GRISIELY YARA STRÖHER NEVES

ESTRESSE SALINO E SEUS EFEITOS NA LIGNIFICAÇÃO DE RAÍZES DE SOJA

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GRISIELY YARA STRÖHER NEVES

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Prof. Dr. Osvaldo Ferrarese-Filho
Orientador

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BIOGRAFIA

Grisiely Yara Ströher Neves, filha de Arnildo Werner Ströher e Nadir Aparecida Zanardo Ströher, nasceu em Mandaguari, estado do Paraná, no dia 11 de outubro de 1977.

Graduou-se em Ciências Biológicas em fevereiro de 2000, pela Universidade Estadual de Maringá (UEM).

Obteve o título de Especialista em Biologia: Células, Tecidos, Organismos - Estrutura, Função e Comportamento Reprodutivo, pela UEM, em 2001.

Concluiu o Mestrado no Programa de Pós-graduação em Agronomia (PGA) - UEM, em 2004, desenvolvendo a dissertação "Estresse salino em soja (*Glycine max* L. Merr.): germinação das sementes e lignificação das raízes", sob orientação da Prof[a] Dr[a] Maria de Lourdes Lucio Ferrarese.

Iniciou o Doutorado no Programa de Pós-graduação em Ciências Biológicas (PBC) - UEM, em 2005, sob a orientação do Prof. Dr. Osvaldo Ferrarese Filho.

Atualmente é docente da Fundação Faculdade de Filosofia Ciências e Letras de Mandaguari.

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Ao meu marido Jean.

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APRESENTAÇÃO

Esta Tese é composta de uma breve revisão e de dois artigos científicos. A revisão descreve o estado atual da arte no que tange ao estresse salino e o papel do cálcio nas plantas. O primeiro artigo descreve os efeitos do estresse salino no crescimento e lignificação das raízes de soja. O segundo artigo descreve os efeitos do cálcio no estresse causado pelo NaCl nas raízes de soja. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, os artigos foram redigidos de acordo com as revistas Journal of Arid Environments (Qualis A2) e Plant Growth Regulation (Qualis B1).

G.Y.S. Neves. Estresse salino e cálcio.

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RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – A salinidade é um dos estresses abióticos mais importantes que limita diretamente a produtividade das culturas em muitas regiões do mundo. A resposta das plantas ao estresse salino é complexa, e envolve mudanças na sua morfologia, fisiologia e metabolismo, podendo alterar-se entre variedades de um mesmo tipo de planta e nos diferentes estágios de crescimento. A resposta imediata ao estresse salino é a redução na taxa de expansão da superfície foliar com a diminuição da expansão conforme aumento da concentração de sal. O estresse salino também resulta em decréscimo considerável nas biomassas frescas e secas das folhas, caules e raízes. O crescimento radicular é caracterizado pela alta atividade metabólica e, por essa razão, as raízes são altamente suscetíveis aos estresses ambientais. No caso do estresse salino, a raiz é o único órgão diretamente exposto ao excesso de sal. A lignificação (processo de impermeabilização de uma célula vegetal pela deposição de lignina) é necessária ao desenvolvimento em todos os tecidos das plantas, e é um passo importante no crescimento radicular. A lignina é um componente complexo da parede celular formada por heteropolímeros fenólicos, covalentemente ligados a polissacarídeos e proteínas. Está localizada principalmente nos condutos de transporte impermeáveis à água do xilema e outros tecidos de apoio. É sintetizada pela via de fenilpropanóides, que está envolvida na síntese de compostos fenólicos, bem como uma vasta gama de produtos secundários nas plantas. A primeira enzima limitante desta via é a fenilalanina amônia-liase (PAL) que, em associação com outras enzimas, leva à síntese dos aldeídos *p*-cumaríl, coniferíl e sinapíl. Na última etapa da via, as peroxidases (POD) catalisam a polimerização dos monolignóis levando à síntese de lignina.

O cálcio (Ca^{2+}) é um macronutriente essencial para a planta. Apresenta uma série de funções fisiológicas e é indispensável ao funcionamento normal das membranas vegetais, sendo apontado como um segundo mensageiro para inúmeros sinais ambientais e hormonais das plantas. O Ca^{2+} mantém a integridade da membrana e a composição da parede celular uma vez que proporciona rigidez através das ligações das cadeias de pectina da lamela média. É sabido que a expansão celular é um processo primário na morfogênese da raiz, e o influxo do Ca^{2+} extracelular armazenado é necessário para o alongamento das células nas raízes. Como um componente estrutural de macromoléculas, várias funções estão relacionadas à sua capacidade de coordenação e formação de ligações intermoleculares estáveis, predominantemente na parede celular e na membrana plasmática. Paredes celulares sofrem lignificação quando a planta está sob estresse. No entanto, controvérsias acerca do estresse provocado pelo NaCl na lignificação das raízes têm sido relacionadas, indicando aumento, diminuição ou nenhum efeito do estresse salino sobre a produção de lignina. Da mesma forma, alguns estudos sobre o papel do Ca^{2+} em

relação ao metabolismo de compostos fenólicos e enzimas relacionadas têm sido relatados. Para alguns pesquisadores, o Ca^{2+} aumenta as atividades da POD solúvel e da PAL acumulando ou reduzindo compostos fenólicos. Outros verificaram que o Ca^{2+} diminui as atividades da POD solúvel e da PAL reduzindo (ou não afetando) a produção de compostos fenólicos ou, ainda, suprimindo as perdas de compostos fenólicos a partir das raízes.

Entre as diferentes espécies vegetais, a soja (*Glycine max* L. Merrill) é considerada sensível a moderadamente tolerante à salinidade. Embora os efeitos do NaCl sobre o crescimento desta planta sejam amplamente divulgados, o seu efeito sobre a lignificação da raiz não tem sido explorado. Com base nisto, o presente trabalho foi planejado para estudar 1) os efeitos do NaCl sobre o crescimento radicular, atividades da PAL e das POD solúveis e ligadas a parede celular, peróxido de hidrogênio (H_2O_2), teor de lignina e sua composição monomérica em soja; 2) a influência do Ca^{2+} exógeno sobre os parâmetros relacionados com a lignificação em raízes de soja sob estresse provocado pelo NaCl.

MÉTODOS – Plântulas de três dias foram cultivadas em solução nutritiva (pH 6,0) sem ou com 5 mM $Ca(NO_3)_2$ e NaCl (50, 100, 150 e 200 mM). Os experimentos foram efetuados em câmara de germinação, a 25ºC, em ciclo de 12 horas claro/12 horas escuro e irradiação de 280 μmol m^{-2} s^{-1}, por 24 horas. Após a incubação as raízes foram medidas e preparadas para os ensaios bioquímicos. A viabilidade celular, as atividades das POD solúveis e ligadas à parede celular, os conteúdos de H_2O_2 e de lignina foram determinados espectrofotometricamente, enquanto as atividades da PAL e a composição monomérica da lignina foram determinadas por HPLC. ANOVA foi aplicada para verificar a significância das variações observadas. Os resultados foram expresssos como valores médios de quatro a sete experimentos independentes ± erro padrão da media. As diferenças entre os parâmetros foram avaliadas pelo teste de Scott-Knott e consideradas significativas quando apresentaram valor de $P \leq 0,05$.

RESULTADOS E DISCUSSÃO – Nos tratamentos com 50 a 200 mM de NaCl, o comprimento das raízes de soja, as biomassas fresca e seca e a atividade da PAL diminuíram, as POD solúveis e ligadas à parede celular aumentaram e o conteúdo de H_2O_2 diminuiu significativamente. Estes resultados estão de acordo com os relatados em outras espécies de plantas sob estresse provocado pelo NaCl. Neste trabalho, o aumento da atividade enzimática foi associado ao decréscimo do conteúdo de H_2O_2 nas raízes estressadas pelo sal. Como uma consequência da exposição ao NaCl, o conteúdo de lignina aumentou nos tratamentos com 150 e 200 mM de NaCl em relação ao controle, sem alterar, contudo, a composição monomérica. É conhecido que a biossíntese de lignina é acompanhada por um aumento na atividade da POD ligada à parede

celular, e este processo envolve ligação oxidativa, dependente do H_2O_2, o que implica em rápida ligação de polímeros de parede celular. Desta forma, o estresse salino tem sido associado a uma maior deposição de lignina nos tecidos vasculares e/ou desenvolvimento do xilema. Esta lignificação tem sido sugerida como um fator que inibe o crescimento radicular e, consequentemente, representa um mecanismo de adaptação para resistir ao estresse imposto pela salinidade. Além disso, análises morfológicas, histoquímicas e bioquímicas têm reforçado o papel do estresse salino sobre a lignificação e processos relacionados na parede celular. Em suma, é plausível que a lignificação possa desempenhar um papel regulador na redução do crescimento das raízes de soja provocada pelo estresse salino.

Experimentos posteriores mostraram que a ausência de Ca^{2+} reduz o crescimento das raízes e aumenta a lignificação das plântulas de soja cultivadas em solução nutritiva sem NaCl. Como descrito acima, a produção de lignina depende de aumentos nas atividades das POD solúveis e ligadas à parede celular e também de um processo de ligação oxidativa, o qual utiliza H_2O_2 para a rápida ligação de polímeros de parede celular. Também é conhecido que o Ca^{2+} é necessário para manter a integridade da membrana e a composição da parede celular, e seu influxo a partir da reserva extracelular é necessário para o alongamento das células nas raízes. Ruptura das paredes celulares e colapso dos tecidos são sintomas conhecidos da falta de Ca^{2+}. Em parte, os resultados aqui relatados podem ser atribuídos ao estresse causado pela ausência de uma oferta exógena de Ca^{2+}. Além disso, o NaCl reduziu o crescimento radicular e todos os parâmetros relacionados à lignificação, independentemente do Ca^{2+} na solução nutritiva. No entanto, o crescimento radicular, as atividades da PAL, das POD e os conteúdos de H_2O_2 foram mais afetados após o tratamento com NaCl sem Ca^{2+}. Há indícios de que o Ca^{2+} tem um efeito sobre a melhora do crescimento de plantas sob estresse salino. O Ca^{2+} neutraliza os efeitos do Na^+ na alteração da homeostase celular, e sua ação se deve, ao menos parcialmente, à manutenção da integridade da membrana plasmática nas raízes. No entanto, os resultados do presente trabalho indicam que, a 5 mM, o Ca^{2+} não restaura os efeitos deletérios do NaCl. Isto não exclui a possibilidade de respostas diferentes com plântulas crescendo em outras concentrações de Ca^{2+}.

CONCLUSÕES – Os resultados encontrados indicam que 1) a lignificação pode desempenhar papel regulador no crescimento das raízes de soja provocado pela redução do estresse salino. 2) a ausência de Ca^{2+} diminui o crescimento radicular e aumenta a lignificação em plântulas de soja cultivadas em solução nutritiva sem NaCl. Este resultado se deve ao estresse causado pela ausência de uma oferta exógena de Ca^{2+}. 3) o NaCl reduz o crescimento radicular e os parâmetros relacionados à lignificação, independentemente do fornecimento de Ca^{2+}. 4) a 5 mM, o Ca^{2+} não atenua os efeitos deletérios do NaCl sobre os parâmetros relacionados à lignificação.

GENERAL ABSTRACT

INTRODUCTION AND AIMS – Salinity is one of the most important abiotic stresses which directly limit crop productivity in many areas of the world. Plants response to saline stress is complex since it involves changes in their morphology, physiology and metabolism, and may be expected to vary in different varieties of a same type of plant and different growth stages. The immediate response of salt stress is reduction in the rate of leaf surface expansion leading to cessation of expansion as salt concentration increases. Salt stress also results in a considerable decrease in the fresh and dry weights of leaves, stems, and roots. Root growth is characterized by high metabolic activity, and for this reason roots are highly susceptible to environmental stresses. In the case of salinity stress, the root is the only organ directly exposed to excess salt. Lignification, the process of sealing a plant cell by lignin deposition, is a necessary developmental process in all tissues of plants, and it is an important step during root growth. Lignin is a complex cell wall component comprised of phenolic heteropolymers that are covalently bound to both polysaccharides and proteins. It is mainly localized in the impermeable water transport conduits of the xylem and other supporting tissues. Lignin is synthesized by the phenylpropanoid pathway, which is involved in the synthesis of phenolic compounds as well as a wide range of secondary products in plants. The first rate-limiting enzyme of this pathway is phenylalanine ammonia-lyase (PAL), which, in association with other enzymes, leads to the synthesis of *p*-coumaral-, coniferal- and sinapaldehydes. In the last step of the pathway, peroxidase (POD) catalyzes monolignol polymerization, leading to the lignin synthesis.

