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Butyric acid tolerance of rice mutant M_4 families

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ABSTRACT - *Hydromorphic soils have a low drainage capacity and are used mainly for the cultivation of irrigated rice. This condition favors the development of anaerobic microorganisms that produce phytotoxic substances. The objective of this study was to evaluate the response of rice mutants to the phytotoxicity caused by butyric acid under anaerobic conditions. The experiment consisted of four treatments arranged in a randomized block design. Plants of 40 families were grown in a hydroponic system and the measured variables were root length and length of aerial part (LAP), number of roots (NR) and root dry matter (RDM) and aerial part dry matter (DMAP). The analysis of variance was performed, the relative performance calculated and linear regressions were fitted. Only the treatment effect for NR and effect of interaction for LAP were not significant. Root length was most affected by the acid and the regressions expressed positive as well as negative effects for acid tolerance in the mutant families.*

Key words: *Oryza sativa*, mutation, toxicity, organic acids.

INTRODUCTION

The chief characteristic of irrigated rice is the continuously flooded soil during most part of the crop development, establishing an anaerobic environment. This condition favors anaerobic microorganisms that ferment organic matter (Ponnamperuma 1965). During anaerobic fermentation, intermediary phytotoxic substances are produced, principally short-chained aliphatic organic acids of low molecular weight, such as acetic, propionic and butyric acid; they usually occur in a concentration range of 0.1 to 14 mM (Camargo et al. 1993) at a ratio of 6:3:1, respectively (Bohnen et al. 2005).

The adoption of no-till and minimum tillage systems for irrigated rice, in which plant residues are not removed from the soil surface increases the acid production, limiting crop growth and yield (Camargo et al. 2001). In the initial phases of rice development, organic acid toxicity is manifested in reduced germination, root growth, weight and height of the plantlets (Sousa and Bortolon 2002). In cases of more severe toxicity, the damage to the growth is reflected in other phases, resulting in less tillering, lower nutrient uptake and lower grain yield (Camargo et al. 2001). Butyric acid is less concentrated in the soil solution, but has a higher phytotoxicity level; the larger the size of its carbon chain, the greater is the phytotoxicity (Rao and Mikkelsen 1977a). The damage potential of butyric acid is particularly high during the initial establishment of rice plantlets in the field, impairing the final crop yield in no-till or minimum tillage systems.

The identification and characterization of the genetic variability for tolerance to organic acids is of

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fundamental importance to obtain promising genetic constitutions for genetic breeding programs of this cereal. Mutation is frequently used to amplify genetic variability (Maki 2002, Pandini et al. 1997, Tulmann Neto et al. 1995a, Tulmann Neto et al. 1995b), and has contributed to increase the world grain production. Banks of mutants have been generated for many traits (Maluszynski et al. 1998). Induced mutations are used to increase the frequency of spontaneous mutations and can be provoked by chemical agents, such as alquilant substances, or physically, as by ionizing radiations (Coimbra et al. 2004). Independent of the methodology, the identification of the generated variability is a decisive but difficult step, mainly in traits related to the root system.

The genetic variability can be identified in three environments: i) under field conditions; ii) in semicontrolled conditions of a greenhouse and iii) in the laboratory, using nutrient solutions in controlled environmental conditions (Duncan and Baligar 1990). While the evaluation of genotypes in artificial environments does not take the real pressures of the medium into account (Duncan and Baligar 1990), the selection in field trials unites numerous uncontrolled variables, such as different tolerance to climate, biotic or nutritional stresses (Wright 1989). In the case of cultivated grasses, studies show significant correlations between parameter data of field tests and of artificial environments, in soil or nutrient solution (Bilinski and Foy 1987).

The identification of mutants in hydroponic systems has contributed significantly to studies of genetic variability, function, regulation and action, especially by the new available technologies (Ahloowalia and Maluszynski 2001), besides, mutants are used in the gene incorporation in superior cultivars. Gene incorporation responsible for tolerance to organic acids in high-yielding cultivars may contribute to increase the area cultivated with no-till irrigated rice. This would in turn increase the yield, reducing the environmental impacts of the conventional cultivation and the production costs of this cereal.

