Plant Physiology and Biochemistry 142 (2019) 275–282

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Exogenous application of rosmarinic acid improves saccharification without affecting growth and lignification of maize

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ARTICLE INFO

Keywords:
Bioethanol
Biomass alkaline pretreatment
Lignification
Maize growth
Phenylpropanoid pathway

ABSTRACT

Biomimetically incorporated into the lignin structure, rosmarinic acid improves *in vitro* maize cell wall saccharification; however, no *in planta* studies have been performed. We hypothesized that rosmarinic acid, itself, could inducer saccharification without disturbing plant growth. Its effects on growth, enzymes of the phenylpropanoid pathway, lignin, monomeric composition, and saccharification of maize were evaluated. In a short-term (24 h) exposure, rosmarinic acid caused deleterious effects on maize roots, inhibiting the first enzymes of the phenylpropanoid pathway, phenylalanine ammonia-lyase and tyrosine ammonia-lyase, altering lignin composition and slightly increasing saccharification. In a long-term (14 d) exposure, rosmarinic acid increased saccharification of maize stems by about 50% without any deleterious effects on plant growth, the phenylpropanoid pathway and lignin formation. This demonstrated that exogenous application of rosmarinic acid on maize plants improved saccharification, and represented an interesting approach in facilitating enzymatic hydrolysis of biomass polysaccharides and increasing bioethanol production.

1. Introduction

To substitute fossil fuels in a sustainable manner is a major challenge facing the world today, and lignocellulosic biomass is considered one of the more promising reservoirs for producing biofuels. This biomass consists of cellulose, hemicelluloses, and lignin, which accounts for 20–30% of dry weight, depending on the plant species (Van Acker et al., 2017).

Like many secondary compounds such as gallotannins, flavonoids, coumarins, suberins, lignans, and neolignans, lignin is an end-product of the phenylpropanoid pathway (Vanholme et al., 2012, Fig. 1A). The firsts reactions of this metabolic pathway involve the non-oxidative deamination of L-phenylalanine by phenylalanine ammonia-lyase (PAL) yielding *t*-cinnamate, which is then hydroxylated by cinnamate 4-hydroxylase (C4H) to form *p*-coumarate. In monocots, tyrosine ammonia-lyase (TAL) converts L-tyrosine directly to *p*-coumarate. The next step in the pathway is the activation of *p*-coumarate to a thioester by 4-coumarate:CoA ligase (4CL). The *p*-coumaroyl-CoA formed is transesterified and hydroxylated by 4-hydroxycinnamoyl-CoA: shikimate/quinate 4-hydroxycinnamoyltransferase (HCT), *p*-coumarate 3-hydroxylase (C3H), and caffeoyl shikimate esterase (CSE) to generate caffeate initially, and then ferulate. While caffeoyl-CoA is formed from caffeoyl-shikimate by the action of HCT, feruloyl-CoA is synthesized from caffeoyl-CoA by caffeoyl-CoA *O*-methyltransferase (CCoAOMT). Both caffeoyl-CoA and feruloyl-CoA are also generated from caffeate and ferulate, respectively, by 4CL. By the sequential action of cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeate 3-*O*-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD), the CoA thioesters are converted to three main monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols. Finally, peroxidases/laccases polymerize these monolignols in *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of lignin. In addition, several other aromatic compounds can act as lignin building blocks (Vanholme et al., 2019), including tricin, a methylated flavone formed from *p*-coumaryl-CoA (Fig. 1A).

Lignin is crucial for plant growth, providing rigidity to cell walls, acting as a glue, keeping different cells together, and making the cell wall hydrophobic (Calvo-Flores et al., 2015). However, these functions represent recalcitrance factors in biofuel production as they hinder the enzymatic degradation of cell wall polysaccharides into

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https://doi.org/10.1016/j.plaphy.2019.07.015
Received 27 June 2019; Accepted 11 July 2019
Available online 12 July 2019