Calcium (Ca^{2+}) is an essential plant macronutrient. It has a number of physiological functions, and it is essential for the normal functioning of plant membranes and has been implicated as a second messenger for a number of plant responses to both environmental and hormonal signals. Ca^{2+} maintains membrane integrity and cell wall composition since that it provides cell wall rigidity by cross linking the pectic chains of the middle lamella. It is thought that cell expansion is a primary process in root morphogenesis, and Ca^{2+} influx from the extra-cellular store is required for cell elongation in roots. As a structural component of macromolecules most functions are related to its capacity for coordination, by which it provides stable intermolecular linkages, predominantly in cell walls and at plasma membrane. Cell walls suffer lignification when the plant is under stress. However, controversial reports about NaCl stress on root lignification have been related by indicating increase, decrease or no effect of salt stress on

lignin production. Similarly, some studies on the role of Ca^{2+} in relation to phenolic compounds metabolism and related enzymes have been reported. For some researchers, Ca^{2+} augmented the soluble POD and PAL activities accumulating or reducing the phenolic compounds. Others verified that Ca^{2+} decreased soluble POD and PAL activities reducing (or not affecting) the phenolic compounds production or suppressing the leakage of phenolic compounds from roots.

Among different plant species, soybean (*Glycine max* L. Merrill) has been considered as a salt-sensitive to moderately salt-tolerant crop. Although the effects of NaCl on soybean growth have been widely reported, the effects on root lignification have not been exploited. Based on this, current work has been planned to study 1) the effects of NaCl on root growth, PAL and soluble and cell wall-bound POD activities, hydrogen peroxide (H_2O_2) level and lignin content and its monomeric composition in soybean; 2) the influence of exogenous Ca^{2+} on these lignification-related parameters in soybean roots under NaCl stress.

METHODS – Three-day-old seedlings were cultivated in nutrient solution (pH 6.0) with or without 5 mM $Ca(NO_3)_2$ and NaCl (50, 100, 150 and 200 mM). Experiments were carried out in a growth chamber (25°C, 12-h light/12-h dark cycle, irradiance of 280 μmol m^{-2} s^{-1}) during 24 hours. Roots were then measured and prepared for assays. Cell viability, soluble and cell wall-bound POD activities, H_2O_2 and lignin contents were determined spectrophotometrically, whereas PAL activities and lignin monomeric composition were determined by high performance liquid chromatography (HPLC). ANOVA was applied to test the significance of measured differences. Data are expressed as means of four to seven independent experiments ± S.E. Whereas variance tested the significance of the observed differences, the difference between parameters was evaluated by Scott-Knott´s test and *P* values ≤ 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION – After 50 to 200 mM NaCl treatments, soybean root length, fresh and dry weights decreased, PAL activity decreased, soluble and cell wall-bound POD activities increased and H_2O_2 content decreased significantly. These results are in agreement with those reported in other plant species under NaCl stress. In the current research, the increased enzyme activities were associated with decreased H_2O_2 content in the salt-stressed roots. As a consequence of NaCl exposure, lignin content increased after 150 and 200 mM NaCl treatments respect to controls without, however, to alter the monomeric composition. It is known, lignin biosynthesis is accompanied by an increase in the activities of cell wall-bound

POD, and this process involves oxidative coupling, dependent on H_2O_2, which causes a rapid cross-linking of cell wall polymers. In this way, salinity stress has been associated with a greater deposition of lignin in vascular tissues and/or xylem development. This lignification has been suggested to be a factor that inhibits root growth and, consequently, represents an adaptation mechanism in resisting salinity-imposed stress. Moreover, coordinated morphological, histochemical and biochemical analyzes have been reinforcing the role of salt stress on lignification and related processes in cell walls. In brief, it is plausible that lignification may play a regulatory role in the soybean root growth reduction caused by salt stress.

Subsequent experiments revealed that the absence of Ca^{2+} reduced root growth and increased lignification of soybean seedlings grown in NaCl-free nutrient solution. As discussed above, lignin production depends of increases in the activities of soluble and cell wall-bound POD and of an oxidative coupling process, which uses H_2O_2 for rapid cross-linking of cell wall polymers. It is also known that Ca^{2+} is necessary to maintain the membrane integrity and cell wall composition, and its influx from the extracellular store is required for cell elongation in roots. Disruption of cell walls and collapse of tissues are known symptoms of the absence of Ca^{2+}. In brief, results reported here may be attributed to the stress caused by the absence of an exogenous Ca^{2+} supply. In addition, NaCl reduced the root growth and all lignification-related parameters, independently of Ca^{2+} in the nutrient solution. However, root growth, PAL and POD activities and H_2O_2 contents were more affected after NaCl treatments without Ca^{2+}. There is evidence that Ca^{2+} has an ameliorating effect on the growth of plants under salt stress. Supplemented Ca^{2+} counteracts the effects of Na^{+} on disturbance of the cell Ca^{2+} homeostasis, and its alleviating action may at least partly be due to maintenance of plasma membrane integrity in roots. However, findings of the present work indicate that, at 5 mM, Ca^{2+} did not restore the deleterious effects of NaCl. This does not rule out the possibility of different responses with seedlings growing in other Ca^{2+} concentrations.

CONCLUSIONS – Current results indicate that 1) the lignification may play a regulatory role in the soybean root growth reduction caused by salt stress. 2) the absence Ca^{2+} reduces the root growth and increases lignification of soybean seedlings grown in NaCl-free nutrient solution. This finding is due to the stress caused by the absence of an exogenous Ca^{2+} supply. 3) NaCl reduces the root growth and lignification-related parameters, independently of Ca^{2+} supply. 4) At 5 mM, Ca^{2+} did not alleviate the deleterious effects of NaCl on lignification-related parameters.

ESTRESSE SALINO E CÁLCIO

Grisiely Yara Ströher Neves

Estresse Salino

Estresses abióticos, como o salino, estão entre os fatores mais limitantes na produção de soja (*Glycine max* L. Merrill). Em regiões áridas e semi-áridas do mundo, o aumento da temperatura, as chuvas escassas, a evaporação elevada, a transpiração excessiva e o manejo inadequado da água são agravantes que têm contribuído para o aumento da salinidade (Meloni et al., 2003).

Em condições naturais, as plantas encontram altas concentrações de sais próximas ao litoral e em estuários, mas nas terras afastadas do mar, infiltrações naturais por depósitos de sal podem tornar áreas inutilizáveis ao plantio (Taiz e Zeiger, 2006). Um problema muito maior para a agricultura é o acúmulo de sais na água destinada à irrigação, que pode alcançar rapidamente níveis que são prejudiciais às espécies sensíveis ao sal, degradando a estrutura do solo, diminuindo a porosidade deste e a permeabilidade à água (Barros et al., 2004).

O aumento da concentração de sais em áreas irrigadas é um problema de ordem mundial. Milhões de hectares de terra em todo o mundo são salinos e a cada ano se tornam mais improdutivos (Luo et al., 2005). O manejo inadequado da água de irrigação aliado ao uso intensivo de fertilizantes tem contribuído para o aumento de áreas agricultáveis com problemas de salinidade (Carneiro et al., 2002). Esse fato é particularmente importante nas regiões áridas e semiáridas, devido à escassez da precipitação pluvial e à alta demanda evaporativa, que dificultam a lixiviação dos sais localizados na camada arável do solo (Távora et al., 2001). Estima-se que no Brasil existam, aproximadamente, nove milhões de hectares com problemas de salinidade, a maior parte dessa área localizada nos perímetros irrigados do Nordeste (Cruz et al., 2006).

Em condições naturais e agricultáveis as plantas estão frequentemente expostas ao estresse ambiental, que desempenha um papel importante na determinação de como o solo e o clima limitam a distribuição das espécies vegetais. Assim, a compreensão dos processos fisiológicos subjacentes aos danos provocados por estresse e dos mecanismos de adaptação e

aclimatação de plantas a estresses ambientais é de grande importância para a agricultura e o meio-ambiente (Ribeiro et al., 2001).

De modo geral, define-se estresse como um fator externo que exerce influência desfavorável sobre a planta. Na maioria das vezes, ele é avaliado em relação à sobrevivência da espécie, cultivo e crescimento (acúmulo de biomassa), ou aos processos primários de assimilação (entrada de CO_2 e minerais), que estão relacionados ao desenvolvimento completo (Taiz e Zeiger, 2006). Com base na sua resposta às altas concentrações de sais, as plantas podem ser divididas em dois grupos: halófitas (nativas de solos salinos, onde completam o seu ciclo de vida) e glicófitas (sensíveis à salinidade). Entre as espécies cultivadas, o milho (*Zea mays*), a cebola (*Allium cepa*), o feijão (*Phaseolus vulgaris*) e a soja estão entre as culturas sensíveis à salinidade do solo (Parida e Das, 2004).

Segundo Torres (2007), o excesso de sais no solo afeta negativamente a germinação, o estande das plantas, o desenvolvimento vegetativo, a produtividade e, nos casos mais graves, causa a morte das plântulas. A água é osmoticamente retida em solução salina, de forma que o aumento da concentração de sais torna-a cada vez menos disponível para as plantas. A capacidade de adaptação dos vegetais superiores aos solos salinos depende de alguns fatores, destacando-se a constituição fisiológica e o seu estádio de desenvolvimento (Cramer et al., 1994). Algumas espécies, como sorgo (*Sorghum bicolor*), milho, feijão e trigo (*Triticum aestivum*) são menos afetadas durante a fase inicial de seu ciclo. Porém, em arroz (*Oriza sativa*), a sensibilidade à salinidade aumenta durante a floração e a frutificação (Moterle et al., 2006).

O potencial osmótico de uma solução salina apresenta valor mais negativo do que o apresentado pelas células do embrião, dificultando, portanto, a absorção da água necessária à germinação (Machado-Neto et al., 2006). Esta diminuição na capacidade de germinar é atribuída à redução das atividades enzimáticas. A salinidade afeta a germinação, não só por dificultar a absorção de água, mas também por facilitar a entrada de íons em quantidades tóxicas nas sementes durante a embebição (Amorim et al., 2002). Dos fatores externos que interferem no processo germinativo a hidratação da semente é o mais importante, pois a água constitui a matriz onde ocorre a maioria dos processos bioquímicos e fisiológicos que resultam na protrusão da raiz primária (Moraes et al., 2005).

A sensibilidade ao estresse salino é variável de acordo com a espécie e a fase de desenvolvimento de cada cultura. De acordo com Teixeira et al. (2008), a redução na absorção de água, a toxicidade de íons específicos e os efeitos indiretos dos sais nos processos

fisiológicos, são fatores responsáveis pela redução do crescimento das plantas. Segundo Azevedo e Tabosa (2000), o rendimento das culturas cai significantemente quando o teor de sais na solução do solo prejudica a absorção de água e nutrientes pelas culturas, resultando em perdas no crescimento, desenvolvimento e produção. Em geral, esses decréscimos estão associados à redução na capacidade fotossintética, devido ao declínio na condutância estomatal (Ziska et al., 1990). Lima e Bull (2008) consideram a germinação e o desenvolvimento inicial as fases mais sensíveis aos efeitos da salinidade.

A emergência e o crescimento inicial podem ser severamente afetados pelo excesso de sal, principalmente em culturas sensíveis (Carmo et al., 2003). Diferentes estudos relatam os efeitos da salinidade sobre a germinação em arroz, milho, feijão e soja (Campos e Assunção, 1990a; Hilal et al., 1998; Lin e Kao, 2001a; Lin e Kao, 2001b). Em alfafa (*Medicago sativa*), Campos e Assunção (1990b) demonstraram que quanto maior a concentração do sal (NaCl), menor a percentagem de germinação. Em plantas de arroz, observou-se estreitamento das raízes, confirmando que o crescimento é um dos processos fisiológicos mais atingidos pelo estresse salino, sendo a sua redução a mais imediata e sensível resposta a quase todos os estresses (Freitas e Camargo, 1988). Blanco et al. (2007) verificaram que a emergência e o crescimento da soja foram reduzidos pela salinidade, sendo as plântulas mais resistentes na fase de emergência do que no desenvolvimento inicial. Queiroga et al. (2006) observaram que a salinidade reduziu a área foliar e o crescimento em plântulas de melão (*Cucumis melo*). Em pepino (*Cucumis sativus*), Torres et al. (2000) verificaram que a diminuição progressiva do potencial osmótico do substrato pelo NaCl prejudicou a germinação e o desenvolvimento das plântulas. Em aroeira (*Myracrodruon urundeuva*), a 100 mM de NaCl foi observado redução superior a 40% na biomassa seca de raízes, caules e folhas quando comparados ao controle (Silva et al., 2000; Melloni et al., 2000). Em feijão, Moraes et al. (2005) verificaram que o vigor das sementes é mais afetado pelo NaCl do que a germinação, e que a porcentagem de plântulas anormais e o diâmetro da radícula das plântulas normais aumenta na presença do sal, sendo o mesmo observado em soja (Braccini et al., 1998).