The objectives of this study were to evaluate the initial development of rice plantlets subjected to the toxic action of butyric acid, determine the most stress-responsive variable and to evaluate the efficiency of mutation induction with gamma rays (^{60}Co) to generate genetic variability for the trait butyric-acid tolerance in hydroponic rice.

MATERIAL AND METHODS

The experiment was carried out in a laboratory of the Centro de Genômica e Fitomelhoramento (CGF) in cooperation with the Regional Centro of Oncology of the Universidade Federal de Pelotas (UFPel), in the county of Pelotas, Rio Grande do Sul, in 2002.

Rice seed of cultivar BRS 7-Taim on a 30 x 30 field was irradiated with gamma rays (^{60}Co) by an Eldorado 78 teletherapy unit, at 37.9 cGy (centigrays) min^{-1}, at 25 cm from the source.

To establish the most adequate dose, a preliminary study with varied doses was conducted to determine the effect of ionizing radiation (^{60}Co). In this study, six treatments were carried out with the doses 0, 25, 50, 100, 200, and 400 Gy. Each treatment consisted of a 200 seed sample that was soaked in distilled water for one hour, then irradiated and thereafter rinsed in tap water for another hour. The seeds were then sown on sand-filled trays and evaluated after 21 days for germination and abnormalities in the root system and aerial part. In this study the dose of 250 Gy was determined to induce mutation in rice cultivar BRS 7-Taim. This radiation dose induced most phenotypic abnormalities, which can be considered a parameter to estimate the efficiency of the dose to induce variability at the genetic level, while loss by seed death was lowest.

Thereafter, 3000 seeds previously hydrated as described above were irradiated (250 Gy) to constitute the M_1 population. These seeds, after washing in tap water for one hour, were sown on an experimental field to advance the generations. In the M_2 and M_3 generations mutants were selected for diverse traits originating some of the families used here (Zimmer et al. 2003) (Table 1).

To evaluate the M_4 families for responsiveness to butyric acid, 40 families of the genebank of the Centro de Genômica e Fitomelhoramento (CGF) - UFPel were selected in order to best represent the main mutations identified in previous studies (Table 1) in which the root system was specifically evaluated. So far, no association between mutations in the root system and tolerance to abiotic stresses to butyric acid has been reported. As control, the cultivar of origin (BRS 7-Taim) of the mutant families was also evaluated. In hydropony,

Table 1. Morphological traits of the 40 rice mutant M_4 families used in the study of tolerance to butyric acid toxicity

Family	Accession CGF*	Description
1	CGF-M_4-Ar-10	Irregular seminal root growth
2	CGF-M_4-Ar-12-1	Excess of pilosity in the root system
3	CGF-M_4-Ar-12-2	Irregular root growth
4	CGF-M_4-Ar-12-3	Absence of seminal roots
5	CGF-M_4-Ar-12-4	Irregular root growth
6	CGF-M_4-Ar-13	Irregular root growth
7	CGF-M_4-Ar-16	Irregular root growth
8	CGF-M_4-Ar-17	Absence of seminal roots
9	CGF-M_4-Ar-18	Absence of main root (excess of secondary roots)
10	CGF-M_4-Ar-19	Excess of pilosity in the root system
11	CGF-M_4-Ar-20-1	Absence of main root (excess of secondary roots)
12	CGF-M_4-Ar-20-2	Irregular root growth
13	CGF-M_4-Ar-23	Irregular root growth and absence of seminal roots
14	CGF-M_4-Ar-24	Irregular root growth
15	CGF-M_4-Ar-27	Irregular root growth
16	CGF-M_4-Ar-44	Atrophied seminal roots
17	CGF-M_4-Ar-50	Excess of pilosity in the root system
18	CGF-M_4-Ar-51	Excess of pilosity in the root system
19	CGF-M_4-Ar-68	Irregular root growth
20	CGF-M_4-Ar-78	Absence of seminal roots
21	CGF-M_4-Ar-87	Absence of seminal roots
22	CGF-M_4-Ar-92	Excess of pilosity in the root system
23	CGF-M_4-Ar-105	Excess of pilosity in the root system
24	CGF-M_4-Ar-108	Irregular root growth
25	CGF-M_4-Ar-125	Absence of seminal roots
26	CGF-M_4-Ar-208	Absence of seminal roots
27	CGF-M_4-Ar-213-1	Absence of seminal roots
28	CGF-M_4-Ar-213-2	Excess of pilosity in the root system
29	CGF-M_4-Ar-237	Absence of seminal roots
30	CGF-M_4-Ar-238	Absence of main root (excess of secondary roots)
31	CGF-M_4-Ar-240	Absence of seminal roots
32	CGF-M_4-Ar-245	Absence of seminal roots
33	CGF-M_4-Ar-247	Excess of pilosity in the root system
34	CGF-M_4-Ar-252	Excess of pilosity in the root system
35	CGF-M_4-Ar-253	Absence of seminal roots
36	CGF-M_4-Ar-300	Irregular root growth
37	CGF-M_4-Ar-309	Irregular root growth
38	CGF-M_4-Ar-436	Atrophied seminal roots
39	CGF-M_4-Ar-444-1	Absence of main root (excess of secondary roots)
40	CGF-M_4-Ar-444-2	Absence of seminal roots

