	-	-	-
xj120-12	1d	830.5	-	143.9	-	-	-	-
	2d	1209.2	-	148.2	-	-	-	543.5
	3d	1402.4	-	156.3	-	-	-	201.7
	4d	1299.3	-	151.2	-	-	-	-
	5d	1205.3	-	148.4	-	-	-	-
xj120-31	1d	893.2	-	121.5	-	-	-	-
	2d	1022.3	-	134.2	-	-	-	-
	3d	1329.4	-	145.6	-	-	-	-
	4d	1293.8	-	142.8	-	-	-	-
	5d	1221.6	-	138.7	-	-	-	-
xj180-22	1d	784.3	-	161.4	-	-	-	-
	2d	973.2	-	165.4	-	-	-	-
	3d	1394.2	-	172.9	-	-	-	-
	4d	1302.5	-	170.4	-	-	-	-
	5d	1259.5	-	162.8	-	-	-	-