A resposta das plantas à salinidade é um fenômeno complexo, envolvendo alterações morfológicas e de crescimento, além de processos fisiológicos e bioquímicos (Kerepesi e Galiba, 2000). O estresse salino pode estimular ou inibir enzimas como as peroxidases (POD), que estão associadas às mudanças nos processos fisiológicos de plantas submetidas aos estresses. Plântulas de feijão mantidas sob o efeito do NaCl (100 mM), durante 10 dias, mostraram aumento nas

atividades das POD e diminuição dos teores de proteínas, com folhas enrugadas evidenciando sinais de clorose seguida de necrose (Lima et al., 1999). De acordo com Navari-Izzo et al. (1990), a hidrólise de proteínas poderia contribuir para o ajuste osmótico, uma vez que o teor maior de aminoácidos livres seria um mecanismo importante de tolerância à seca. O aumento observado nas atividades das POD pode ser uma adaptação do tecido vegetal à condição proposta, pois é conhecido que ocorre aumento da oxidação dos lipídios de membrana sob condições de estresse, indicando um possível papel protetor das POD nestas condições (Lima et al., 1999).

As POD encontram-se amplamente distribuídas nos vegetais, exercendo importantes funções no crescimento, diferenciação, desenvolvimento e lignificação da parede celular, podendo, em alguns casos, ter o seu efeito acentuado quando associado a fatores bióticos e abióticos (Menezes et al., 2004). Sua ação constitui uma proteção antioxidativa para os vegetais, implicando no aumento de sua atividade em diferentes tipos de estresses (Rodrigues et al., 2002). Caverzan (2008) constatou decréscimo na atividade das POD presentes no endosperma de variedades de arroz tolerantes às condições de salinidade, enquanto nas variedades sensíveis foi observado aumento. A salinidade pode provocar a inibição da absorção dos íons potássio (K^+) e cálcio (Ca^{2+}) pelas raízes, provocando sintomas de deficiências como a redução no crescimento e diminuição no teor de proteínas, uma vez que poderia estar ocorrendo competição pelos sítios de absorção em nível de membrana (Carmo et al., 2003). Enzimas como a fenilalanina amônia-liase (PAL), fosfatase ácida e desidrogenase glutâmica também revelam atividade aumentada em plantas tratadas com NaCl (Rossi et al., 1997).

Neumann et al. (1994) demonstraram que a inibição do crescimento das raízes, decorrente da salinidade, estava associada com a expansão da parede celular, atribuindo papel primordial para as POD ligadas à parede celular (Fry, 1986). Do mesmo modo, Lin e Kao (2001a) mostraram que o aumento das concentrações de NaCl (50 a 150 mM), resultou em progressiva redução no crescimento das raízes de arroz, com aumento da atividade das POD ionicamente ligada à parede celular. Além disso, observaram que a redução do crescimento das raízes estava diretamente associada com o aumento dos níveis de peróxido de hidrogênio (H_2O_2), essencial para a atividade das POD. Segundo os autores, os resultados encontrados reforçam a idéia de que as POD e o H_2O_2 participam na regulação da redução do crescimento das raízes de arroz, sob estresse salino.

Alla et al. (2002) relataram que o tratamento salino de raízes e caules de milho provocou significativo acúmulo de compostos fenólicos, com aumento nas atividades da PAL e tirosina amônia liase (TAL). Deve-se ressaltar que estas duas enzimas estão associadas ao metabolismo dos compostos fenólicos os quais, geralmente, se acumulam nas plantas sob condições de estresse.

No que se refere aos estudos com soja, Essa (2002) analisou a sensibilidade à salinidade em três cultivares diferentes. A percentagem de germinação, o comprimento das plantas e o acúmulo de cálcio e magnésio nas folhas foram significativamente reduzidos com o aumento dos níveis de salinidade. Hosseini et al. (2002) compararam a germinação das sementes e o crescimento das plântulas em condições salinas e observaram que a germinação decresceu cerca de 80% quando as sementes foram tratadas com NaCl 330 mM. Com NaCl 420 mM, somente 40% das sementes germinaram enquanto que, sob tratamento com NaCl 500 mM, não ocorreu germinação. Em relação ao crescimento das plântulas, observaram que esta variável foi drasticamente reduzida com o aumento da salinidade: com 220 mM de NaCl o crescimento foi reduzido para 5%, enquanto que em 330 mM, foi praticamente zero.

Importante aspecto a ser levado em conta, ainda, é a possibilidade de o estresse salino alterar o metabolismo de compostos secundários presentes nos vegetais. Neste aspecto, é imprescindível destacar que o metabolismo secundário nas plantas está intimamente associado à resposta das mesmas aos diferentes estresses bióticos e abióticos. Destaque-se, neste caso, a via de fenilpropanóides. Como componente terminal desta via, a lignina é, após a celulose, o composto mais abundante na biosfera terrestre, e o maior componente estrutural da parede celular (Moraes et al., 2005). É um polímero complexo de unidades de fenilpropanos, hidroxilados e metoxilados, associados por ação de enzimas como as POD e as lacases. A incorporação de lignina na parede celular ocasiona rigidez estrutural e resistência dos tecidos das plantas (Polle et al., 1994). Isto se deve ao mecanismo de espessamento, o qual diminui a extensibilidade da parede celular em decorrência da formação de pontes difenil entre polímeros da parede, por ação das POD (Sánchez et al., 1996). Ainda nesta linha de raciocínio, é importante ressaltar que a biossíntese de lignina é um processo altamente complexo, e envolve inúmeras etapas enzimáticas que compõem o metabolismo secundário nas plantas, o qual, por seu lado, confere várias funções fisiológicas para sobrevivência e adaptação a perturbações ambientais (Strack, 1997).

Segundo Abreu (1994), as reações durante a biossíntese de lignina compreendem cinco etapas fundamentais: aromatização, com a formação do ácido fenilpirúvico; aminação, desaminação e redução formando os precursores primários por hidroxilação e metilação, e a última etapa, a de formação da unidade molecular polimérica. A sequência reacional da biossíntese de uma substância do metabolismo secundário está relacionada à origem do precursor e com a via metabólica. Desta forma, os metabólitos secundários usam rotas biossintéticas determinadas como as vias do ácido acético, do ácido mevalônico e do ácido chiquímico. Esta última representa o caminho de um dos precursores intermediários de importância relevante em todos os vegetais superiores. Em nível metabólico, é interessante destacar que a formação do ácido chiquímico ocorre a partir do processo regenerativo do carboidrato eritrose 4-fosfato, sintetizado durante a fotossíntese, além do ácido fosfoenolpirúvico, derivado da glicólise. Ambos são iniciadores de uma via biossintética que, através de reação de condensação e de várias outras reações transformadoras, forma o ácido chiquímico. Subsequentemente, esta mesma via culminará com a formação de aminoácidos aromáticos como triptofano, tirosina e fenilalanina. A partir da transformação do ácido prefênico, duas vias diferentes levam a fenilalanina e a tirosina, respectivamente. Em ambos os casos, os ácidos hidroxilados e carbonilados são totalmente convertidos em ácidos aminados sendo que a interconversão desses ácidos é observada somente no sentido fenilalanina – tirosina.

A conversão, pelo processo de desaminação da fenilalanina em ácido cinâmico é catalisada pela PAL e da tirosina, através da TAL. Muito do que se sabe a respeito da lignificação nas plantas relaciona-se ao estudo das ações de enzimas envolvidas no processo, principalmente as óxido-redutase (catalase – CAT, POD solúvel e ligada à parede celular, PAL, cinamil álcool desidrogenase – CAD, além de outras oxidases) (Rós Barceló et al., 2003).

As plantas desenvolvem mecanismos moleculares e bioquímicos para sobreviver ao estresse salino, entre eles, a capacidade de acumular íons no vacúolo e/ou solutos orgânicos de baixo peso molecular no citoplasma, em um processo denominado ajustamento osmótico. Isto permite a manutenção da absorção de água e da turgescência celular; controle da captação dos íons através das raízes e transporte até as folhas, mudança na via fotossintética, alteração na estrutura da membrana, indução de enzimas antioxidantes, e síntese de hormônios, principalmente o ácido abscísico (ABA) (Parida e Das, 2004; Cruz et al., 2006). Estudos indicam que o ABA é responsável pela indução de genes com papel importante na tolerância ao estresse salino em arroz (Gupta et al., 1998). O ABA ameniza o efeito inibitório do NaCl sob a

fotossíntese, crescimento, translocação de assimilados e promove fechamento estomático por rápida alteração no fluxo de íons nas células-guarda em condições de estresse. Evidências experimentais também mostram que o ABA está associado com o aumento da captação do cálcio, o que contribui para a manutenção da integridade da membrana a longo prazo (Chen et al., 2001).

O estresse salino é complexo e leva à deficiência hídrica, com produção de espécies reativas de oxigênio (ROS). A atividade citotóxica das ROS pode causar sérios danos oxidativos aos lipídios, proteínas e ácidos nucléicos (Delledone et al., 2002). A atividade das enzimas antioxidantes como a CAT, POD, glutationa redutase (GR) e superóxido dismutase (SOD) aumentam nas plantas sob estresse salino, existindo correlação entre os níveis destas enzimas e a tolerância ao sal (Sun et al., 2007). Os vegetais possuem diferentes sistemas de defesa contra os estresses ambientais, o que lhes permite adaptar-se para minimizar os danos que podem ocorrer pela ação das ROS. Neste contexto, o sistema natural de defesa antioxidante nos vegetais atua na proteção das plantas submetidas aos estresses (Kopyra e Gwózdz, 2003).

Cálcio

O cálcio (Ca^{2+}) é importante fator na manutenção da integridade da membrana, regulação e transporte de íons (Dunn et al., 1998). É fundamental na síntese da parede celular, em particular da lamela média, do fuso mitótico durante a divisão celular (Rosolem e Marcello, 1998). É requerido para o funcionamento normal das membranas vegetais e foi-lhe atribuído o papel de mensageiro secundário em várias respostas das plantas, tanto aos sinais ambientais quanto aos hormonais. Sintomas característicos da deficiência de cálcio incluem a necrose de regiões meristemáticas jovens, como os ápices radiculares ou folhas jovens, nas quais a divisão celular e a formação de parede são mais rápidas (Taiz e Zeiger, 2006).

O Ca^{2+} é necessário para o crescimento e desenvolvimento da planta, sendo mediador de muitas reações químicas (Silveira et al., 2008). A maioria das atividades desse nutriente está relacionada à sua capacidade de coordenação, providenciando estabilidade e ligações moleculares reversíveis, predominantes na membrana celular. É considerado nutriente estrutural da planta, pois é um dos principais integrantes da parede celular, onde os pectatos de cálcio ajudam na formação da lamela média, responsável pela ligação de células adjacentes e a consequente extensibilidade da parede celular (Silveira e Monteiro, 2007).

Lacerda e colaboradores (2004), avaliando o crescimento de sorgo sob estresse salino, observaram redução nos teores de Na^+ nas folhas dos genótipos sensível e tolerante com o aumento do Ca^{2+} na solução nutritiva. Isso se deve, provavelmente, ao melhor controle na absorção radicular e no transporte de Na^+ para a parte aérea que ocorre em níveis adequados de Ca^{2+} no meio de nutrição, o que tem sido apontado como um dos principais efeitos benéficos do Ca^{2+} em plantas sob condições de estresse salino. De acordo com Reid e Smith (2000), o aumento na concentração de Ca^{2+} no meio de nutrição não afeta igualmente o crescimento vegetativo e a absorção de Na^+. Segundo eles, a concentração de Ca^{2+} requerida para afetar o crescimento é menor do que a necessária ao controle da absorção de Na^+, em plantas sob estresse salino. Aparentemente, os efeitos benéficos do Ca^{2+} na redução da toxidez do Na^+ podem ser sobrepujados pelos efeitos osmóticos associados ao aumento da concentração total de sais. Isto se deve, possivelmente, ao fato do Ca^{2+} ser essencial à seletividade de canais de cátions no plasmalema de células das raízes tendo, consequentemente, efeito sobre o influxo passivo de Na^+ (Davenport et al., 1997; Lacerda et al., 2004). Resultados como estes evidenciam que um dos principais papéis do Ca^{2+}, em plantas cultivadas sob ambientes salinos, pode ser o controle da absorção radicular de Na^+ e de K^+, atuando na membrana celular e favorecendo a manutenção de teores mais adequados desses íons nos tecidos fotossintetizantes. O Ca^{2+} tem, portanto, influência decisiva na manutenção da seletividade K^+/Na^+, como sugerido por outros autores (Colmer et al., 1996).

Outros estudos têm demonstrado que a salinidade diminui os teores de Ca^{2+} na parte aérea das plantas de milho (Azevedo e Tabosa, 2000). Semelhantemente a este trabalho, os autores reportaram que o aumento do teor de Na^+ do meio externo ocasiona a diminuição dos teores de Ca^{2+} nos tecidos das plantas, pois o Na^+ desloca o Ca^{2+} do plasmalema das células radiculares, o que resulta em uma perda da integridade da membrana e efluxo citosólico de solutos orgânicos e inorgânicos. Eklund e Eliasson (1990) *apud* Manfredini (2008), trabalhando com hipocótilos de *Pinus*, observaram que a falta de suprimento de Ca^{2+} reduziu a síntese de parede celular, evidenciando o papel deste nutriente na manutenção da estrutura celular.