* Code of the accession in the genebank of the Centro de Genômica e Fitomelhoramento

the development of the main seminal root is strong, with few secondary roots and on average four well-distributed adventitious roots, with homogenous growth and thinly pilose roots until 14 days of development. For the experimental plots, 80 seeds of each genotype were scattered on water-soaked filter paper to germinate at 25 ± 1°C for 72 hours. Of these seedlings, 60 with a homogenous root length of approximately 5mm were selected and transferred to the hydroponic system. The experiment was arranged in completely randomized blocks with three replications and each experimental unit consisted of 10 seeds per replication.

The hydroponic system consisted of 5.5 L pots in which a nylon mesh was fixed to the lid of each pot permitindo the plantlets support and growth of the root system to the cultivation medium. The pots were placed in a water bath pool (25 ± 1 °C), under controlled artificial illumination and aeration of the nutrient solution for oxygen supply, for the development of the root system.

The nutrient solution contained: calcium nitrate - $Ca(NO_3)_2$ 4 mM, magnesium sulphate – 2 mM $MgSO_4$, potassium nitrate - 4 mM KNO_3, ammonium sulphate - 0.435 mM $(NH4)_2SO_4$, potassium phosphate - 0.5 mM KH_2PO_4, boric acid - 10 μM H_3BO_3, sodium molybdate - 0.10 μM $NaMoO_4$, sodium chloride – 30 μM NaCl, zinc sulphate - 0.8 μM $ZnSO_4$, copper sulphate - 0.3 μM $CuSO_4$, manganese sulphate - 2 mM $MnSO_4$, iron-EDTA – 10 μM Fe SO_4 + Na (Camargo and Oliveira 1981).

The treatments consisted of three butyric acid concentrations added to the nutrient solution: 0 (control); 2; 4; and 6 mM, and the pH was adjusted to 4.7 with HCl 1N or NaOH 1N, controlled daily and corrected when necessary; according to Rao and Mikkelsen (1977a) the pH of the nutrient solution in experiments with organic acids is variable and interferes with the acid toxicity.

The seedlings were kept in nutrient solution together with the treatments for 14 days. After this period they were removed from the nylon mesh and evaluated for the following traits: root length (RL) and length of aerial part (LAP) in cm; number of roots (NR); root dry matter (RDM) and dry matter of aerial part (DMAP) in mg, weighed after drying to constant mass in a hot air oven (60 °C).

The data were evaluated in factorial analysis of variance (Steel and Torrie 1980) analysis of simple linear regression of the most responsive variable, according to a methodology proposed by Camargo and Ferreira (1992). The relative performance of each evaluated variable was computed (Vasconcelos et al. 2002). All analyses were carried out using the statistical software SAS (Statistical Analysis System 2002).

RESULTS AND DISCUSSION

The results of the analysis of variance (Table 2) showed significant effects by the F test at 5 % probability, for treatment, family and interaction (treatment x family), with exception of the effect of interaction for length of aerial part (LAP). These results confirmed that the induced mutation efficiently generated genetic variability for the study traits, in view of the differentiated responses of the families to the treatment levels used here.

