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Effect of Lactic Acid on *α*-Amylase Activity and Phytic Acid Content in Germination of Rice (*Oryza sativa* L.)

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Abstract. Lactic acid has been known as one of compounds to cause cellular harm in waterlogged tissues through the process of cytoplasmic acidosis. The effects of lactic acid on *α*-amylase activity and phytic acid (myo-inositol 1,2,3,4,5,6-hexaphosphate) content using an assay for high phosphate in the germination stage of rice were evaluated. It is showed that lactic acid inhibited rice germination at every treated dose. The reduction of *α*-amylase content attributed to lactic acid at 24 h after germination of rice seeds was observed. The findings highlighted the effects of lactic acid on *α*-amylase activity and phytic acid content and suggested that this compound may play a potent role as a germination regulator in rice.

Introduction

Lactic acid (LA) is one of the products produced from anaerobic respiration which accumulates in seeds [1]. LA has been reported as a major factor to cause cellular damage when cytoplasm is acidic infected in waterlogged condition [2]. The physiological roles of LA on seed germination have been documented [3]. According to Kulkarni and Chavan [1], LA treatment showed greater inhibition on growth of radicals than coleoptiles of millets. However, the influence of LA on photosynthesis, metabolite, and allelopathy has not been much known.

Several reports have shown that *α*-amylase can reserve carbohydrate catabolism during seed germination and starch mobilization [1, 4]. Starch, a primary product of photosynthesis in higher plants, is a storage carbohydrate that supported seed growth in the dark [5-7], whereas *α*-amylase normally correlated with the primary original on starch rich seeds [8]. The *α*-amylase activity was improved by extended darkness [9], virus infection in the leaf [10], and water stress [11]. Particularly, higher levels of this enzyme activity were required at an increased level of heat stress [12].

The major storage compound of phosphorus in plants tissue is phytic acid [13]. This compound can soak up irons in foods and animal system and it decreases the absorption capacity of zinc, manganese, copper, molybdenum, calcium, magnesium, iron as well as protein [14]. Phytic acid content appeared to differ among levels of phosphorous is also controlled by inorganic phosphate concentration in developing seeds and seedling [8]. Loreti et al. [15] showed that during germination, phytates were broken down by process of phytate degradation and released phosphorous, minerals, and myo-inositol, which promoted rice at germination and seedling stages.

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population [16]. Rice is cultivated under different ecosystems, extending from irrigated to rainfed lowland to deep-water. Germination is a natural process of seeds in which seeds require the lowest condition for plant growth and development [8]. One of the major limitations for rice production is low germination rate in stress condition as a flooding event. Thus, this study was carried out to examine the effects of lactic acid on *α*-amylase activity and content of phytic acid during germination of rice. It also examined whether lactic acid plays any role as a regulator during seed germination of rice.

Materials and Methods

There were seven rice varieties were used (Table 1). Information of these rice is listed in Table 1.

Table 1. Rice lines/varieties

Codes	Varieties/lines	Subtypes
I13	OM5629	Indica
I8	F7 (OM6162/Swanasub1)	Indica
I34	Line 54	Indica
I5	Line 45	Indica
I49	F7 (IR75499-29-2-B/IR64 Sub1)	Indica
J65	K5	Indica
J25	Xn	Indica

Reagents

DNS (3,5 dinitrosalicylic acid): The colour reagent was prepared by dissolving an amount of 1.0 g of 3,5 dinitrosalicylic acid in 50 ml distilled water, then 30.0 g of sodium potassium tartrate tetrahydrate were slowly added in 20 ml of 2 N NaOH and volume was makeup till 100 ml with distilled water.

Starch solution: This solution was prepared by dissolving 1.0 gm soluble starch in 100 ml of 0.02 M sodium phosphate buffer and 0.006 M sodium chloride (pH 6.9). The mixture was subsequently heated in water bath and after cooling the volume was prepared till 100 ml with distilled water.

The released maltose was estimated from a standard curve of a graph represent different concentrations (0, 0.1, 0.3, 0.5, 0.8 and 1.0 ml of a maltose solution containing 5.0 mg maltose/ml)

Methods

Lactic acid treatment

Four concentrations of LA (0.1, 0.2, 0.4 and 0.5%) were prepared in distilled water. The seeds were surface-sterilized by soaking in 0.1% NaOCl for 30 min and washed in distilled water. A total of 10 rice seeds were placed in each Petri dish, lined up on filter paper moistened with 10 ml of each applied concentrations of LA, whereas the control was used with distilled water only. The germination percentage was recorded after every 24 h. The shoot height were recorded in each 48 h, 72 h, and 96 h. For the enzyme activity, one concentration of LA 0.1% was assayed from seed germination after every 24 h.