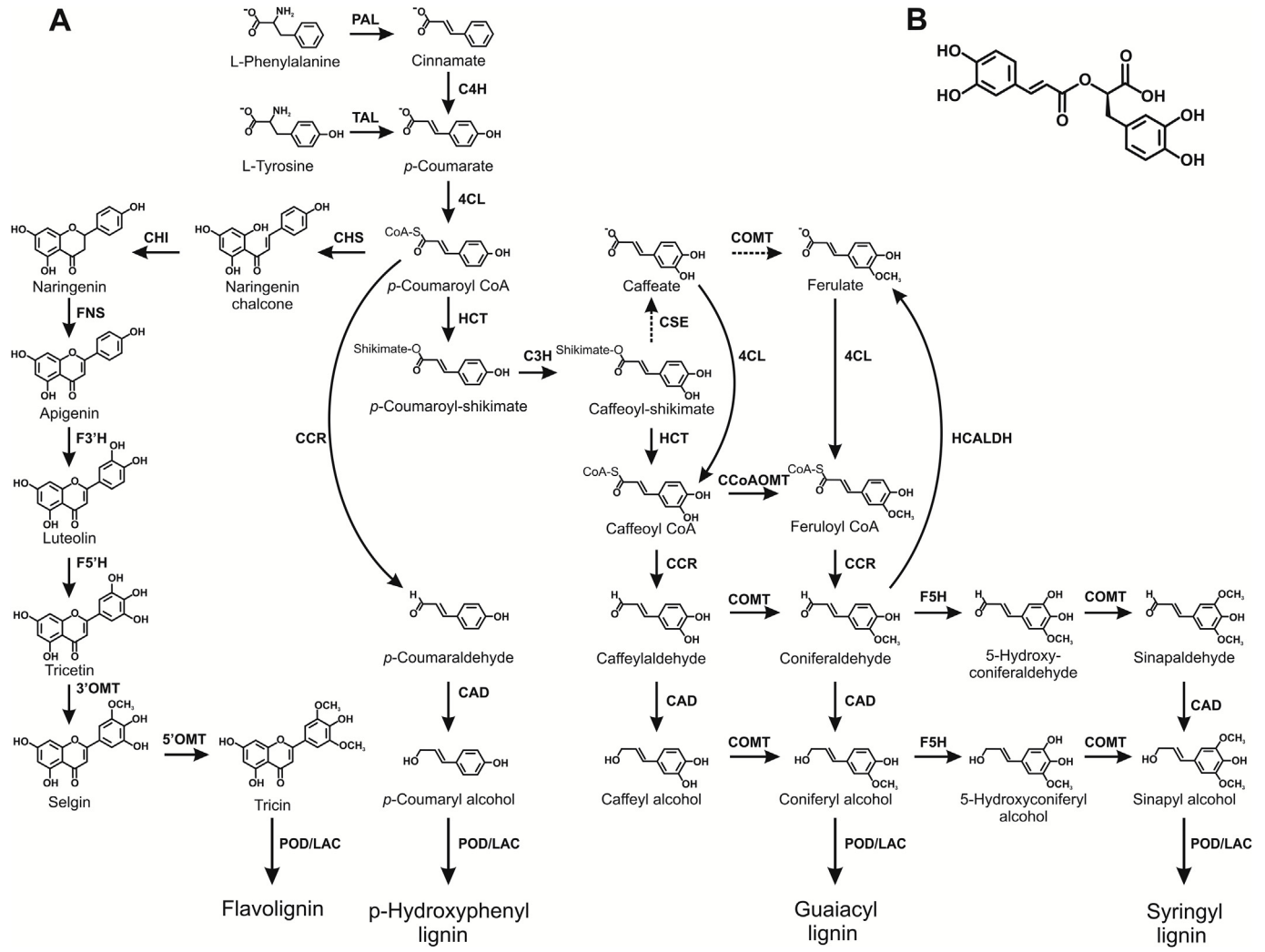

Fig. 1. A) Lignin biosynthesis. PAL, phenylalanine ammonia-lyase; TAL, tyrosine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, 4-hydroxycinnamoyl-CoA: shikimate/quinate 4-hydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeate 3-*O*-methyltransferase, CAD, cinnamyl alcohol dehydrogenase; POD, peroxidase; LAC, laccase; HCALDH, coniferaldehyde dehydrogenase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3′H, flavonoid 3-hydroxylase; F5′H, flavonoid 5-hydroxylase; 3′OMT, 3-*O*-methyltransferase; 5′OMT, 5-*O*-methyltransferase. B) Molecular structure of rosmarinic acid [(*R*)-*O*-(3,4-dihydroxycinnamoyl)-3-(3,4- dihydroxyphenyl)lactic acid].

monosaccharides, and consequently, saccharification (Guo et al., 2014; Souza et al., 2018). Therefore, the reduction of lignin recalcitrance is a biotechnological bottleneck. Currently, several strategies are proposed to overcome this obstacle, including manipulation of biosynthetic pathways, composition, structure, and quantity of lignin, typically by genetic-based metabolic engineering (Umezawa, 2018). However, genomic changes can affect other phenylpropanoid-derived pathways, cell wall composition and plant growth (Bonawitz and Chapple, 2013; Van Acker et al., 2013; Eudes et al., 2014; Kalluri et al., 2014; Mottiar et al., 2016; Meents et al., 2018). Therefore, other approaches are necessary to facilitate lignin depolymerization and improve saccharification efficiency of sugars from biomass. As such, the redesign of lignin by the *in vitro* biomimetic incorporation of alternative monomers into the polymer backbone is a promising strategy (Tobimatsu et al., 2012; Grabber et al., 2015; Mottiar et al., 2016). Several compatible compounds have been reported, including monomers that facilitate lignin fragmentation during mild pretreatments, monomers that block cross-linking between lignin and polysaccharides, and hydrophilic monomers (Grabber et al., 2019).

