

## CALCIUM PARTIALLY RELIEVES THE DELETERIOUS EFFECTS OF HYPOXIA ON A MAIZE CULTIVAR SELECTED FOR WATERLOGGING TOLERANCE

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**ABSTRACT** - Low soil oxygen concentrations due to waterlogging or transient flooding severely reduce maize production. However, genotypes with tolerance or even resistance to hypoxia develop morphological and biochemical adaptation mechanisms which may prove to be useful criteria for the selection and breeding of new improved genotypes. The objective of this work was to evaluate the effect of levels and modes of application of calcium sources on biomass yield, the contents of total soluble sugars and reduced sugars and on the activities of invertase isoforms and phosphoenolpyruvate carboxylase, in a maize variety (BRS 4154) derived from the 14th selection cycle for tolerance to transient soil flooding conditions, when cultivated under waterlogging conditions. The study was carried out in a greenhouse, utilizing 20 kg pots, filled with a lowland soil. The effects of two sources of calcium (CaCl<sub>2</sub>, 300 kg ha<sup>-1</sup> and CaSO<sub>4</sub>, 500 and 1.500 kg ha<sup>-1</sup>) and three modes of application (applied 8 cm below surface, mixed with the whole soil volume and surface application) were tested in soils kept at field capacity and waterlogged after plants reached growth stage V6. Above ground biomass yield, the contents of total soluble sugars and reducing sugars and the activities of the neutral citosol (INC), acid vacuolar (IAV), acid cell wall invertases and phosphoenolpyruvate carboxylase were determined at the onset of flowering. Calcium application partially relieved the deleterious effect of waterlogging on biomass yield and the activities of INC, IAV and PEPcase.

**Key words:** BRS 4154, transient waterlogging, invertase, PEPcase

## O CÁLCIO ALIVIA PARCIALMENTE OS EFEITOS DELETÉRIOS DA HIPOXIA EM UMA CULTIVAR DE MILHO SELECIONADA PARA TOLERÂNCIA AO ENCHARCAMENTO

**RESUMO** - Baixas concentrações de oxigênio no solo, causadas pelo encharcamento ou alagamento temporário, reduzem a produtividade do milho. Entretanto, genótipos com tolerância ou resistência à hipoxia desenvolvem adaptações morfológicas e bioquímicas que podem ser utilizadas como marcadores para seleção de novos genótipos melhorados. O objetivo deste trabalho foi avaliar o efeito de níveis, fontes e formas de aplicação do

cálcio na produção de biomassa, teores de açúcares solúveis totais, açúcares redutores e na atividade da invertase e da carboxilase do fosfoenolpiruvato, em uma variedade de milho (BRS 4154) derivada do 14º ciclo de seleção para tolerância ao encharcamento transiente do solo, quando cultivada em solo encharcado. O experimento foi conduzido em casa-de-vegetação, em vasos de 20 kg contendo solo de várzea. Os tratamentos utilizados foram: duas fontes de cálcio ( $\text{CaCl}_2$ , 300 kg ha<sup>-1</sup> e  $\text{CaSO}_4$ , 500 e 1.500 kg ha<sup>-1</sup>) e três formas de aplicação (incorporado a 8 cm da superfície, incorporado em todo o solo, e localizado na superfície), em solo mantido na capacidade de campo e sob encharcamento. A imposição do encharcamento foi iniciada no estágio de crescimento V6. Quando as plantas entraram em floração, determinou-se a produção de matéria seca da parte aérea e, nas raízes, os teores de açúcares solúveis totais e açúcares redutores, a atividade das invertases neutra do citosol (INC), ácida do vacúolo (IAV), ácida da parede celular (IAPC) e da carboxilase da fosfoenolpiruvato (PEPcase). A aplicação de cálcio aliviou parcialmente os efeitos deletérios do encharcamento sobre a produção de biomassa e na atividade da INC, IAPC e PEPcase.

**Palavras-chave:** BRS 4154, encharcamento temporário, invertase, PEPcase,

Different plant species are native to highly contrasting environments with distinct conditions for growth and development. In environments with excess water in the soil, low oxygen concentration produces stressful conditions for most species (Jackson & Drew, 1984) causing metabolic changes leading to unfavorable growth and development. (Kozłowski & Pallardy, 1984; Crane & Davies, 1988). Low soil oxygen concentration (hypoxia) or total absence of oxygen (anoxia) affects nutrient uptake, synthesis and translocation of growth regulators, photosynthesis, respiration and carbohydrate partitioning, decreasing the productivity of crops grown in soils with inadequate drainage or subjected to transient flooding. Moreover, depending on the intensity and duration of stress conditions, plant death may occur (Kawase, 1987).