As alterações nas membranas onde o Ca^{2+} é um íon estabilizante podem conduzir a uma sensibilidade maior da cultura ao estresse salino, tendo em vista a seletividade das membranas nos processos de absorção e compartimentação iônica (Azevedo e Tabosa, 2000). Trabalhos com plantas sob estresse salino têm demonstrado que a suplementação de Ca^{2+} reduz o acúmulo de

sódio e mantém os níveis de potássio e metabólitos fosfatados nos tecidos radiculares, além de reduzir as perdas de fósforo (Garcia et al., 2007).

Indiretamente, o menor crescimento das plantas, devido à salinidade, também tem sido atribuído à redução na absorção de Ca^{2+}, que pode levar à perda da integridade da membrana plasmática, com consequente diminuição da capacidade de absorção de alguns íons, principalmente o potássio (Lacerda et al., 2001; Cachorro et al., 1994). Variedades tolerantes tendem a apresentar maiores taxas de transferência de potássio, e apenas leve redução na transferência de Ca^{2+} para a parte aérea, visando manter uma relação positiva entre esses nutrientes e os íons Na^+ e Cl^- (Niu et al., 1995; Cruz, et al., 2006).

Aplicado exogenamente, o Ca^{2+} diminui o estresse salino por meio de função desconhecida que preserva a seletividade K^+/Na^+ e inibe sítios de absorção de K^+, o que pode reduzir o influxo de Na^+ mediado pelo componente de baixa afinidade de absorção de K^+. O cálcio é usualmente mantido no citoplasma a 100 – 200 mM, por transporte ativo e o NaCl promove um rápido aumento na sua concentração no citoplasma que, provavelmente, atua como um sinal geral de estresse. Embora não haja confirmação de que esse aumento seja um efeito de tolerância à salinidade, as maiores concentrações de Ca^{2+} no citoplasma podem ser transitórias. Resultados sugerem que esse aumento, em função da exposição ao NaCl, pode ser reduzido pelo aumento da atividade da Ca–ATPase (Melloni et al., 2000). Na planta, o Ca^{2+} move-se com a água, sendo sua translocação e seu teor nos tecidos sujeitos à taxa de transpiração (Barta e Tibbitts, 2000). Uma vez depositado não apresenta redistribuição para outras partes da planta, sendo acumulado principalmente em tecidos que transpiram mais facilmente (Millaway e Wiersholm, 1979 *apud* Beninni et al., 2003).

Estudos efetuados em solos salinos têm revelado que o aumento na concentração de Na^+ é acompanhado pelo decréscimo na concentração de Ca^{2+} trocável, resultando em desequilíbrio iônico que pode afetar o crescimento das plantas. Cereais como o sorgo, o milho, o arroz e a cevada (*Hordeum vulgare*) são particularmente sensíveis à elevação na relação Na^+/Ca^{2+} em solos afetados por sais, exibindo redução no crescimento e deficiências de Ca^{2+} (Lacerda et al., 2004).

A elevação da concentração de Ca^{2+} no ambiente radicular tem sido apontada como uma alternativa para amenizar os efeitos negativos da salinidade sobre o crescimento das plantas. O Ca^{2+}, além de reduzir a absorção de íons potencialmente tóxicos, como o Na^+, favorece a absorção de íons essenciais como o K^+ e o próprio Ca^{2+} (Garcia et al., 2007). Recentemente

admitiu-se que o Ca^{2+} também promove o acúmulo de solutos orgânicos, como a prolina e a glicinabetaina, os quais possibilitariam o estabelecimento de um equilíbrio osmótico no citoplasma mais compatível com o metabolismo celular, favorecendo o crescimento das plântulas sob condições de estresse salino (Girija et al., 2002).

Considerações Finais

É interessante destacar que entre os agentes osmolizantes, a solução de NaCl tem sido a mais empregada na indução do estresse salino. A resposta das plantas ao excesso de NaCl é complexa, e envolve mudanças na sua morfologia, fisiologia e metabolismo. Embora várias pesquisas tenham sido descritas, os mecanismos pelos quais a salinidade inibe o crescimento das plantas ainda são desconhecidos, o que evidencia a necessidade de mais estudos para entendimento desses problemas. Além disso, avaliar qual o papel do Ca^{2+} no estresse salino é outro desafio a ser vencido. Neste aspecto, algumas questões podem ser destacadas: 1) O processo de lignificação nas raízes das plantas sofreria a ação do NaCl? 2) Esta ação envolveria alterações nas atividades de enzimas relacionadas à lignificação, como, por exemplo, POD e PAL? 3) Estas enzimas poderiam ser indicadoras do estresse salino nas plantas? 4) Poderia o Ca^{2+} atenuar o efeito do NaCl sobre as plantas? A obtenção de respostas satisfatórias a estas questões cruciais poderá conduzir a melhor compreensão dos mecanismos pelos quais o estresse salino afeta o crescimento das plantas.

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Soybean root growth inhibition and lignification induced by salt stress

G. Y. S. Neves, R. Marchiosi, M. L. L. Ferrarese, R. C. Siqueira-Soares, O. Ferrarese-Filho

Correspondence to:

Osvaldo Ferrarese-Filho
Department of Biochemistry
University of Maringá
Av. Colombo, 5790
87020-900, Maringá, PR
BRAZIL

E-mail: oferrarese@uem.br
Fax: +55 44 2633655

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G. Y. S. Neves, R. Marchiosi, M. L. L. Ferrarese, R. C. Siqueira-Soares, O. Ferrarese-Filho

Department of Biochemistry, University of Maringá, Av. Colombo, 5790, 87020-900, Maringá, PR, Brazil

ABSTRACT

The effects of NaCl were tested on root growth, phenylalanine ammonia-lyase (PAL) and soluble and cell wall-bound peroxidase (POD) activities, hydrogen peroxide (H_2O_2) level and lignin content and its monomeric composition in soybean (*Glycine max*) roots. Three-day-old seedlings were cultivated in half-strength Hoagland's solution (pH 6.0), with or without 50 – 200 mM NaCl in a growth chamber (25°C, 12/12 h light/dark photoperiod, irradiance of 280 μmol m^{-2} s^{-1}) for 24 h. In general, root length, fresh and dry weights decreased after NaCl treatments. Whereas PAL activity decreased, soluble and cell wall-bound POD activities increased and H_2O_2 content decreased significantly after NaCl exposure. As a consequence, lignin content increased without to alter the monomeric composition. Taken together, these results suggest that the lignification caused by NaCl is due to the enhanced cell wall-bond POD activity and lignin production solidifying the cell wall and restricting the root growth.

Keywords: Hydrogen peroxide, Lignin, Peroxidases, Phenolic compounds, Phenylalanine ammonia-lyase, Soybean root, Salinity.

1. Introduction

Salinity, a severe problem that affects 2 million km^2 of land used for agricultural production, is one of the most important abiotic stresses which directly limits crop productivity in many areas of the world. The increased salinization of arable land is expected to have devastating global effects, resulting in 30% land loss within the next 15 years, and up to 50% by the year 2050 (Wang et al., 2003; Bartels and Sunkar, 2005). Plants response to saline stress is complex since it involves changes in their morphology, physiology and metabolism, and may be expected to vary in different varieties of a same type of plant (Jbir et al., 2001a; Essa, 2002; Luo et al., 2005; Azevedo Neto et al., 2006; Gulen et al., 2006) and different growth stages (Rios-Gonzales et al., 2002; Davenport et al., 2003; Meloni et al., 2003; Talano et al., 2006; Kim et al., 2007; Aghaei et al., 2009; Ghanem et al., 2009).

Root growth is characterized by high metabolic activity, and for this reason roots are highly susceptible to environmental stresses. In the case of salinity stress, the root is the only organ directly exposed to excess salt (Karahara et al., 2004). It is well known that lignification, the process of sealing a plant cell by lignin deposition, is an important step during root growth. Lignin is a complex cell wall component comprised of phenolic heteropolymers that are covalently bound to both polysaccharides and proteins. It is mainly localized in the impermeable water transport conduits of the xylem and other supporting tissues (Donaldson, 2001). Lignin is synthesized by the phenylpropanoid pathway, which is involved in the synthesis of a wide range of secondary products in plants such as phenolic acids, flavonoids, tannins, coumarins (Kovácik et al., 2007) and lignin (Boerjan et al., 2003). The first step in this pathway is the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL) to produce cinnamate. By action of subsequent enzymes, cinnamate is converted in different derivatives metabolites which are, further, converted to the corresponding monolignols. In the last step of the pathway, peroxidase (POD) within the cell wall, in bound state, catalyzes the oxidative polymerization of the three *p*-hydroxycinnamil alcohols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol). They give rise to the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the lignin polymer, respectively. As the main structural component of secondarily thickened plant cell walls, lignin contributes to the compression strength of stems. Lignin imparts mechanical support and allows for the efficient conduction of water and solutes over long distances within the vascular systems (Boerjan et al., 2003).

In general, cell walls suffer lignification when the plant is under stress (Christensen et al., 1998). However, controversial reports about NaCl stress on root lignification have been related. For example, Cachorro et al. (1993) demonstrated lignin deposition in vascular tissues of bean (*Phaseolus vulgaris*) roots in response to salinity. No effect of NaCl on lignin content has been verified in tomato (*Solanum lycopersicon*) roots (Peyrano et al., 1997). On the other hand, Lin and Kao (2001a) showed that lignin levels decreased in NaCl-treated root of rice (*Oryza sativa*) seedlings. Among different plant species, soybean (*Glycine max*) has been considered as a salt-sensitive to moderately salt-tolerant crop (Luo et al., 2005; Kao et al., 2006). Although the effects of NaCl on soybean growth has been widely reported (Hilal et al., 1998; Essa, 2002; Kao et al., 2006; Tuncturk et al., 2008; Aghaei et al., 2009), its effects on root lignification has not been exploited. To test the hypothesis that exogenously applied NaCl may to increase lignin production reducing root growth, the aim of the current research was to analyze its effects on PAL and POD activities, hydrogen peroxide (H_2O_2) level and lignin contents of soybean seedlings.

2. Materials and methods

2.1. General procedures

Soybean (*Glycine max* L; Merrill, cv BRS-133), surface-sterilized with 2% sodium hypochlorite for 2 min and rinsed extensively with deionized water, were dark-germinated (at 25°C) on three sheets of moistened filter paper. Twenty-five 3-day-old seedlings of uniform size were supported on an adjustable acrylic plate and dipped into a glass container (10 × 16 cm) filled with 200 mL of half-strength Hoagland's solution (pH 6.0) with or without NaCl (50, 100, 150 and 200 mM).

The container was kept in a growth chamber (25 °C, 12/12 h light/dark photoperiod, irradiance of 280 μmol m^{-2} s^{-1}) for 24 h. Roots were measured before incubation and at the end of experiments, and the lengths were obtained by difference among them. Fresh root weight was determined immediately after incubation, and the dry weight was estimated after oven-drying at 80 °C until it reached a constant weight. All reagents used were of the purest grade available or chromatographic grade.

2.2. Enzymatic assays

PAL was extracted as described by Ferrarese et al. (2000). Fresh roots (2 g) were ground at 4 ºC in 0.1 M sodium borate buffer (pH 8.8). Homogenates were centrifuged (2,200 ×*g*, 15 min) and the supernatant was used as the enzyme preparation. The reaction mixture (100 μmoles sodium borate buffer pH 8.7 and a suitable amount of enzyme extract in a final volume of 1.5 mL) was incubated (40 °C, 5 min) for the PAL activity assay. Fifteen μmoles of L-phenylalanine were added to start the reaction, which was arrested after 1 h by the addition of 50 μL of 5 N HCl. Samples were filtered through a 0.45 μm disposable syringe filter and analyzed (20 μL) with a Shimadzu® Liquid Chromatograph. A reversed-phase Shimpack® CLC-ODS column (150 × 4.6 mm, 5 μm) was used at 30 ºC. The mobile phase was methanol:water (70%:30%) with a flow rate of 0.5 mL min^{-1} for an isocratic run of 10 min. Absorption was measured at 275 nm. *t*-Cinnamate, the product of PAL, was identified by comparing its retention time with standard values. PAL activity was expressed as μmol *t*-cinnamate h^{-1} g^{-1} fresh weight.