Rosmarinic acid (Fig. 1B) is a hydrophilic compound that has been artificially copolymerized with normal monolignols in maize cell walls. As an ester of caffeic acid and 3,4-dihydroxyphenyl-lactic acid, rosmarinic acid forms new benzodioxane structures during β-O-4 coupling with canonical monolignols by means of *in vitro* peroxidase-catalyzed reactions that model *in vivo* lignin polymerization. Included into the lignin backbone, these less resilient ester linkages are more easily cleaved by mild alkali treatment, facilitating lignin depolymerization and increasing saccharification (Tobimatsu et al., 2012).

Although *in vitro* biomimetic approaches have been demonstrated to be a useful tool (Grabber et al., 2019), *in planta* studies are necessary to verify that while redesigned lignin might improve saccharification it does not prejudice normal plant growth. In this study, we evaluated the effects of rosmarinic acid itself on plant growth, the activities of the main enzymes of the phenylpropanoid pathway, lignin, monomeric composition, and saccharification of maize. For this purpose, two experimental conditions were established. Because roots were characterized by high metabolic activity, and were the first organ susceptible to stress signals (Komatsu et al., 2010), we first evaluated the effects of rosmarinic acid on seedling roots for 24 h. In addition, we analyzed the effects of the compound on stems and leaves of plants grown for 14 d.

2. Material and methods

2.1. Plant material and general procedures

Maize (*Zea mays* L. cv. IPR-114) seeds, surface-sterilized with 2% sodium hypochlorite for 5 min and rinsed abundantly with deionized water, were dark-germinated at 25 °C on two sheets of moistened filter paper. Seedlings were grown for two experimental times, 24 h and 14 d. For the shorter-term experiments, three-day-old seedlings were selected for uniformity, supported on an adjustable acrylic plate, and dipped into 10 × 15-cm glass containers filled with 350 mL nutrient solution, pH 6.0 (Dong et al., 2006), with or without 0.25, 0.75 or 1.5 mM rosmarinic acid. The containers were kept in a growth chamber for 24 h at 25 °C, with a cool white fluorescent 14 h light/10 h dark photoperiod and a photon flux density of 280 μmol m^{-2} s^{-1}. Roots were measured before incubation and at the end of the experiments, and the difference in lengths (in cm) were calculated. Fresh root weights were determined immediately after incubation, and dry weights were estimated after drying in an oven at 60 °C to constant weight.

For the longer-term experiments, two-day-old seedlings of uniform size were placed in boxes containing 400 g vermiculite, and were cultivated in a growth room at 25 °C, with a 12 h light/dark photoperiod. The plants were watered with water until the 5th day. On this day, and every other day, the plants were then watered with 90 mL nutrient solution without or with 0.25, 0.75 or 1.5 mM rosmarinic acid. On the 14th day, the lengths of stems and leaves were measured, and the results were expressed in cm. The organs were excised and immediately weighted using an analytical balance. The plant material was dried in an oven at 60 °C to constant weight, and then weighed, with mean values of the replicates being expressed in grams. Rosmarinic acid was purchased from Sigma-Aldrich (St Louis, MO, USA), and all other reagents used were chromatographic grade or the purest grade available.