α-Amylase activity

Germinated seeds (0.3 g) were ground to a fine powder in 3 ml of 50 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 15,000 rpm for 20 min at 4 °C to yield a supernatant for the assay of enzyme activity. The α-amylase activity was determined according to the method of Bernfeld [17]. An aliquot of 0.5 ml of respective enzyme dilutions in a series of test tubes was incubated at 25 °C for 3–4 min and thereafter 0.5 ml (1% w/v) of starch solution was added. Furthermore, an amount of 1 ml dinitrosalicylic acid were added to stop the reaction. Finally, it was cooled to room temperature, and a volume of 5 ml distilled water was added in each test tube and mixed well. A blank was prepared with 0.5 ml distilled water. The absorbance of samples was measured at 540 nm using HACH DR/4000U spectrophotometer (HACH Company, Loveland, CO, USA). Maltose was used as standard solution in concentrations of 0.1, 0.3, 0.5, 0.8

and 1.0 ml containing 5.0 mg maltose/ml to establish the calibration curve. One unit of α-amylase was calculated as one mM of reducing maltose released from soluble starch at 25 °C and pH of 6.9.

Phytic acid content assay

Seeds of rice varieties (0.05 g) were ground to a fine powder, mixed in 2 ml of 0.4 M HCl and incubated at 4 °C for overnight. The solution was mixed and 100 µl of the mixture was transferred to a cuvette. A volume of 1 ml was maintained by adding 900 µl distilled water. After that, 1 ml of Chen's reagent [(6N H_2SO_4:2.5% ammonium molybdate:10% ascorbic acid:distilled water (1:1:1:2)) was added to a cuvette, covered with parafilm and mixed well by inversion. A blank was used as control having 1ml Chen's reagent and 1 ml water [18]. The samples were then incubated at 37 °C for 1.5 h. The absorbance of the reaction was measured at 820 nm. The phytic acid content was determined using a phosphate standard curve that established in triplication of 1 mM KH_2PO_4 ranging from 25, 50, 100, 150, to 200 µl.

Statistical analysis

All experiments were carried out with three replications. Comparisons of the differences in germination percentage and shoot length were evaluated using two-way analysis of variance (ANOVA) by the SPSS grogram applied to Duncan's multiple range test. A significant difference was determined at $p < 0.01$. The data on α-amylase activity and phytic acid content assays were analysed by the Minitab 16 software. Comparisons with $p < 0.05$ were considered significantly different.

Results and Discussion

Effect of LA on germination and shoot growth

The results showed that lactic acid significantly affected the percentage of seed germination and shoot height (Tables 2-5). The germination ratio was remarkably reduced by concentration of LA at 0.2 %, especially after 24 h (Table 2).

Table 2. Effect of LA concentrations on germination rate of rice varieties (%) (24 h)

Varieties	Concentration (%)				
	Control	0.1	0.2	0.4	0.5
I13	90.00 bc	40.00 i	26.67 kl	0.00 n	0.00 n
I8	86.67 cd	40.00 i	23.33 klm	16.67 m	0.00 n
I34	100.00 a	50.00 g	30.00 k	0.00 n	0.00 n
I5	100.00 a	40.00 i	20.00 lm	0.00 n	0.00 n
I49	93.33 ab	30.00 k	20.00 lm	0.00 n	0.00 n
J65	100.00 a	56.67 f	20.00 lm	0.00 n	0.00 n
J25	100.00 a	83.33 d	73.33 e	46.67 gi	40.00 i
CV (%)	9.97				

Values in a column with similar letters are not significantly different ($p<0.01$)

The shoot lengths declined on the increase of LA concentrations and at doses of 0.4 and 0.5%, the seeds failed to germinate, except for I8 and J25. In general, the J25 variety was least affected as compared with other varieties. The results showed that the percentage of germination was proportional to the LA concentrations. At 0.5% LA concentration, seeds of all varieties were not germinated. The increased flooding period caused a significant reduction in lactic acid content [19].

Table 3. Effect of LA on shoot height of rice varieties (mm) (48 h)

Varieties	Concentration (%)	
	Control	0.1 (%)
I13	6.1 b	2.2 g
I8	3.6 e	1.9 g
I34	4.2 e	2.1 g
I5	5.5 cd	2.3 g
I49	5.0 d	2.0 g
J65	6.9 a	2.1 g
J25	5.8 bc	2.9 f
CV (%)	8.4	

Values in a column with similar letters are not significantly different ($p<0.01$)

It was observed that LA treatments were significantly affected seed development after 48 h, 72 h and 96 h stages of germination (Tables 3, 4, 5). The effect of 0.1% LA concentration on seed germination and shoot growth was high as compared to the control and higher concentrations. By 48 h, 72 h and 96 h, the shoot growth was almost inhibited. It indicated that lactic acid caused a reduction in seed germination and shoot height in rice. Statistically, the cultivars J65 and J25 were the most inhibited (Table 5).