POD was extracted from fresh roots (0.5 g) with 67 mM phosphate buffer (5 mL, pH 7.0). The extract was centrifuged (2,200 ×*g*, 5 min, 4 °C), and the supernatant was used to determine the activity of soluble POD. The pellet was incubated in 1 M NaCl (2 mL, 1 h, 4 °C). The homogenate was centrifuged (2,200 ×*g*, 5 min, 4 °C), and the supernatant contained the cell wall-(ionically)-bound POD. Enzyme activities were determined according to dos Santos et al. (2008). The reaction mixture (3 mL) contained 25 mM sodium phosphate buffer, pH 6.8, 2.58 mM guaiacol and 10 mM H_2O_2. The reaction was started by adding the enzyme extract. The guaiacol oxidation was followed for 5 min at 470 nm, and enzyme activity was calculated from the extinction coefficient (25.5 mM^{-1} cm^{-1}). POD activities were expressed as μmol tetraguaiacol min^{-1} g^{-1} fresh weight.

2.3. Hydrogen peroxide quantification

Fresh roots (1 g) were homogenized in 3 mL of 0.1% trichloroacetic acid (Alexieva et al., 2001). The homogenate was centrifuged at 2200 *g* for 20 min. An aliquot (0.5 mL) of supernatant was added to 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 0.2 mL of 5 M potassium iodide. Absorbance was followed for 1 min at 390 nm. The blank consisted of a reaction mixture without potassium iodide, and its absorbance was subtracted from the mixture

with H_2O_2 extract. H_2O_2 content was calculated using a standard curve prepared with known concentrations of H_2O_2. Results were expressed as nmol H_2O_2 g^{-1} fresh weight.

2.4. Lignin quantification and monomeric composition

After removal of other compounds by phosphate buffer, Triton® X-100, NaCl and acetone, root lignin content was determined from the protein-free cell wall fraction by lignin-thioglycolic acid (LTGA) reaction (Ferrarese et al., 2002). Lignin was expressed as mg LTGA g^{-1} dry weight. Alkaline nitrobenzene oxidation was used to determine lignin monomeric composition (Zanardo et al., 2009). The protein-free cell wall fraction (50 mg) obtained above was sealed in a Pyrex® ampule containing 1 mL of nitrobenzene and heated to 170 °C for 90 min, while the sample was occasionally shaken during the reaction. The sample was cooled at room temperature, washed twice with chloroform, acidified to pH 2 with 2 N HCl, and extracted twice with chloroform. The organic extracts were combined, dried, re-suspended in 1 mL of methanol, and diluted in methanol/acetic acid 4% in water (20:80, v/v). All samples were filtered through a 0.45-μm disposable syringe filter and analyzed by HPLC. The mobile phase was methanol/acetic acid 4% in water (20:80, v/v), with a flow rate of 1.2 mL min^{-1} for an isocratic run of 20 min. Quantification of the monomeric aldehyde (*p*-hydroxybenzaldehyde, vanillin and syringaldehyde) products released by nitrobenzene oxidation was performed at 290 nm using the corresponding standards. Results were expressed as μg monomer mg^{-1} cell wall.

2.5. Statistical design

The experimental design was completely randomized, and each plot was represented by one glass container with 25 seedlings. Data are expressed as the mean of four to seven independent experiments ± S.E. The one-way variance analysis to test the significance of the observed differences was performed with Sisvar® package (Version 4.6, UFLA, Brazil). Differences between parameters were evaluated by the Scott-Knott test, and *P* values ≤0.05 were considered as statistically significant.

3. Results and discussion

3.1. Effects of NaCl on root growth

To evaluate the salt effects on root growth, soybean seedlings were grown during 24 h in nutrient solution containing 50 to 100 mM NaCl (Table 1). Increased NaCl levels resulted in a significant decrease in root lengths that were from 44.7% to 96% shorter than control roots after 100 to 200 mM treatments. These effects were also evident in root fresh and dry weights, which significantly decreased after 50 to 200 mM NaCl exposures: 12.7% to 27.9% for fresh and 10.6% to 34.3% for dry weights. NaCl exposure inhibited seedling growth and biomass of bean, cotton (*Gossypium hirsutum*), lettuce (*Lactuca sativa*), maize (*Zea mays*), rice and sunflower (*Helianthus annuus*) as demonstrated by several authors (Cachorro et al., 1993; Lin and Kao, 2001b; Rios-Gonzales et al., 2002; Alla et al., 2002; Sohn et al., 2005; Tas et al., 2005). In the case of soybean, Hilal et al. (1998) reported that roots of plants treated with 120 mM NaCl were shorter and had fewer secondary roots than controls, with a decrease of growth rate of seedlings. Essa (2002) and Hosseini et al. (2002) alike established that seedling growth rate decreased with increasing salinity (almost zero at 330 mM). Dry weight and plant growth of different cultivars decreased after 150 mM NaCl exposure (Tuncturk et al., 2008). At 200 mM, NaCl severely decreased the length and fresh weight of the hypocotyl and root (Aghaei et al., 2009). Reported studies agree with our results, which confirm that soybean is a salt-sensitive crop.

3.2. Effects of NaCl on PAL and POD activities

NaCl-affected PAL activities were significantly different from controls (Fig. 1). The data revealed that PAL activities decreased about 35% regardless of its concentration as compared to the control. Little information is available about PAL activity influenced by NaCl stress. Jbir et al. (2001b) demonstrated that the application of NaCl to wheat seedling of a salt-tolerant (*Triticum durum*) and a salt-sensitive (*Triticum aestivum*) species increased PAL activity. The effects were slightly more pronounced in salt-sensitive species. Similarly, Alla et al. (2002) reported that salt treatments significantly increased the enzyme activity in maize roots. Results obtained in the present research are not consistent with data obtained by those authors. It is well known that PAL is the entry enzyme into the phenylpropanoid pathway and, therefore, it has

multiple roles in aromatic secondary metabolism that may not be related to only lignin biosynthesis. At least for NaCl-treated soybean roots, the impact of PAL on lignin biosynthesis may not be critical.

At 150 and 200 mM, NaCl substantially increased (37% and 30%, respectively) soluble POD activities, relative to the control (Fig. 2A). Salt treatment also increased cell wall-bound POD activities, surpassing the control value by about 27% and 26.4% for 150 and 200-mM NaCl exposures, respectively (Fig. 2B). Activities of antioxidant enzymes (catalase, superoxide dismutase, glutathione reductase and soluble POD) increased in several plant species, such as rice, cucumber (*Cucumis sativus*), wheat, maize, cotton, sunflower and tomato (Fadzilla et al., 1997; Lechno et al., 1997; Meneguzzo et al., 1999; Rios-Gonzales et al., 2002; Meloni et al., 2003; Mittova et al., 2004; Azevedo Neto et al., 2006; Gapińska et al., 2008). Cited authors suggests that the action of the antioxidant system protects the plant against reactive oxygen species (ROS) and, therefore, against salt stress. In rice roots, Lin and Kao (2001b) have pointed out that NaCl (up to 150 mM) increased cell wall-(ionically)-POD activity indicating that this enzyme participates in the regulation of root growth reduction under salinity condition. One of the noteworthy features of peroxidases is its involvement in controlling plant cell growth by promoting cell wall rigidity through lignin synthesis and cross-linking of polysaccharide components. In this regard, cell wall-bound POD is often held to be most directly involved in the lignification process (Passardi et al., 2005). Results reported in the present research are in agreement with those authors since that POD activities increased under NaCl exposure. In addition, Gulen et al. (2006) and Talano et al. (2006) demonstrated significant increase in a basic isoperoxidase associated with the ligno-suberization of cell walls in leaf of strawberry (*Fragaria vesca*) and in tomato hairy roots, respectively.

3.3. Effects of NaCl on H_2O_2 and lignin content

In addition to earlier findings, subsequent experiments showed the effects of NaCl on H_2O_2 and lignin content and its monomeric composition (Figs. 3 to 5). The increased POD activities (Fig. 2) were associated with decreased H_2O_2 content in the salt-stressed roots. In fact, roots exposed to 50 – 200 mM NaCl significantly reduced H_2O_2 contents from 31% to 47% respect to control (Fig. 3). As a consequence of NaCl exposure, lignin content increased 72% and 90% after 150 and 200 mM NaCl treatments respect to control (Fig. 4). The analysis of alkaline

nitrobenzene oxidation products (Fig. 5) revealed that lignin monomer content (*p*-hydroxyphenyl + guaiacyl + syringyl; H + G + S) increased slightly (only G monomer) when compared to that in untreated roots.

Current study has clearly shown that soybean root growth decreased while cell wall-bound POD activity and lignin contents increased after NaCl treatments. Lignin biosynthesis is accompanied by an increase in the activities of cell wall-bound POD, and this process involves oxidative coupling, dependent on H_2O_2, which causes a rapid cross-linking of cell wall polymers (Boerjan et al., 2003; Passardi et al., 2005). So, it seems feasible that these facts explain decreases of H_2O_2 contents, reported in this paper (Fig. 3). In agreement with these findings, Kim et al. (2005) earlier demonstrated that the enhanced POD activity is associated with reduced H_2O_2 content in barley (*Hordeum vulgare*) roots.

The discovery that NaCl stimulated lignin production in soybean roots is of particular interest. Cell walls are know to become lignified when cell expansion decreases, when the cell is under stress, and when it differentiates to particular specialization, notably the xylem (Christensen et al., 1998). Salinity stress has been associated with a greater deposition of lignin in vascular tissues and/or xylem development. In bean root vascular, NaCl caused an earlier and stronger lignification which has been suggested to be a factor that inhibits root growth and, consequently, represents an adaptation mechanism in resisting salinity-imposed stress (Cachorro et al., 1993).

Coordinated morphological, histochemical and biochemical analyzes have been reinforcing the role of salt stress on lignification and related processes in cell walls. For example, Neumann et al. (1994) reported that long-term salinization inhibited elongation of primary root growth by inducing a hardening of cell walls in the growing tips of maize. Additionally, Hilal et al. (1998) related that saline stress retarded primary xylem differentiation and induced acceleration of the development of secondary xylem in soybean roots. In central cylinder of NaCl-treated wheat roots, the cell walls are much thicker than control seedling, which is consistent with an increased process of lignification (Jbir et al., 2001a). In the same way, Sánchez-Aguayo et al. (2004) established that salinity treatment increased the number of lignified vessels in roots and stems of tomato plants. Authors concluded that tomato under salt stress undergo extensive development and lignification of the xylem, with the most evident response exhibited by roots. In earlier study, Sancho et al. (1996) had demonstrated that the increase in POD activity was parallel to an increase of lignin-like compounds in tomato suspension cells. These high values of POD activity

reflect the changed mechanical properties of the cell wall related to a possible salt adaptation process.

4. Conclusions

The present study shows that salt stress induced lignification in soybean roots. This metabolic process is, as a rule, accompanied by increases in the POD activities and lignin production associated with cell wall rigidification and root growth reduction. Then, at least for soybean, it is plausible that lignification may play a regulatory role in the root growth reduction caused by salt stress.

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Table 1. Changes in root length, root fresh and dry weights of soybean seedlings treated with NaCl for 24 h.

Fig. 1. Effects of NaCl on phenylalanine ammonia-lyase (PAL) activities. Means (N=7 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 2. Effects of NaCl on soluble (A) and cell wall-bound (B) peroxidases (POD) activities. Means (N=4 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 3. Effects of NaCl on H_2O_2 contents. Means (N=5 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 4. Effects of NaCl on lignin contents. Means (N=4 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 5. Effects of 150 mM NaCl on lignin monomer composition. H, *p*-hydroxyphenyl; G, guaiacyl, and S, syringyl monomers. Mean±S.E. values (N=4 ± S.E.) followed by different letters are significantly different according to the Scott–Knott test ($P \leq 0.05$).

Table 1

NaCl (mM)	Root length (cm)	%	Fresh weight (g)	%	Dry weight (g)	%
0	2.48 ± 0.073^{a}		3.22 ± 0.067^{a}		0.198 ± 0.004^{a}	
50	2.36 ± 0.110^{a}		2.81 ± 0.086^{b}	12.7	0.177 ± 0.001^{b}	10.6
100	1.37 ± 0.087^{b}	44.7	2.73 ± 0.069^{b}	15.2	0.166 ± 0.003^{b}	16.2
150	0.18 ± 0.047^{c}	92.7	2.80 ± 0.025^{b}	13.0	0.151 ± 0.003^{c}	23.7
200	0.10 ± 0.028^{c}	96.0	2.32 ± 0.089^{c}	27.9	0.130 ± 0.006^{d}	34.3

Means (N=5 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$). The column % represents inhibition of statistically significant means in comparison to control (0 mM).