Some arboreal and herbaceous species are able to survive in soils with low oxygen concentration during their initial growth period,

whereas other species die after a few weeks under waterlogging (Hall & Smith, 1955; Crawford & Tyler, 1969). Tolerance to hypoxia varies among species (Gibbs & Leitão Filho, 1978; Kozłowski, 1984) and among genotypes of the same species (Gill, 1970). Plant species with tolerance or even resistance to hypoxia develop morphological and biochemical adaptation mechanisms which may prove to be useful criteria for the selection and breeding of genotypes with increased resilience to waterlogging.

Low soil oxygen concentration due to waterlogging or transient flooding severely reduces maize yield. Nonetheless, recent work has identified maize cultivars with some degree of tolerance to hypoxia and this has been attributed to biochemical and physiological adaptations leading to stomatal closing during the stress period, and to morphological modifications, including formation of adventitious roots and aerenchymas (Drew *et al.*, 1979; Dantas *et al.*, 2001). In tolerant species, hypoxia is known to

alter gene expression and to induce the synthesis of anaerobic polypeptides shuttling plant metabolism to an anaerobic route (Chang *et al.*, 2000; Alves *et al.*, 2000; Liao & Lin, 2001).

Work done with the maize cultivar Saracura, selected for increased adaptation to transient flooding (Parentoni *et al.*, 1995) showed that 42% of the seedlings survived hypoxic conditions for three days. In this work, sensitive plants developed a translucent mesocotyl which evolved to a constriction zone, resulting in seedling death. The appearance of this constricted zone was attributed to an enhanced activity of enzymes involved in cell wall degradation, with the root cell wall fraction showing an increased presence of low molecular weight peptides (Vitorino *et al.*, 2001).

Taking into consideration the fact that calcium acts as a structural element in cell wall stability (Damarty *et al.*, 1984; Grant *et al.*, 1973) further research was undertaken to determine a possible role of this element in the tolerance mechanism to hypoxia. In these experiments, addition of  $\text{CaCl}_2$  to the germination solution increased the survival of both tolerant (Saracura) and sensitive (BR 107) maize cultivars (Vitorino *et al.* 2001) and concomitantly reduced root growth by 37% in relation to control plants (Alves *et al.*, 2002). These authors confirmed the role of calcium as a structural element when they compared the effect of manitol and  $\text{CaCl}_2$  and observed a reduction in seedling growth in the presence of calcium. Similar results were also observed in another experiment designed to study the effect of calcium in the germination and initial growth of maize cultivar Saracura seedlings (Purcino *et al.* 2001a and b). These studies demonstrated a positive relationship between reduction in seedling growth and increased tolerance to transient flooding. Besides attributing

the reduction of seedling growth to a possible decrease in cellular extensibility, these authors suggested that calcium is involved in the metabolism of carbohydrate reserves.

The role of calcium in the partitioning of seed reserves during germination of cv. Saracura, suggests it initially increased the degradation and translocation of endosperm starch, but this effect was followed by a decreased utilization of these carbohydrates, resulting in reduced plant growth (Fries 2003; Purcino *et al.* 2001a and b). During hypoxia, the activity of neutral and acid invertases decreased, but this effect seemed to be calcium-independent (Ricard *et al.*, 1998).

Despite the fact that calcium addition to the germination solution increased the tolerance of cv. Saracura to hypoxia during germination and initial seedling growth, so far no experimental evidence has been obtained to support the hypothesis that this effect is relevant under field conditions. Therefore, the objective of this study was to determine the effect of calcium on the mechanisms of waterlogging tolerance in fully developed Saracura plants. Specifically, experiments were carried out to determine the effect of sources, levels and mode of calcium application on above ground dry matter yield and invertase isoforms and phosphoenolpyruvate carboxylase activities in the leaves of this cultivar during flowering, in plants cultivated under waterlogging from V6 stage until harvest.