Table 4. Effect of LA concentrations on shoot height of rice varieties (mm) (72 h)

Varieties	Concentration (%)	
	Control	0.1 (%)
I13	21.0 a	8.7 fg
I8	9.0 f	4.3 kl
I34	20.5 ab	5.6 ik
I5	19.4 bc	5.3 hi
I49	18.4 c	7.4 gh
J65	13.0d	4.0 l
J25	11.3 e	4.1 l
CV (%)	7.0	

Values in a column with similar letters are not significantly different ($p<0.01$).

Table 5. Effect of lactic acid concentrations on shoot height of rice varieties (mm) (96 h)

Varieties	Concentration (%)	
	Control	0.1
I13	38.8 a	19.8 d
I8	19.8 d	14.0 e
I34	33.6 b	15.0 e
I5	27.8 c	14.2 e
I49	41.0 a	14.4 e
J65	26.7 c	11.6 e
J25	25.4 c	11.4 e
CV (%)	8.7	

Values in a column with similar letters are not significantly different ($p<0.01$).

Our finding was supported by the study of Kulkarni and Chavan [1] who reported that the shoot elongation was more sensitive than the coleoptile growth of finger millet cultivar GPU28. The inhibitory effect of lactic acid on rice seedling growth was high at 0.1% concentration. Thus, the enzymatic assay was performed by giving the treatment of 0.1% lactic acid to rice varieties after the 24 h of seed germination stage.

Effect of LA on α-amylase activity

The effect of LA on the activity of *α*-amylase was observed at germination stage of rice after 24 h (Table 6). All the varieties exhibited a significant reduction in *α*-amylase activity at 0.1 % LA concentration. The activity of amylase in seed germination was also affected by other sensitive conditions such as salinity and waterlogged stress (24 h). It was reported by Kulkarni and Chavan [1] that LA could decrease in the amylase activity on finger millet cultivar GPU28 at 24 h during germination time of treatment. In this study, the *α*-amylase activity of all varieties was affected by LA at the germination stage. The inhibition of *α*-amylase by LA treatment would certainly decrease the availability of substrate for metabolism during germination of rice.

Table 6. *α*-Amylase activity of seed germination after 24 h (unit/mg fresh weight)

Varieties	Control	0.1% LA
I13	2936.2d ± 36.8	2659.0e ± 54.0
I8	3395.9c ± 106.1	3030.8d ± 31.7
I34	3371.6c ± 38.7	2613.0e ± 123.4
I5	2450.7e ± 91.9	2031.5f ± 28.8
I49	1453.3g ± 5.8	1053.3h ± 5.8
J65	4494.0a ± 33.8	3947.7b ± 111.2
J25	1974.7f ± 112.2	1601.5g ± 71.0

Values with similar letters are not significantly different ($p < .05$) ± SD (standard deviation).

Although the seeds in our experiment were germinated and grown on moist filter paper without the addition of nutrients, the effects of LA could be explained from different physiological roles in the absence of nutritional components. Our results showed that LA was a strong inhibitor of *α*-amylase production in germinating rice.

Phytic acid assay

Phytic acid is the major storage compound of phosphorous (P) in plants [13]. The present study revealed that the highest content of phytic acid was observed in the I49 variety with 41.772 ± 0.126, followed by I13 variety (40.822 ± 0.404) and I34 variety (40.620 ± 0.175). While the lowest content was found in the I5 variety (Table 7). According to Khattak et al. [20] and Beleia [14], phytates played an important role in controlling minerals, which reduced the availability of Fe, Zn, Ca, Mg, Cu, Mn, and Mo. Therefore, low content of phytic acid rice may have higher bioavailable Zn^{2+} and Fe^{3+}.

Table 7. Free phosphate content (μg/ml) in rice seedlings after 48 h germinated

Varieties	Controls	0.1% LA
I13	35.267 d ± 0.121	40.822 b ± 0.404
I8	30.721 f ± 0.061	34.358 d ± 0.369
I34	33.610 e ± 0.153	40.620 b ± 0.175
I5	25.630 g ± 0.182	33.671 d ± 0.070
I49	38.701 b ± 0.093	41.772 a ± 0.126
J65	40.297 a ± 0.264	38.822 c ± 0.153
J25	36.398 c ± 0.438	34.358 d ± 0.369

Values with the same letters are not significantly different ($p < 0.05$; ± values indicate standard errors of the means).