Table 3 shows the mean relative reductions in the traits RL, LAP, NR, RDM and DMAP, for each dose used in the experiment. The highest relative reduction was observed for the variable RL, the most susceptible to butyric-acid toxicity, with up to 31.35% of relative performance at the dose of 6 mM. These results agree with findings of Rao and Mikkelsen (1977b) in rice, who stated that the trait root length is most affected by the organic acid treatments. In a study of the physiologic symptoms related to organic acid toxicity in rice Armstrong and Armstrong (2001) reported that this acid causes cell wall degradation, inhibition of the respiratory functions and consequent reduction of cell division of the root system that is in direct contact with the toxic element. This indicates the main reason for reduced root growth and dry matter accumulation in the analyzed genotypes (Table 3). The only variable that presented increases in absolute values was number of roots (NR). Camargo *et al.* (2001) and Armstrong & Armstrong (2001) observed that adventitious root growth was reduced, causing callus proliferation at the coleoptile base and increasing the number of lateral roots. This is the probable cause of the increase of the number of roots in rice subjected to organic acid treatments.

The results of the evaluation of the relative performance, associated to the results of the analysis of variance allow the conclusion that the families responded differently to the treatment effects due to the significant interactions for the traits root length (RL), number of roots (NR), root dry matter (RDM) and

Table 2. Summary of the analysis of variance, means and coefficient of variation (C.V.) for the rice traits: root growth (RL) and aerial part (LAP), number of roots (NR), root dry matter (RDM) and aerial part dry matter (DMAP) of 40 mutant M_4 families and rice cultivar BRS 7-Taim, evaluated in nutrient solution at four butyric acid concentrations

S.V.	d.f.	Mean squares				
		RL	LAP	NR	RDM	DMAP
Families	40	0.95 *	2.86 *	3.53 *	0.71 *	6.12 *
Treatments	3	1542.00 *	1692.56 *	8.99 *	168.62 *	1085.21 *
Interaction	117	0.94 *	0.74	1.91 *	0.88 *	0.84 *
Residue	312	0.15	0.72	0.41	0.09	0.56
Mean		7.83	13.77	3.68	3.66	9.52
C.V.		4.98	6.15	17.50	8.21	7.87

* Significant at 5% error probability by the F test

Table 3. Relative performance (%) of the traits root length (RL) and aerial part (LAP), number of roots (NR) and root dry matter (RDM) and aerial part dry matter (DMAP), in nutrient solution at four butyric acid concentrations

Variables	Relative performance (%)*		
	2 mM	4 mM	6 mM
RL	77.85	54.47	31.35
LAP	81.23	68.29	62.43
NR	112.91	114.82	125.80
RDM	87.89	69.75	45.45
DMAP	77.42	60.86	48.56

* Relative reductions assuming 1 as absolute value in the control treatment (dose 0 mM)

dry matter of aerial part (DMAP) (Table 2). Simple equations of linear regression were therefore individually fitted for each family, assuming the trait root length (RL) as dependent variable (y) which was most responsive to the phytotoxic action of butyric acid, expressed in higher indices (Table 3).

The parameters of the regression equations established for the responsiveness of the families to the butyric acid levels used in the experiment are shown in Table 4. The relatively high values for the coefficients of determination (R^2) allow the conclusion that the simple linear model was well fit to the observed data. Another factor is that most of the families obtained degree 1 (simple linear) as adjusted equation of higher degree (data not shown), indicating that the selected doses were within a response interval of the crop to the stress effect (Camargo and Ferreira 1992). Very high doses would cause the non-growth or death of the plantlets, while very low doses would not cause any damage to the plantlet development suggesting the fitting of higher degree regressions (quadratic or cubic).

The doses were selected based on previous studies of Rao and Mikkelsen (1977a); Camargo et al. (1993), Sousa and Bortolon (2002) and Kopp et al. (2007) so the average dose would result in a root length reduction of about 50%. According to results presented in Table 3, the relative performance of the variable RL was 54.47%, concordant with the above-cited studies.

To evaluate the tolerance of each family, the t test of the value of the regression coefficient (b) of each family was performed in each experiment, where values of non-significant coefficients, i.e., coefficients significantly equal to zero, determine tolerant families. It can be stated that the regression coefficients of the families 7; 12; 15; 19; 24; 28; 30; 31; 33; 36 and 40 were non-significant for the trait root length under four levels of butyric acid, totaling 27.5% of the families with mutations responsible for the improvement in the index of tolerance to butyric acid (Table 4).