#### Material and methods

Seeds of a maize variety BRS 4154 Saracura – derived from the 14th selection cycle for waterlogging tolerance were cultivated in eighty 20 kg pots filled with a clayey, Eutrophic Fluvic Neossol Tb soil, from a plain phase lowland (Embrapa Solos, 1999). The experiment was carried out in a greenhouse (21° 08' 24" S,

45° W) where maximum and minimum temperatures ranged from 30° - 36 °C and 9.5° - 15.5 °C respectively, and relative humidity varied between 52% and 78%. Each pot was fertilized with 23 g of 5-20-20+Zn and planted with 5 seeds. After germination each pot was thinned down to 2 plants and received weekly applications of 5 g ammonium sulfate. For evaluation of the calcium effect on waterlogging tolerance, 2 sources (CaCl<sub>2</sub> at 300 kg ha<sup>-1</sup> and Ca<sub>2</sub>SO<sub>4</sub> at 500 and 1.500 kg ha<sup>-1</sup>) and 3 modes of application Ca (placed 8 cm below soil surface; mixed with the entire soil volume and placed at the soil surface) were tested. For evaluation of the waterlogging effect on plant growth, sugar content and enzyme activities, 40 pots were waterlogged and 40 pots were kept close to field capacity immediately after plants reached the V6 growth stage (plants with 6 fully developed leaves). Distilled water was used for irrigation. The entire experiment was carried out as a fully randomized design with 4 replicates. The data obtained were analyzed with the aid of the SISVAR software package (Ferreira, 2000), and means were compared by the Tukey test for P < 0,05.

During flowering, dry matter weight was determined for above ground growth. In the roots, total soluble sugars (TSS) and reducing sugars (RS) contents and the activities of cytosolic neutral invertase (CNI), vacuolar acid invertase (VAI), cell wall acid invertase (CWAI) and phosphoenolpyruvate carboxylase (PEPcase) were measured. Before analysis, leaf and root tissues were washed with distilled water, blotted dry, wrapped in aluminum foil, flash frozen in liquid nitrogen and kept at -80 °C until analysis.

Reducing sugars (RS) were determined according to Miller (1959). Extraction was performed by homogenization of 0.1 g lyophilized root tissue in 4 mL distilled water and

centrifugation at 20.000 x g for 20 min. The assay mix contained 500 mL root extract, 1 mL distilled water and 1 mL of the DNS reagent. After heating at 100 °C in a water bath for 5 min and cooling to room temperature, reducing sugar content was determined at 540 nm against a standard curve prepared with pure glucose.

Total soluble sugars were determined according to Yemm & Willis (1954) in assay mixes prepared at 4 °C, and containing 50 µL root extract, 950 mL distilled water and 2 µL anthrone. After heating at 100 °C in a water bath for 3 min and cooling to room temperature, total soluble sugars were determined at 620 nm against a standard curve prepared with pure glucose.

Invertase activity was determined as described by Zeng *et al.*, (1999) and modified by Fries (2003). Extraction of cytosolic neutral invertase and vacuolar acid invertase was done by homogenization of 0.1 g lyophilized root tissue in 4 mL of a 100 mM HEPES extraction buffer (pH 7.5) containing 1 mM PMSF, 5 mM MgCl<sub>2</sub>, 1 mM DTT and 20 mM ascorbic acid, followed by centrifugation at 20.000 x g, 4 °C, for 20 min. The supernatant was collected for soluble invertases analysis, but for determination of cell wall acid invertase, the pellet was further homogenized for 7 min in 4 mL of 200 mM citrate buffer (pH 4,8), containing 1 mM PMSF, 5mM MgCl<sub>2</sub>, 1 mM DTT, 20 mM ascorbic acid and 1 M NaCl. After centrifugation at 20.000 x g, 4 °C, for 20 min, the supernatant was used for enzyme analysis. CNI activity was assayed in 1 mL of 100 mM phosphate buffer (pH 7.5) whereas VAI and CWAI were assayed in 1 mL of 200 mM citrate buffer (pH 4.8). Both buffers contained 5 mM MgCl<sub>2</sub> and 200 mM sucrose and after incubation in a water bath, at 30 °C, for 40 min (t<sub>40</sub>), the reactions were stopped by rapid submersion of the assay mix in liquid nitrogen.

The content of endogenous reducing sugars in the root tissue was determined by stopping the activity of control reactions at time zero ( $t_0$ ).

PEPcase activity was determined essentially as described by Purcino *et al.* (1998).

### Results and discussion

Waterlogging had a deleterious effect on above ground dry matter yield (Table 1). Several authors have indicated that the yield of shoot growth is strongly influenced by waterlogging because this type of stress affects several plant physiological mechanisms (Huang & Johnson, 1995; Huang *et al.*, 1995; Albrecht *et al.*, 1997). According to Summers *et al.* (2000) and Alves *et al.* (2000), waterlogging induces the plant to switch to alternative respiration pathways, with deficient energy output, therefore, negatively influencing plant growth and development. However, in this experiment, it was observed that when plants subjected to waterlogging received either 500 or 1.500 kg ha<sup>-1</sup> CaSO<sub>4</sub> applied to the entire soil root volume (treatments 3 and 6) or 1.500 kg ha<sup>-1</sup> CaSO<sub>4</sub> applied to the soil surface (treatment 7), above ground yield was higher than that observed for control plants subjected to waterlogging (treatment 1). A comparison for above ground dry matter yield between pots kept waterlogged and at field capacity, for the same level and source of calcium, suggests that CaSO<sub>4</sub> significantly relieved the deleterious effect of waterlogging on plant growth. Plants grown under waterlogging in soil previously treated with CaSO<sub>4</sub> produced above ground growth comparable to control plants grown at field capacity (treatments 3, 6 and 7 versus treatment 11). It is noteworthy, however, that plants grown at field capacity also benefited from the application of either 1.500 kg ha<sup>-1</sup> CaSO<sub>4</sub> or 300