Conclusions

Lactic acid was found as an inhibitor on α-amylase production in rice germination. The increase of lactic acid concentration could reduce the phytic acid content. This study provided new information about the effects of lactic acid in seed germination stage and seedling growth of rice.

References

[1] S.S. Kulkarni, P.D. Chavan, Influence of lactic acid on seed germination of finger millet (*Eleusine coracana* Gaertn.), International Journal of Applied Biology and Pharmaceutical Technology. 3 (2012) 242–247.

[2] P.W. Hochachaka, T.P. Mommsen, Protons and anaerobiosis, Science. 219(4591) (1983) 1391–1397.

[3] K.A. Howell, A.H. Millar, J. Whelan, Ordered assembly of mitochondria during rice germination begins with promitochondrial structures rich in components of the protein import apparatus, Plant Molecular Biology. 60(2) (2006) 201–223.

[4] G.B. Fincher, Molecular and cell biology associated with endosperm mobilization in germinating cereal grains, Annual Review of Plant Physiology and Plant Molecular Biology. 40(1) (1989) 305–346.

[5] T. Akazawa, I. Hara-Mishimura, Topographic aspects of biosynthesis, extracellular section and intracellular storage of proteins in plant cells, Annual Review of Plant Physiology. 36(1) (1985) 441–472.

[6] E. Beck, P. Ziegler, Biosynthesis and degradation of starch in higher plants, Annual Review of Plant Physiology and Plant Molecular Biology. 40(1) (1989) 95–117.

[7] M.A. Zayed, I.M. Zeid, Effect of water and salt stress on growth, chlorophyll, mineral ions and organic solutes contents on enzyme activity in mug bean seedlings, Biologia Plantarum. 40(3) (1997) 351–356.

[8] R.N. Trethewey, A.M. Smith, Starch mobilization in leaves, in: R.C. Leegood, T.D. Sharkey, S. von Caemmerer (Eds.), Photosynthesis. Advances in Photosynthesis and Respiration, vol. 9, Springer, Dordrecht, 2000, pp. 205–231.

[9] M. Saeed, S.H. Duke, Amylase in pea tissues with reduce chloroplast density and/or function, Plant Physiology. 94(4) (1990) 1813–1819.

[10] T. Heits et al., Two apoplastic α-amylase are induced in tobacco by virus infection, Plant Physiology. 97(2) (1991) 651–656.

[11] M.Y. Ashraf et al., Alpha-amylase, protease activities and associated changes under water stress condition in wheat seedling, Pakistan Journal of Scientific and Industrial Research. 38 (1995) 430–434.

[12] P.D. Commuri, S.H. Duke, Apoplastic α-amylase in pea is enhanced by heat stress, Plant Cell Physiology. 38(5) (1997) 625–630.

[13] J. Shi et al., The maize low-phytic acid 3 encodes a myo-inositol kinase that plays a role in phytic acid biosynthesis in developing seeds, The Plant Journal. 42(5) (2005) 708–719.

[14] A. Beleia, L.T. Thu Thao, E. I. Ida, Lowering phytic phosphorus by hydration of soybean, Journal of Food Science. 58(2) (1993) 375–377.

[15] E. Loreti et al., Repression of α-amylase activity by anoxia in grains of barley is independent of ethanol toxicity or action of abscisic acid, Plant Biology. 4(2) (2002) 266–272.

[16] M. Haque, et al., Yield contributing characters effect of submerged water levels of boro rice (*Oryza sativa* L.), Scientia Agricultural. 9(1) (2015) 23–29.

[17] P. Bernfeld, Amylases, α and β, in: Methods in enzymology, S.P. Colowick, N.D. Kaplan, (Eds.), Vol. 1, Academic Press, New York, 1955, pp. 149-150.

[18] P.S. Chen, T.Y. Toribara, H. Warner, Microdeteminations of phosphorous, Analytical Chemical. 28 (1956) 1756–1758.

[19] S.S. Kulkarni, P.D. Chavan, Study of some aspects of anaerobic metabolism in roots of finger millet and rice plants subjected to water logging stress, International Journal of Botany. 9(2) (2013) 80–85.

[20] A.B. Khattak et al., Influence of germination techniques on phytic acid and polyphenols content of chickpea *(Cicer arietinum* L.) sprouts, Food Chemistry. 104(3) (2007) 1074–1079.