Figure 1

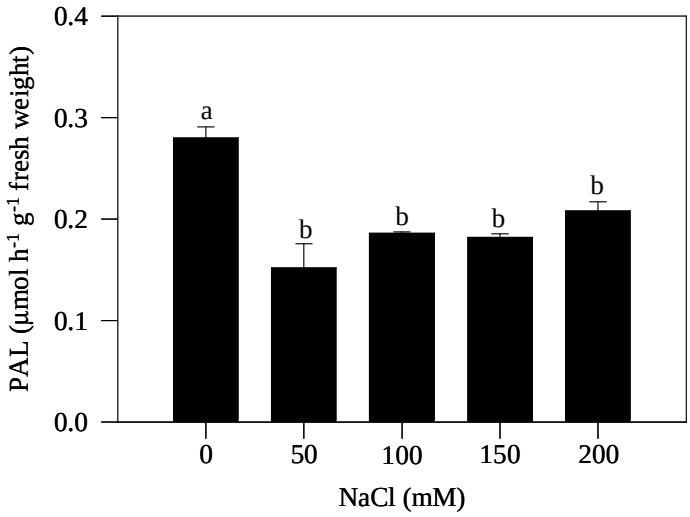

0.4
0.3
0.2
0.1
0.0
PAL (µmol h-1 g-1 fresh weight)
a
b
b
b
b
0
50
100
150
200
NaCl (mM)

Figure 2

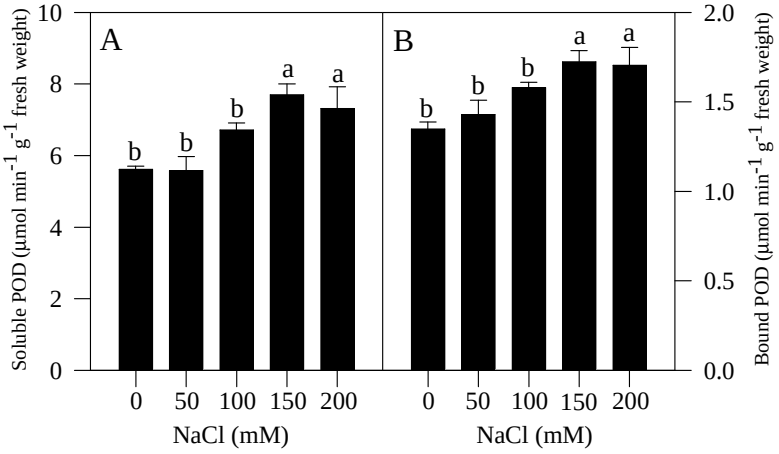

A
B
Soluble POD (µmol min-1 g-1 fresh weight)
Bound POD (µmol min-1 g-1 fresh weight)
0
2
4
6
8
10
0.0
0.5
1.0
1.5
2.0
b
b
b
a
a
b
b
b
a
a
0
50
100
150
200
NaCl (mM)
NaCl (mM)

Figure 3

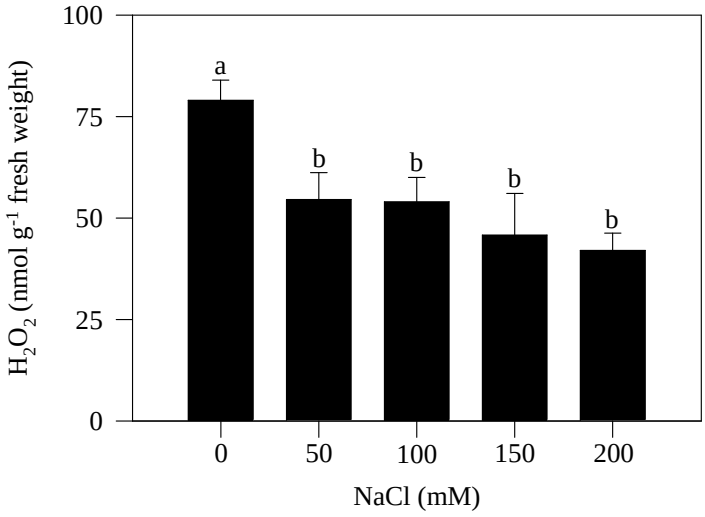

100
75
50
25
0
H_2O_2 (nmol g^{-1} fresh weight)
a
b
b
b
b
0
50
100
150
200
NaCl (mM)

Figure 4

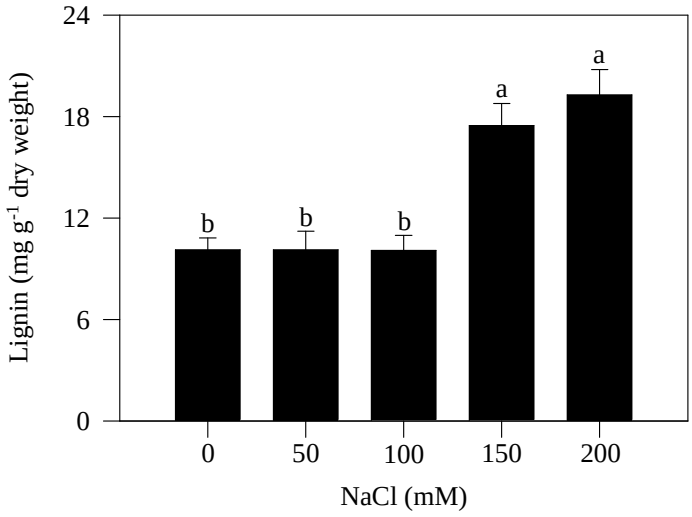
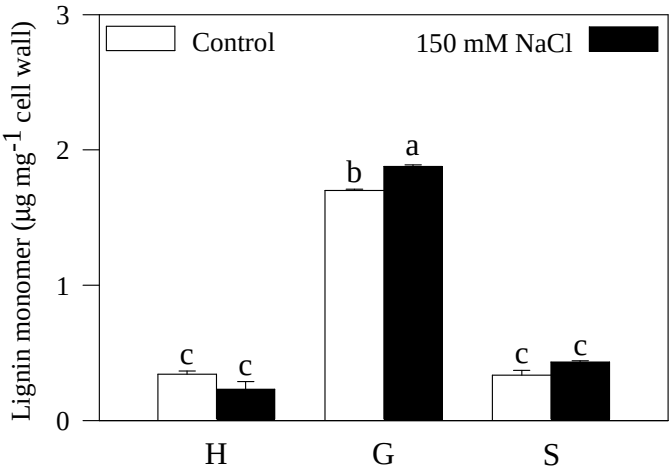

24
18
12
6
0
Lignin (mg g-1 dry weight)
b
b
b
a
a
0
50
100
150
200
NaCl (mM)

Figure 5


Lignin monomer (µg mg-1 cell wall)
3
2
1
0
Control
150 mM NaCl
c
c
b
a
c
c
H
G
S

Effects of calcium on lignification-related parameters in NaCl-stressed soybean roots

G. Y. S. Neves, M. L. L. Ferrarese, O. Ferrarese-Filho

Correspondence to:

Osvaldo Ferrarese-Filho
Department of Biochemistry
University of Maringá
Av. Colombo, 5790
87020-900, Maringá, PR
BRAZIL

E-mail: oferrarese@uem.br
Fax: +55 44 2633655

Effects of calcium on lignification-related parameters in NaCl-stressed soybean roots

G. Y. S. Neves, M. L. L. Ferrarese, O. Ferrarese-Filho

Department of Biochemistry, University of Maringá, Av. Colombo, 5790, 87020-900, Maringá, PR, Brazil

ABSTRACT

The effects of exogenous calcium on root growth and lignification-related parameters – phenylalanine ammonia-lyase (PAL) and peroxidases (POD) activities, hydrogen peroxide (H_2O_2) and lignin contents – in roots of NaCl-stressed soybean (*Glycine max*) seedlings were analyzed. Three-day-old seedlings were cultivated in half-strength Hoagland's solution (pH 6.0) with or without 5 mM $Ca(NO_3)_2$ and 50 to 200 mM NaCl in a growth chamber (25°C, 12/12 h light/dark photoperiod, irradiance of 280 μmol m^{-2} s^{-1}) for 24 h. In general, results showed that the absence of Ca^{2+} reduced root growth and increased lignification of soybean seedlings grown in NaCl-free nutrient solution. NaCl reduced the root growth and all lignification-related parameters. Root growth, PAL and POD activities and H_2O_2 contents were more affected after NaCl treatments without Ca^{2+} in the nutrient solution. At 5 mM, Ca^{2+} did not alleviate the deleterious effects of NaCl on lignification-related parameters.

Keywords: calcium; phenylpropanoid pathway; roots; salt stress; soybean.

1. Introduction

Calcium is an essential plant macronutrient. Taken up by the root system from the soil solution in the cationic form (Ca^{2+}) and delivered to the shoot via the xylem. It may traverse the root to the xylem either through the cytoplasm of cells linked by plasmodesmata or through the extra-cellular spaces (White and Broadley, 2003). Outside the cell, Ca^{2+} maintains membrane integrity and cell wall composition since that it provides cell wall rigidity by cross linking the pectic chains of the middle lamella. It is thought that cell expansion is a primary process in root morphogenesis, and Ca^{2+} influx from the extra-cellular store is required for cell elongation in roots (Marschner, 1995). Within the vacuole, Ca^{2+} acts as a counter-cation for inorganic and organic anions (Broadley et al., 2003). As a structural component of macromolecules most functions are related to its capacity for coordination, by which it provides stable intermolecular linkages, predominantly in cell walls and at plasma membrane (Marschner, 1995). There is more and more evidence that Ca^{2+} serves as a messenger in many normal growth and developmental process such as cell division, cell differentiation, cell polarity, cell elongation, photomorphogenesis, plant defense and biotic and abiotic stresses responses (Reddy, 2001; Parida and Das, 2004).

Salinity is a severe problem that affects 2 million km^2 of land used for agricultural production. It is one of the most important abiotic stresses which directly limit crop productivity in many areas of the world (Wang et al., 2003; Bartels and Sunkar, 2005). Plants response to saline stress is complex since it involves changes in their morphology, physiology and metabolism in different varieties of a same type of plant (Jbir et al., 2001; Essa, 2002; Luo et al., 2005; Azevedo Neto et al., 2006; Gulen et al., 2006) and in different growth stages (Rios-Gonzales et al., 2002; Davenport et al., 2003; Meloni et al., 2003; Talano et al., 2006; Kim et al., 2007; Aghaei et al., 2009; Ghanem et al., 2009). Root growth is characterized by high metabolic activity, and for this reason roots are highly susceptible to environmental stresses. The primary effect of salinity is thought to take place in root, since it is the only organ directly exposed to excess salt (Karahara et al., 2004).

Lignification, the process of sealing a plant cell by lignin deposition, is a necessary developmental process in all tissues of plants. Lignin is a complex cell wall component comprised of phenolic heteropolymers that are covalently bound to both polysaccharides and proteins. It is deposited into secondary cell walls of lignifying tissue providing rigidity, structural

support and enabling water transport in the vascular system (Donaldson, 2001). Lignin is synthesized by the phenylpropanoid pathway, which is involved in the synthesis of a wide range of secondary products in plants such as phenolic acids, flavonoids, tannins, coumarins (Kovácik et al., 2007) and lignin (Boerjan et al., 2003). As a fundamental component of the cell wall, lignin contributes to the compression strength of stems, and to the water proofing of conductive elements within the xylem (Donaldson, 2001; Ralph et al., 2004). Phenylalanine ammonia-lyase (PAL) is regarded as the primary enzyme of the phenylpropanoid biosynthetic pathway, and peroxidase (POD) within the cell wall, in either the free or bound state, has been shown to be involved in monolignol polymerization and, therefore, during lignification (Boerjan et al., 2003; Passardi et al., 2005).

In general, cell walls suffer lignification when the plant is under stress (Christensen et al., 1998). However, controversial reports about salt stress on root lignification have been related. For example, Cachorro et al. (1993) demonstrated lignin deposition in vascular tissues of bean (*Phaseolus vulgaris*) roots in response to NaCl stress. By contrary, Lin and Kao (2001) showed that lignin levels decreased in NaCl-treated root of rice (*Oryza sativa*) seedlings. In addition, some studies on the role of Ca^{2+} in relation to phenolic compounds metabolism and related enzymes have been reported. For some researchers, Ca^{2+} augmented the soluble POD and PAL activities accumulating (Castañeda and Pérez, 1996; Kolupaev et al., 2005) or reducing (Ruiz et al., 2003) the phenolic compounds. Others verified that Ca^{2+} decreased soluble POD and PAL activities reducing (or not affecting) the phenolic compounds production (Ke and Saltveit, 1986; Kawai et al., 1995; Tomás-Barberán et al., 1997) or suppressing the leakage of phenolic compounds from roots (Zieslin and Abolitz, 1994).

Among different plant species, soybean (*Glycine max*) has been considered as a salt-sensitive to moderately salt-tolerant crop (Luo et al., 2005; Kao et al., 2006). Although the effects of NaCl on soybean growth has been widely reported (Hilal et al., 1998; Essa, 2002; Tuncturk et al., 2008; Aghaei et al., 2009), its effects on root lignification has not been exploited. Since it is still an open question and due to the importance of Ca^{2+} for plant growth, current work has been planned to study the influence of exogenous Ca^{2+} on PAL and POD activities and hydrogen peroxide (H_2O_2) level and lignin contents in soybean roots under NaCl exposure.