kg ha<sup>-1</sup> CaCl<sub>2</sub> (treatments 16 and 20 versus treatment 11).

During flowering, total soluble and reducing sugars were neither affected by waterlogging nor calcium. In luffa and bitter melon, Su *et al.* (1998) have found that waterlogging initially increased the concentration of root sugars but this effect gradually changed, reaching values similar to or lower than values observed for non-waterlogged control plants. Similar to our present results, these authors concluded that tolerance to waterlogging was not correlated to root sugar levels.

In this experiment, VAI activity was neither affected by waterlogging nor by addition of any source of calcium, but when compared to plants under waterlogging, the activity of this invertase isoform was higher in plants grown at field capacity with addition of either 1.500 kg ha<sup>-1</sup> CaSO<sub>4</sub> or 300 kg ha<sup>-1</sup> CaCl<sub>2</sub> applied at the soil surface (treatments 4 and 10 versus treatments 14 and 20).

Under waterlogging, CNI activity was increased by addition of either 1.500 kg ha<sup>-1</sup> CaSO<sub>4</sub> or 300 kg ha<sup>-1</sup> CaCl<sub>2</sub> when any of these two sources of Ca were mixed with the entire soil volume. Similarly, under waterlogging, CWAI activity was significantly increased in plants that received either 500 kg ha<sup>-1</sup> CaSO<sub>4</sub> or 300 kg ha<sup>-1</sup> CaCl<sub>2</sub> applied at the soil surface. It is noteworthy, that under waterlogging, plants receiving calcium application had higher CWAI activity than plants grown at field capacity.

Very low PEPcase activity was detected in the roots of waterlogged control plants (treatment 1), but this negative effect was relieved by surface application of 1500 kg ha<sup>-1</sup> CaSO<sub>4</sub> (treatment 7) or 500 kg ha<sup>-1</sup> CaSO<sub>4</sub> applied 8 cm below soil surface (treatment 2). Although

**TABLE 1.** Above ground dry matter yield, total soluble sugars (TSS), reducing sugars (RS) and the activities of cytosolic neutral invertase (CNI), vacuolar acid invertase (VAI), cell wall acid invertase (CWAI) and phosphoenolpyruvate carboxylase (PEPcase) evaluated during the flowering period of maize cv. Saracura grown in soils kept at field capacity and soils flooded after plants reached the V6 growth stage and submitted to different levels, doses and mode of calcium application