Figure 1 shows the regression equations of family 38 (sensitive), 36 (tolerant) and the non-mutated cultivar BRS 7-Taim and its graphic representations. The results showed a stronger inclination of the line of family 38 than of cultivar BRS 7-Taim, in confirmation of its greater sensibility to the acid concentrations. For family 36 a weaker inclination of the straight line compared to the cultivar was observed, demonstrating greater tolerance to the increase of the butyric acid levels. Although the root growth of family 38 was superior (13.57 cm) in the absence of the acid (dose 0), the higher acid concentration caused a greater effect on the plantlet, reducing its root development considerably. In the case of family 36 with root length of 10.85 cm in the absence of the acid (dose 0), the growth was not very much affected by the increase of the acid concentration,

Table 4. Parameters of the linear regression equations of the variable root length (RL): interception on axis y (a), regression coefficient (b) and coefficient of determination (R^2) of the 40 mutant M_4 families and rice cultivar BRS 7-Taim evaluated at four butyric acid concentrations

Family	Regression parameters			Relative performance**		
	a	b	R^2	2 mM	4 mM	6 mM
1	12.66	-1.45	0.94	84.27	54.04	32.35
2	11.17	-1.30	0.88	75.83	51.16	31.05
3	11.94	-1.39	0.63	75.55	52.62	30.79
4	12.36	-1.46	0.86	73.53	53.48	29.20
5	12.48	-1.53	0.75	67.55	47.52	27.97
6	12.11	-1.33	0.91	92.36	64.60	30.58
7	11.47	-1.24*	0.95	78.27	57.86	34.93
8	12.58	-1.50	0.64	83.54	50.49	29.73
9	11.84	-1.41	0.91	72.07	52.55	28.69
10	12.26	-1.47	0.86	69.21	51.79	28.50
11	12.01	-1.42	0.75	66.13	54.68	28.16
12	11.12	-1.24*	0.82	77.90	57.12	32.19
13	12.07	-1.38	0.93	67.67	55.77	30.73
14	11.40	-1.29	0.71	78.87	55.51	31.46
15	12.41	-1.47*	0.90	86.30	56.10	27.67
16	11.33	-1.20	0.94	86.31	63.43	34.69
17	11.67	-1.38	0.82	68.95	46.57	32.19
18	11.87	-1.38	0.83	77.47	54.91	29.70
19	11.23	-1.21*	0.73	76.07	58.55	35.19
20	11.74	-1.30	0.84	77.89	61.83	30.86
21	12.30	-1.48	0.95	63.66	45.74	30.44
22	11.97	-1.40	0.68	80.03	51.26	31.25
23	12.35	-1.46	0.72	81.75	49.88	30.45
24	11.01	-1.24*	0.86	77.23	54.16	32.44
25	11.46	-1.31	0.95	72.46	49.75	33.89
26	12.42	-1.55	0.94	78.84	43.72	28.30
27	13.22	-1.64	0.83	73.74	42.42	29.49
28	11.82	-1.27*	0.79	83.52	66.77	31.77
29	13.13	-1.64	0.90	75.29	52.52	23.89
30	11.48	-1.25*	0.93	85.94	59.42	33.82
31	11.62	-1.25*	0.95	81.97	59.84	34.72
32	11.88	-1.32	0.95	84.53	61.87	30.48
33	11.64	-1.20*	0.88	82.50	62.78	36.55
34	12.25	-1.36	0.79	77.53	59.85	31.72
35	12.02	-1.41	0.87	80.21	48.28	32.19
36	10.85	-1.19*	0.82	80.45	54.87	34.55
37	12.00	-1.37	0.86	69.57	53.68	31.42
38	13.57	-1.68	0.90	76.29	45.86	27.94
39	11.43	-1.28	0.96	81.94	49.74	35.61
40	11.32	-1.16*	0.90	94.94	66.62	36.60
BRS 7	12.22	-1.41	0.79	73.81	53.84	31.12

* Non-significant by the t test at 5 % error probability for the regression model at four treatment levels
** Relative performance based on 1 as absolute value in the control treatment (dose 0 mM)