2. Materials and methods

2.1. General procedures

Soybean (*Glycine max* L. Merrill, cv BRS-184) seeds, surface-sterilized with 2% sodium hypochlorite for 2 min and rinsed extensively with deionized water, were dark-germinated (at 25 °C) on three sheets of moistened filter paper. Twenty-five 3-day-old seedlings of uniform size were supported on an adjustable acrylic plate and dipped into a glass container (10×16 cm) filled with 200 mL of half-strength Hoagland's solution (pH 6.0) with or without 5 mM $Ca(NO_3)_2$ and NaCl (50, 100, 150 and 200 mM). The containers were kept in a growth chamber (25 °C, 12/12 h light/dark photoperiod, irradiance of 280 μmol m^{-2} s^{-1}) for 24 h. Roots were measured before incubation and at the end of experiments, and the lengths were obtained by difference among them. Fresh root weight was determined immediately after incubation, and the dry weight was estimated after oven-drying at 80 °C until it reached a constant weight. All reagents used were of the purest grade available or chromatographic grade.

2.2. Cell viability

After 24 h of incubation, all seedlings were removed to determine the loss of cell viability by Evans blue staining spectrophotometric assay (Zanardo et al., 2009). All freshly harvested roots were incubated for 15 min with 30 mL of 0.25% Evans blue solution. Further, the roots were washed in distilled water for 30 min to remove excess and unbound dye. Excised root tips (3 cm) were soaked in 3 mL of *N*,*N*-dimethylformamide for 50 min at room temperature. The absorbance of released Evans blue was measured at 600 nm, using deionized water as a blank. The loss of cell viability was expressed as absorbance at 600 nm of treated roots in relation to untreated roots (control).

2.3. Enzymatic assays

PAL was extracted as described by Ferrarese et al. (2000). Fresh roots (2 g) were ground at 4 ºC in 0.1 M sodium borate buffer (pH 8.8). Homogenates were centrifuged (2,200 ×*g*, 15 min) and the supernatant was used as the enzyme preparation. The reaction mixture (100 μmoles

sodium borate buffer pH 8.7 and a suitable amount of enzyme extract in a final volume of 1.5 mL) was incubated (40 °C, 5 min) for the PAL activity assay. Fifteen μmoles of L-phenylalanine were added to start the reaction, which was stopped after 1 h by the addition of 50 μL of 5 N HCl. Samples were filtered through a 0.45 μm disposable syringe filter and analyzed (20 μL) with a Shimadzu® Liquid Chromatograph. A reversed-phase Shimpack® CLC-ODS column (150 × 4.6 mm, 5 μm) was used at 30 ºC. The mobile phase was methanol:water (70%:30%) with a flow rate of 0.5 mL min^{-1} for an isocratic run of 10 min. Absorption was measured at 275 nm. *t*-Cinnamate, the product of PAL, was identified by comparing its retention time with standard values. PAL activity was expressed as μmol *t*-cinnamate h^{-1} g^{-1} fresh weight.

POD was extracted from fresh roots (0.5 g) with 67 mM phosphate buffer (5 mL, pH 7.0). The extract was centrifuged (2,200 ×*g*, 5 min, 4 °C), and the supernatant was used to determine the activity of soluble POD. The pellet was incubated in 1 M NaCl (2 mL, 1 h, 4 °C). The homogenate was centrifuged (2,200 ×*g*, 5 min, 4 °C), and the supernatant contained the cell wall-(ionically)-bound POD. Enzyme activities were determined according to dos Santos et al. (2008). The reaction mixture (3 mL) contained 25 mM sodium phosphate buffer, pH 6.8, 2.58 mM guaiacol and 10 mM H_2O_2. The reaction was started by adding the enzyme extract. The guaiacol oxidation was followed for 5 min at 470 nm, and enzyme activity was calculated from the extinction coefficient (25.5 mM^{-1} cm^{-1}). POD activities were expressed as μmol tetraguaiacol min^{-1} g^{-1} fresh weight.

2.4. Hydrogen peroxide quantification

Fresh roots (1 g) were homogenized in 3 mL of 0.1% trichloroacetic acid (Alexieva et al., 2001). The homogenate was centrifuged at 2,200 ×*g* for 20 min. An aliquot (0.5 mL) of supernatant was added to 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 0.2 mL of 5 M potassium iodide. Absorbance was followed for 1 min at 390 nm. The blank consisted of a reaction mixture without potassium iodide, and its absorbance was subtracted from the mixture with H_2O_2 extract. H_2O_2 content was calculated using a standard curve prepared with known concentrations of H_2O_2. Results were expressed as μmol H_2O_2 g^{-1} fresh weight.

2.5. Lignin quantification

After the incubation period, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 mL, pH 7.0) with a mortar and pestle and transferred into a centrifuge tube (Ferrarese et al., 2002). The pellet was centrifuged (1,400 ×g, 4 min) and washed by successive stirring and centrifugation as follows: twice with phosphate buffer pH 7.0 (7 mL); 3× with 1% (v/v) Triton® X-100 in pH 7.0 buffer (7 mL); 2× with 1 M NaCl in pH 7.0 buffer (7 mL); 2× with distilled water (7 mL); and 2× with acetone (5 mL). The pellet was dried in an oven (60 °C, 24 h) and cooled in a vacuum desiccator. The dry matter was defined as a protein-free cell wall fraction. Further, all dry protein-free tissue was placed into a screw-cap centrifuge tube containing the reaction mixture (1.2 mL of thioglycolic acid plus 6 mL of 2 N HCl) and heated (95 °C, 4 h). After cooling at room temperature, the sample was centrifuged (1,400 ×g, 5 min), and the supernatant was discarded. The pellet contained the complex lignin–thioglycolic acid (LTGA). The pellet was washed three times with distilled water (7 mL) and the LTGA extracted by shaking (30 °C, 18 h, 115 oscillations per minute) in 0.5 M NaOH (6 mL). After centrifugation (1,400 ×g, 5 min), the supernatant was stored. The pellet was washed again with 0.5 M NaOH (3 mL) and mixed with the supernatant obtained earlier. The combined alkali extracts were acidified with concentrated HCl (1.8 mL). After precipitation (0 °C, 4 h), LTGA was recovered by centrifugation (1,400 ×g, 5 min) and washed two times with distilled water (7 mL). The pellet was dried at 60 °C, dissolved in 0.5 M NaOH, and diluted to yield an appropriate absorbance for spectrophotometric determination at 280 nm. Lignin was expressed as mg LTGA g^{-1} dry weight.

2.6. Statistical design

The experimental design was completely randomized, and each plot was represented by one glass container with 25 seedlings. Data are expressed as the mean of four to six independent experiments ± S.E. The one-way variance analysis to test the significance of the observed differences was performed with Sisvar® package (Version 4.6, UFLA, Brazil). Differences between parameters were evaluated by the Scott-Knott test, and *P* values ≤0.05 were considered as statistically significant.

3. Results and Discussion

3.1. Absence of Ca^{2+} affects root growth, lignification and related enzymes

Roots grown in nutrient solution free of Ca^{2+} were significantly different those grown with Ca^{2+} (Table 1). Root length and dry weight decreased 82.9% and 14.3%, respectively. PAL, soluble and cell wall-bound POD activities increased by 30.1%, 18.5% and 26.8%, respectively. Whereas H_2O_2 content reduced by 9.12%, lignin content increased 48% respect to Ca^{2+} exposure. No changes have been observed in root fresh weight and cell viability.

It is well established that lignin biosynthesis is accompanied by an increase in the activities of soluble and cell wall-bound POD, and this process involves oxidative coupling, dependent on H_2O_2, which causes a rapid cross-linking of cell wall polymers (Boerjan et al., 2003; Passardi et al., 2005). It is also well known that Ca^{2+} maintains membrane integrity and cell wall composition, and its influx from the extracellular store is required for cell elongation in roots. Cell walls are known to become lignified when the plant is under stress (Christensen et al., 1998), and disruption of cell walls and collapse of tissues are known symptoms of the absence of an exogenous Ca^{2+} supply (Marschner, 1995). This possibility is supported by an earlier observation that, in the absence of Ca^{2+}, increase in PAL activity produced phenolic compounds; further lignin synthesis of cell walls and high POD activity have been linked with restricted growth of soybean roots (Teixeira et al., 2006). Clearly, results showed herein may be attributed to the stress caused by the absence of an exogenous Ca^{2+} supply.

3.2. NaCl affects root growth and lignification-related enzymes

To evaluate the salt effects on root growth, soybean seedlings were grown during 24 h in nutrient solution containing 50 to 100 mM NaCl, with or without Ca^{2+} (Fig. 1). In both conditions of growth, increased NaCl levels resulted in significant decreases in root length when compared to the corresponding controls (Fig. 1A). At 5 mM Ca^{2+}, root length reduced from 11% to 84%. A similar trend was also evident in the absence of Ca^{2+}, *i.e.*, significant decreases from 38% to 89% respect to control. At 5 mM Ca^{2+}, root fresh and dry weights decreased from 5% to 24% (Fig. 1B) and from 16% to 43% (Fig. 1C), respectively. Similar effects were also seen after NaCl exposures without Ca^{2+}: 24% to 27% for fresh (Fig. 1B) and 31% to 59% for dry (Fig. 1C)

weights. Viability of root cells was significantly affected by NaCl in the absence of Ca^{2+} (Fig. 2). Uptake of Evans blue in roots exposed to 50 – 200 mM NaCl was 10 to 17–fold higher than that observed in the control. With Ca^{2+}, roots exposed to 100 – 200 mM NaCl significantly reduced cell viability from 2.4 to 4.5-fold respect to control.

Inhibition of root growth is a general response of plants exposed to the NaCl (Karahara et al., 2004). There are evidences supporting the findings of the current research. In soybean, Hilal et al. (1998) reported that 120 mM NaCl-treated soybean roots were shorter and had fewer secondary roots than controls. Essa (2002) and Hosseini et al. (2002) alike established that seedling growth rate decreased after 330 mM NaCl-treatments. Dry weight and plant growth of different cultivars decreased after 150 mM NaCl exposure (Tuncturk et al., 2008). At 200 mM, NaCl severely decreased the length and fresh weight of the hypocotyl and root (Aghaei et al., 2009).

As a consequence of reduced root growth, salt stress affects several processes such as growth, water relations, ion levels, protein synthesis, lipid metabolism, nitrogen metabolism and photosynthesis (Parida and Das, 2004). NaCl not only significantly reduced the root growth of soybean seedlings (Fig. 1) but also affected all other parameters (Figs. 2 to 6), independently of Ca^{2+} in the nutrient solution. Under Ca^{2+} action, NaCl decreased PAL activity about 25% regardless of its concentration, when compared to control (Fig. 3). Without Ca^{2+}, enzyme activities were also reduced from 48% to 87% with increasing concentrations of NaCl. Salt stress decreased soluble POD activities about 25% (with Ca^{2+}) and 70% (without Ca^{2+}) regardless of its concentration when compared to the corresponding controls (Fig. 4A). Cell wall bound POD activities decreased about 21% (with Ca^{2+}) and 60% (without Ca^{2+}) respect to controls (Fig. 4B). Roots exposed to NaCl strongly reduced H_2O_2 contents about 70% regardless of its concentration, when compared to the corresponding control containing 5 mM Ca^{2+} (Fig. 5). When compared to the Ca^{2+}–free control, NaCl reduced H_2O_2 contents about 95%. In general, comparative analysis between the two groups reveals that root (length and weights) growth, PAL and POD activities and H_2O_2 contents were more affected after NaCl treatments without Ca^{2+} in the nutrient solution. Finally, 100 – 200 mM NaCl treatments decreased lignin contents about 27% and 42% when compared to the corresponding controls, respectively (Fig. 6). A fact revealed herein is that all lignification-related parameters decreased after NaCl-treatments. These findings were not unexpected because lignin biosynthesis is associated to the enzymatic activities of PAL and POD and the use of H_2O_2 for polymerization of monolignols in the cell

wall (Boerjan et al., 2003; Passardi et al., 2005). Results reported in current research agree with this possibility since low PAL activity reduces the synthesis of phenolic acids in the phenylpropanoid pathway. Taking into account that phenolic acids are not available for POD, a subsequent decrease in the lignin production is evident (Fig. 6).

Little information is available about effects of Ca^{2+} on lignification-related parameters of NaCl stressed plants, but evidence supports these findings. Ca^{2+} decreased soluble POD and PAL activities reducing the phenolic compounds production (Ke and Saltveit, 1986; Kawai et al., 1995; Tomás-Barberán et al., 1997) or suppressing its leakage from roots (Zieslin and Abolitz, 1994). In addition, Lin and Kao (2001) showed that lignin levels decreased by action of NaCl in rice seeds germinated in the absence of Ca^{2+}.