Source and level of calcium Soil Environment	Dry matter yield	TSS	RS	CNI	VAI	CWAI	PEPcase
	g	mg glucose g <sup>-1</sup> dry matter	mmol glucose g <sup>-1</sup> dry matter	μmol glucose g <sup>-1</sup> dry matter	μmol glucose g <sup>-1</sup> dry matter	μmol glucose g <sup>-1</sup> dry matter	μmol CO <sub>2</sub> g <sup>-1</sup> dry matter min <sup>-1</sup>
1 - Control, without Ca application Waterlogged	38,6b*	3,19a	0,22a	32,6c	97,8a	59,2b	0,72b*
2 - 500 kg ha <sup>-1</sup> CaSO <sub>4</sub> applied 8 cm below soil surface Waterlogged	37,0b*	8,26a	0,42a	48,0bc*	122,1a	68,3ab	5,21a*
3 - 500 kg ha <sup>-1</sup> CaSO <sub>4</sub> mixed with the entire soil volume Waterlogged	106,6a	6,13a	0,36a	68,2abc	88,9a	66,1ab	3,76ab
4 - 500 kg ha <sup>-1</sup> CaSO <sub>4</sub> applied at the soil surface Waterlogged	45,3b*	3,93a	0,24a	56,9abc	106,3a	80,0a	3,47ab
5 - 1.500 kg ha <sup>-1</sup> CaSO <sub>4</sub> applied 8 cm below soil surface Waterlogged	39,0b*	5,98a	0,29a	50,8abc	87,1a*	41,8b*	2,02ab
6 - 1.500 kg ha <sup>-1</sup> CaSO <sub>4</sub> mixed with the entire soil volume Waterlogged	97,0a*	7,74a	0,49a	89,5a	78,1a	69,3ab	3,32ab
7 - 1.500 kg ha <sup>-1</sup> CaSO <sub>4</sub> applied at the soil surface Waterlogged	95,3a*	7,40a	0,37a	59,8abc	124,0a	65,3ab	4,92a
8 - 300 kg ha <sup>-1</sup> CaCl <sub>2</sub> applied 8 cm below soil surface Waterlogged	38,3b*	7,77a	0,41a	82,7ab	92,8a*	67,8ab	3,04ab
9 - 300 kg ha <sup>-1</sup> CaCl <sub>2</sub> mixed with the entire soil volume Waterlogged	30,3b*	6,02a	0,34a	78,3ab*	106,7a	68,2ab	3,32ab
10 - 300 kg ha <sup>-1</sup> CaCl <sub>2</sub> applied at the soil surface Waterlogged	59,3ab	6,73a	0,34a	51,8abc	77,0a	80,1a*	2,31ab
Mean	58,7*	6,32	0,35	61,9	98,1	66,6	3,21
CV (%)	28,4	30,5	30,7	27,6	33,7	21,5	35,5
11 - Control, without Ca application field capacity	110,1c	5,76a	0,32a	46,9a	101,3a	65,9a	3,32a
12 - 500 kg ha <sup>-1</sup> CaSO <sub>4</sub> applied 8 cm below soil surface field capacity	104,9c	6,23a	0,35a	96,9a	143,6a	64,9a	3,03a
13 - 500 kg ha <sup>-1</sup> CaSO <sub>4</sub> mixed with the entire soil volume field capacity	101,8c	5,35a	0,36a	73,2a	93,8a	59,5a	3,47a
14 - 500 kg ha <sup>-1</sup> CaSO <sub>4</sub> applied at the soil surface field capacity	102,8c	4,77a	0,24a	67,7a	142,0a	46,8a	3,18a
15 - 1.500 kg ha <sup>-1</sup> CaSO <sub>4</sub> applied 8 cm below soil surface field capacity	114,2c	6,42a	0,36a	52,0a	140,2a	72,7a	3,42a
16 - 1.500 kg ha <sup>-1</sup> CaSO <sub>4</sub> mixed with the entire soil volume field capacity	196,9a	7,32a	0,37a	71,9a	106,5a	69,6a	4,82a
17 - 1.500 kg ha <sup>-1</sup> CaSO <sub>4</sub> applied at the soil surface field capacity	180,2ab	7,54a	0,49a	60,9a	111,5a	61,4a	3,37a
18 - 300 kg ha <sup>-1</sup> CaCl <sub>2</sub> applied 8 cm below soil surface field capacity	171,1ab	7,54a	0,38a	86,0a	143,6a	59,9a	3,76a
19 - 300 kg ha <sup>-1</sup> CaCl <sub>2</sub> mixed with the entire soil volume field capacity	158,7b	7,35a	0,38a	42,0a	142,0a	39,0a	3,32a
20 - 300 kg ha <sup>-1</sup> CaCl <sub>2</sub> applied at the soil surface field capacity	153,9b	6,14a	0,31a	46,1a	112,3a	59,3a	3,04a
Mean	139,5	6,67	0,36	63,8	123,7	59,9	3,47
CV (%)	6,8	29,3	22,3	36,2	22,1	24,2	31,1

Column means followed by the same letter are not significantly different for each soil environment by the Tukey test at P < 0,05.  
\* Indicates significant differences for comparisons between soil environments (waterlogged x field capacity) for the same level and source of calcium

differences observed were not significant at  $P < 0.05$ , the data presented in Table 1 indicate that application of either  $\text{CaSO}_4$  or  $\text{CaCl}_2$  can relieve the deleterious effect of waterlogging on PEPcase activity. This may be interpreted as suggesting that adequate levels of calcium increases PEPcase activity in waterlogged plants, therefore, allowing an enhanced regeneration of reducing power ( $\text{NAD}^+$ ) as proposed by Crawford (1966; 1967). Under these conditions, an unusually high Pasteur effect favors the activation of alternative metabolic pathways capable of enhancing plant tolerance to hypoxia, as observed for the Saracura maize cultivar used in this study.

Taken together, these results suggest that application of  $\text{CaSO}_4$  relieved the deleterious effect of waterlogging on above ground dry matter yield of maize cultivar Saracura, where hypoxia treatments were imposed after the plants reached the V6 growth stage.

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