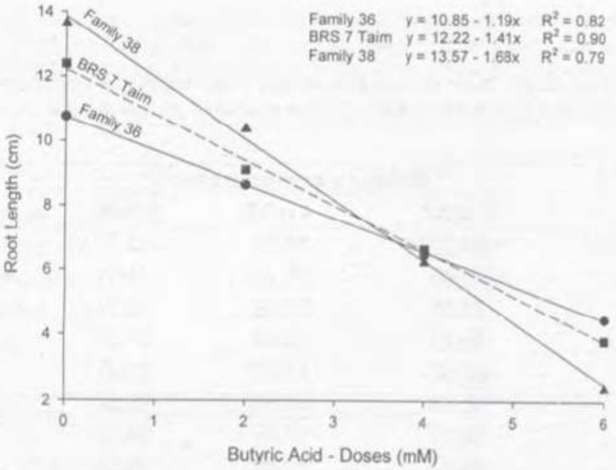

Figure 1. Effect of butyric acid concentrations on root growth of 38 (sensitive) and 36 (tolerant) families and the non-mutated original cultivar BRS 7-Taim

resulting in a greater root length at the dose 6 mM compared to family 38, initially superior in root growth.

Contrasting with the data presented in Table 1, it was possible to associate these families to the morphologic characterization carried out previously in the root system. The families 7; 12; 15; 19; 24 and 36 presented irregular root growth with diverse abnormalities, families 28 and 33 excess of pilosity in the root system and 31 and 40 absence of seminal roots. Thus, one may suppose that these traits are correlated with acid tolerance, suggesting the need for further studies related to the physiology and biochemistry of acid effect on the root system. On the effect of organic acids in rice Armstrong and Armstrong (2001) reported that the acids cause degradation of the cell membranes and loss of the cell content to the medium. Tolerant plants must therefore have genes that confer a greater capacity of cell membrane formation to tolerate these acids, independent of the morphology of the root system. Another conceivable possibility would be the capacity of a plant to produce new roots in substitution of the damaged ones, so that the toxic elements would be neutralized or degraded, since, according to Armstrong and Armstrong (2001) one of the marked effects of organic acids on rice plantlets is the emission of secondary roots.

Mutation induction with gamma rays (^{60}Co) also reduced the tolerance index measured by the values of the regression coefficients for some families when compared to the non-mutated cultivar. In the study with butyric acid 12 families (1, 4, 5, 8, 10. 11, 21, 23, 26, 27, 29 and 38) were relatively more acid-sensitive than cultivar BRS 7-Taim.

CONCLUSIONS

Mutation induction with ^{60}Co gamma rays at the dose of 250 Gy generated variability responsible for tolerance to butyric acid in the rice cultivar BRS 7-Taim. Eleven M_4 families (27.5%) performed superior to the non-mutated cultivar BRS 7-Taim.

Mutation induction also affected the cultivar response to phytotoxic action of butyric acid negatively, with 12 (30%) less tolerant M_4 families to acid concentrations.

Root length is the most affected trait by the phytotoxic effect of butyric acid.

The butyric acid levels 2, 4 and 6 mM caused efficient relative reductions for tolerance studies in hydroponic systems.

Tolerância ao ácido butírico em famílias mutantes M_4 de arroz

RESUMO - *Solos do tipo hidromórfico apresentam uma reduzida capacidade de drenagem, sendo utilizados principalmente para cultivo de arroz irrigado. Esta condição favorece o desenvolvimento de microrganismos anaeróbios que produzem substâncias fitotóxicas. O objetivo deste trabalho foi avaliar 40 famílias mutantes de arroz a ação fitotóxica do ácido butírico produzido nestas condições. O trabalho foi executado em sistema hidropônico com 4 tratamentos e o delineamento utilizado foi blocos casualizados. As variáveis mensuradas foram comprimento de raízes (CR), parte aérea (CPA), número de raízes (NR), matéria seca de raízes (MSR) e parte aérea (MSPA). Foram procedidas análise de variância, desempenho relativo e ajuste de regressões. Apenas o efeito de tratamentos para a variável (NR) e efeito de interação para (CPA) não revelaram significância. A variável (CR) foi a mais afetada pelo ácido e as regressões estabelecidas mostraram efeitos positivos e negativos para tolerância ao ácido nas famílias mutadas.*

Palavras-chave: *Oryza sativa*, mutação, toxicity, ácidos orgânicos.

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