It is well known that Ca^{2+} plays a crucial role in plant metabolic processes (Marschner, 1995), and it has an ameliorating effect on the growth of plants under salt stress (Cachorro et al., 1994; Davenport et al., 1997). Supplemented Ca^{2+} counteracts the effects of Na^{+} on disturbance of the cell Ca^{2+} homeostasis, and its alleviating action may at least partly be due to maintenance of plasma membrane integrity in roots (Rengel, 1992). Although root growth and lignification-related parameters have been more affected by absence of Ca^{2+}, at 5 mM in the nutrient solution, Ca^{2+} did not restore the deleterious effects of NaCl when compared to the corresponding controls (Figs. 1 to 6). This does not rule out the possibility of different responses with seedlings growing in other Ca^{2+} concentrations. There is evidence that 20 mM Ca^{2+} ameliorates root and shoot growth of NaCl-stressed cucumber (*Cucumis sativus*) (Akinci and Simsek, 2004). However, further studies are still needed to confirm this possibility in soybean.

4. Conclusions

The main conclusions that can be drawn from the present study are that 1) the absence Ca^{2+} reduces the root growth and increases lignification of soybean seedlings grown in NaCl-free nutrient solution. This may be due to the stress caused by the absence of an exogenous Ca^{2+} supply. 2) NaCl reduces the root growth and lignification-related parameters. Reduced lignin production is, as a rule, accompanied by decreases in the PAL and POD activities and H_2O_2 levels for polymerization of monolignols in the cell wall. 3) Root (length and weights) growth, PAL and POD activities and H_2O_2 contents were more affected after NaCl treatments without Ca^{2+} in the nutrient solution. The present experiments provide no indication that 5 mM Ca^{2+}

alleviates the deleterious effects of NaCl on all lignification-related parameters.

Acknowledgements

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Table 1. Changes in root growth and lignification-related parameters of soybean seedlings grown in nutrient solution with or without 5 mM Ca^{2+} for 24 h.

Fig. 1. Changes in root length, root fresh and dry weights of soybean seedlings treated with NaCl for 24 h. Means (N=6 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 2. Loss of cell viability in roots of soybean seedlings treated with NaCl. Means (N=6 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 3. Effects of NaCl on phenylalanine ammonia-lyase (PAL) activities. Means (N=3 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 4. Effects of NaCl on soluble (A) and cell wall-bound (B) peroxidases (POD) activities. Means (N=4 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 5. Effects of NaCl on H_2O_2 contents. Means (N=4 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Fig. 6. Effects of NaCl on lignin contents. Means (N=5 ± S.E.) followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$).

Table 1

	$+Ca^{2+}$	$-Ca^{2+}$	%
Root length (cm)	2.526 ± 0.090^{a}	0.431 ± 0.007^{b}	–82.9
Root fresh weight (g)	2.503 ± 0.032^{a}	2.328 ± 0.096^{a}	
Root dry weight (g)	0.161 ± 0.003^{a}	0.138 ± 0.005^{b}	–14.3
Loss of cell viability (absorbance)	0.026 ± 0.003^{b}	0.028 ± 0.002^{a}	
PAL (nmol h^{-1} g^{-1})	230.5 ± 16.08^{b}	299.8 ± 16.12^{a}	+30.1
Soluble POD (nmol min^{-1} g^{-1})	7.317 ± 0.150^{b}	8.670 ± 0.089^{a}	+18.5
Cell wall-bound POD (nmol min^{-1} g^{-1})	1.973 ± 0.046^{b}	2.501 ± 0.045^{a}	+26.8
H_2O_2 (µmol g^{-1})	1.722 ± 0.069^{a}	1.565 ± 0.006^{b}	–9.12
Lignin (mg g^{-1})	8.087 ± 0.389^{b}	11.97 ± 1.601^{a}	+48.0

$+Ca^{2+}$ = 5 mM $Ca(NO_3)_2$ in the nutrient solution; $-Ca^{2+}$ = $Ca(NO_3)_2$ –free nutrient solution. In line, means ± S.E. followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$). The symbol % represents inhibition (−) or activation (+) of statistically significant means between treatments.

Figure 1

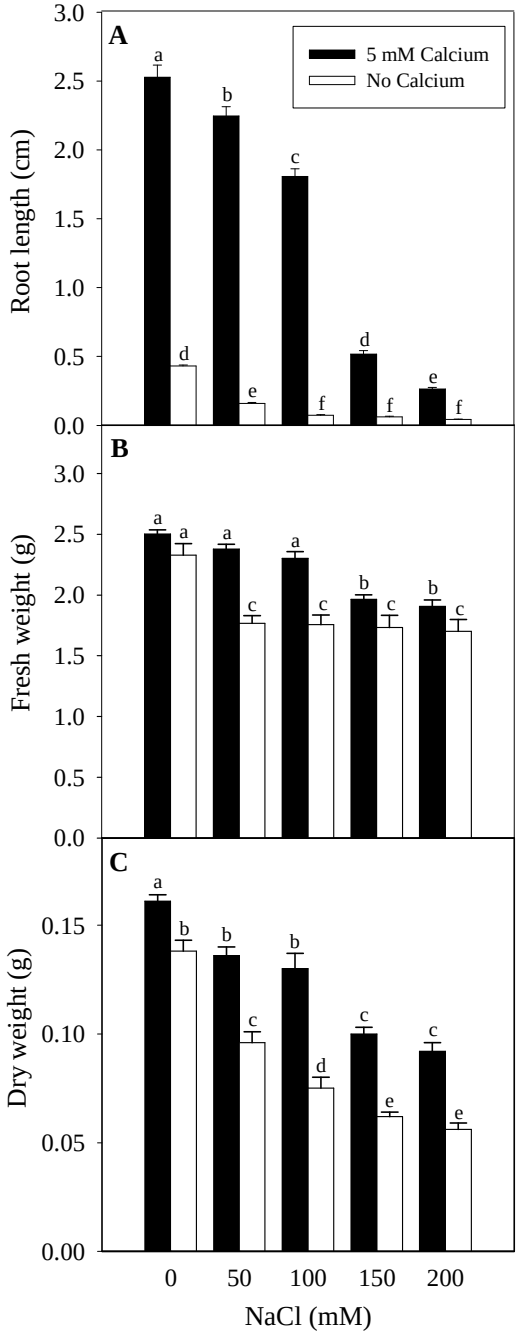

A
B
C
5 mM Calcium
No Calcium
Root length (cm)
Fresh weight (g)
Dry weight (g)
NaCl (mM)
0
50
100
150
200

Figure 2

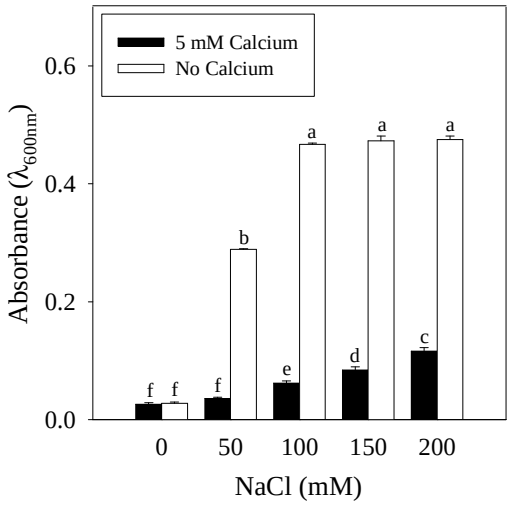

5 mM Calcium
No Calcium
Absorbance (λ_{600nm})
0.0
0.2
0.4
0.6
f f
f
e
d
c
b
a a a
0
50
100
150
200
NaCl (mM)

Figure 3

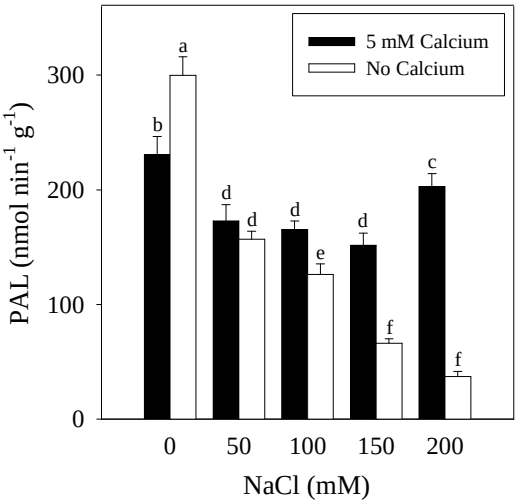

5 mM Calcium
No Calcium
PAL (nmol nin-1 g-1)
300
200
100
0
a
b
d
d
d
e
d
f
c
f
0
50
100
150
200
NaCl (mM)

Figure 4

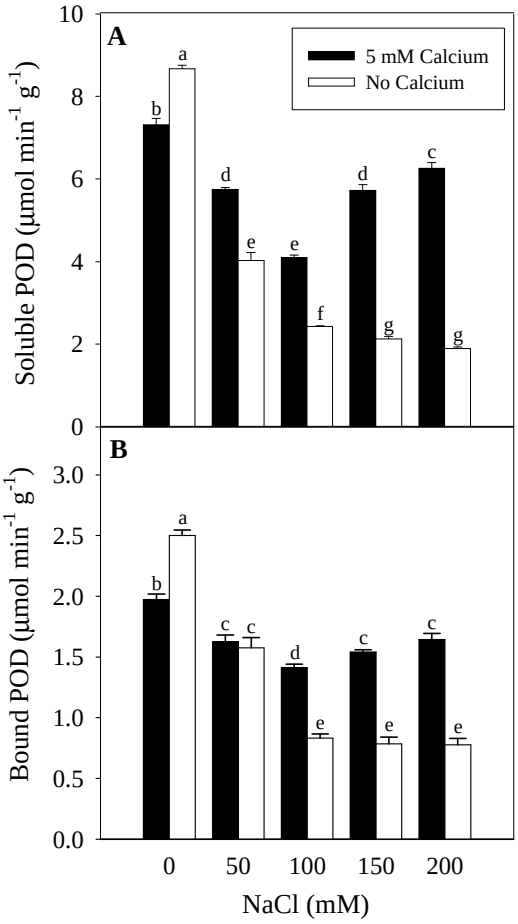

A
B
5 mM Calcium
No Calcium
Soluble POD (µmol min-1 g-1)
Bound POD (µmol min-1 g-1)
NaCl (mM)
0
50
100
150
200

Figure 5

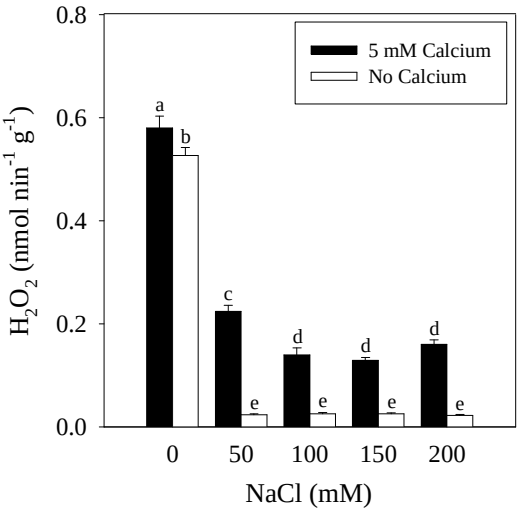
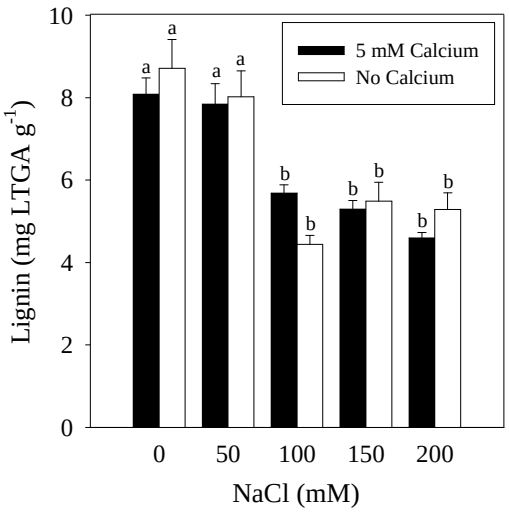

0.8
0.6
0.4
0.2
0.0
5 mM Calcium
No Calcium
a
b
c
d
d
d
e
e
e
e
0
50
100
150
200
NaCl (mM)
H2O2 (nmol nin-1 g-1)

Figure 6


10
8
6
4
2
0
Lignin (mg LTGA g-1)
a
a
a
a
b
b
b
b
b
b
5 mM Calcium
No Calcium
0
50
100
150
200
NaCl (mM)

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Baixar livros de Comunicação
Baixar livros do Conselho Nacional de Educação - CNE
Baixar livros de Defesa civil
Baixar livros de Direito
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Baixar livros de Literatura
Baixar livros de Literatura de Cordel
Baixar livros de Literatura Infantil
Baixar livros de Matemática
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Baixar livros de Medicina Veterinária
Baixar livros de Meio Ambiente
Baixar livros de Meteorologia
Baixar Monografias e TCC
Baixar livros Multidisciplinar
Baixar livros de Música
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Baixar livros de Química
Baixar livros de Saúde Coletiva
Baixar livros de Serviço Social
Baixar livros de Sociologia
Baixar livros de Teologia
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Baixar livros de Turismo