2.2. Enzymatic assays

Enzymes were extracted from the roots, stems and leaves of treated and untreated seedlings. PAL was extracted, and its activity assayed as described by Ferrarese et al. (2000), being expressed as μmol *t*-cinnamate h^{-1} g^{-1} fresh weight. Tyrosine ammonia-lyase (TAL) was extracted as described by Khan et al. (2003), with modifications. One gram of fresh roots was ground in 50 mM Tris-HCl buffer (pH 8.5) at 4 °C. The homogenate was then centrifuged (2200 × *g*, 10 min), and the resulting supernatant was used as the enzyme preparation. For the TAL activity assay, the reaction mixture, consisting of 100 μmoles Tris-HCl buffer, pH 8.0, and a suitable amount of enzyme extract, in a final volume of 0.95 mL, was incubated at 40 °C for 5 min. L-Tyrosine (5.5 μmoles) was added to start the reaction, which was stopped after 1 h of incubation by the addition of 50 μL 5 M HCl. Samples were then filtered through a 0.45-μm disposable syringe filter (Hamilton® Co., Reno, NV, USA) and 20 μL aliquots were analyzed in a 10AVP high-performance liquid chromatography (HPLC) system (Shimadzu®, Tokyo, Japan) equipped with a LC-10AD pump, a Rheodyne® injector, an SPD-10A UV detector, a CBM-101 communications bus module, and a Class-CR10 workstation system. A reversed-phase Shimpack® CLC-ODS column (150 mm × 4.6 mm, 5 μm), protected with an equivalent pre-column, was used at 30 °C. The mobile phase was methanol:4% acetic acid (30%:70%) with a flow rate of 1.0 mL min^{-1} for an isocratic run of 15 min, and UV detection was carried out at 320 nm. *p*-Coumarate, the product of the TAL reaction, was identified by comparing its retention time with a standard compound. TAL activity was expressed as μmol *p*-coumarate h^{-1} g^{-1} fresh weight.

C4H was extracted as described by Myton and Fry (1995) and Krochko et al. (1998), with modifications. Two grams of fresh roots were ground at 4 °C in an extraction medium containing 100 mM phosphate buffer (pH 7.2), 1.0 mM dithiothreitol (DTT), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.4 mM sucrose and 0.5% bovine albumin. The homogenate was centrifuged at 20000 × *g* for 20 min, and the resulting supernatant was centrifuged again at 100000 × *g* for 60 min. The microssomal pellet was then resuspended in 0.5 mL of extraction medium containing 100 mM phosphate buffer (pH 7.2), 1.0 mM DTT and 1.0 mM EDTA, and was used as the enzyme preparation. For the C4H activity assay, the reaction mixture contained 100 mM phosphate buffer (pH 7.6), 0.2 mM *t*-cinnamic acid, 1.0 mM nicotinamide adenine dinucleotide phosphate (NADPH) and a suitable amount of enzyme extract in a final volume of 1.0 mL. The mixture was incubated at 36 °C, and the reaction was stopped after 20 min by the addition of 50 μL 5 M HCl. Samples were passed through a 0.45-μm disposable syringe filter (Hamilton® Co, Reno, NV, USA) and 20 μL of the filtrate was analyzed (by HPLC, as described above. A reversed-phase Shimpack® CLC-ODS column (150 mm × 4.6 mm, 5 μm) was used at room temperature, together with an equivalent pre-column. The mobile phase was 70% methanol (v/v), with a flow rate of 1.0 mL min^{-1} for an isocratic run of 15 min. UV detection was carried out at 309 nm. *p*-Coumarate, the product of the C4H reaction, was identified by comparing its retention time with a standard compound. C4H activity was expressed as nmol *p*-coumarate formed h^{-1} mg^{-1} protein. The protein concentration in the enzyme extract was determined according to Bradford (1976), using bovine-serum albumin as a standard.

4CL was extracted as described by Meng and Campbell (1997), with modifications. Two grams of fresh roots were ground in an extraction medium containing 200 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT and 20% glycerol (v/v). The extract was centrifuged (10000 × *g*, 20 min, 4 °C), and the resulting supernatant was used for enzyme assays. For the 4CL activity assay, the reaction mixture contained 4 mM ATP, 0.2 mM coenzyme A (CoA) and 0.4 mM *p*-coumaric acid, and a suitable amount of enzyme extract, for a final volume of 1.0 mL. The mixture was incubated at 35 °C, with the reaction being started by adding CoA. The production of *p*-coumaroil-CoA was followed spectrophotometrically at 333 nm for 10 min, against a blank without CoA and ATP. 4CL activity was expressed as μmol *p*-coumaroil-CoA formed h^{-1} mg^{-1} fresh weight.

COMT was extracted as described by Inoue et al. (1998), with modifications. Two grams of fresh roots were ground in an extraction medium containing 100 mM Tris/HCl (pH 7.2), 0.2 mM $MgCl_2$, 2 mM DTT and 10% glycerol (v/v). The extract was centrifuged (10000 × *g*, 10 min, 4 °C), with the resulting supernatant being collected and used as the enzyme preparation. For the COMT activity assay, the reaction mixture contained 100 mM Tris/HCl (pH 7.2), 0.2 mM $MgCl_2$, 2 mM DTT, 0.3 mM *S*-adenosylL-methionine (SAM) and 0.2 mL of enzyme extract. The mixture was incubated at 30 °C, with the reaction being initiated by the addition of 50 μM caffeic acid, in a final volume of 1 mL. The reaction was stopped after 30 min by the addition of 50 μL 3 M HCl. The samples were then passed through a 0.45-μm disposable syringe filter (Hamilton® Co, Reno, NV, USA), and 20 μL of the filtrate was analyzed on a 10AVP HPLC system (Shimadzu®, Tokyo, Japan) equipped with a LC-20AT pump, SIL-20A auto injector, SPD-M20A photodiode array detector (PDA), a CBM-20A communication module and DGU-20A5 degasser. A reversed-phase Shimpack® CLC-ODS (M) column (250 × 4.6 mm, 5 μm) was used at 40 °C together with an equivalent pre-column (10 × 4.6 mm). The mobile phase was methanol: 4% acetic acid (30:70, v/v) with a flow rate of 1.0 mL min^{-1}, for an isocratic run of 25 min. UV detection was carried out at 322 nm. Ferulic acid, the product of the COMT reaction, was identified by comparing its retention time with that of a standard compound. Parallel controls were made to eliminate any endogenous ferulic acid in the samples. COMT activity was expressed as μmol ferulate h^{-1} g^{-1} fresh weight.

CAD was extracted and its activity assayed as previously described (dos Santos et al., 2006). CAD activity was assayed by HPLC following the reduction of sinapaldehyde to sinapyl alcohol. CAD activity was expressed as μmol sinapaldehyde h^{-1} g^{-1} fresh weight.

2.3. Quantification of lignin and monomer composition

Acetyl bromide lignin quantification in roots, stems and leaves was performed as described by Moreira-Vilar et al. (2014). Lignin levels were expressed as mg g^{-1} dry weight.

Alkaline nitrobenzene oxidation was used to determine lignin monomeric composition, following the procedure described in detail by Zanardo et al. (2009). Quantification of the monomeric aldehyde products (*p*-hydroxybenzaldehyde, vanillin and syringaldehyde) released by nitrobenzene oxidation was performed by HPLC, with detection at 290 nm, using the corresponding standards. Results were expressed as μg monomer g^{-1} cell wall.

2.4. Analysis of saccharification

To remove soluble sugars, dry samples (0.2 g) of roots, stems or leaves were ground using a mortar and pestle and placed into 15 mL conical centrifuge tubes containing 8 mL 80% ethanol. The samples were then incubated in an orbital shaker (55 °C, 200 rpm, 4 h). After centrifugation (2200 × *g*, 5 min, 4 °C), the supernatant was discarded and the pellet was resuspended in 80% ethanol. The shaking procedure, centrifugation and resuspension of the pellet were repeated until no total soluble sugar was detected in the supernatant by the phenol-sulfuric acid method of Dubois et al. (1956). The remaining material was then dried in an oven at 60 °C and was considered to be the alcohol insoluble residue (AIR).

Before alkaline pretreatment, saccharification was assayed (Gonçalves et al., 2012; Delabona et al., 2013) with some modifications. Dry material (0.015 g) was transferred to a 2-mL screw capped Eppendorf tube, and resuspended with 0.9 mL 50 mM sodium acetate buffer (pH 5.0). After addition of 20 U mL^{-1} of an *Aspergillus niveus* xylanase-rich lignocellulolytic enzymes cocktail, the samples were incubated in a dry bath at 50 °C for 24 h. An enzyme-free experimental control was performed to detect spontaneous release of sugars. After incubation, the samples were centrifuged (2200 × *g*, 5 min), and the supernatant was analyzed for reducing sugars using the dinitrosalicylic acid method (Miller, 1959).

For alkaline pretreatment, 0.2 g AIR was incubated with 8 mL 0.25 mM NaOH in a dry bath at 130 °C for 40 min (Lima et al., 2014). After cooling, the samples were centrifuged (4000 × *g*, 15 min) and the supernatant discarded. To remove residual NaOH in the samples, pellets were washed with 80% ethanol until the pH of the mixtures was 6–7. Lastly, alkaline insoluble residues were dried in an oven at 60 °C. Saccharification was performed as described above. Saccharification was expressed as mg reducing sugars g^{-1} AIR.

2.5. Statistical analysis

Data were expressed as the means of three or five independent measurements ± the standard error of the mean. One-way analysis of variance (ANOVA) was performed to test the significance of the observed differences using the GraphPad Prism® package version 6.0 (GraphPad Software Inc., San Diego, CA, USA). The differences between the parameters were evaluated using Dunnett's test, and P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Effects of rosmarinic acid after short-term exposure

The fresh and dry weights of maize roots and their lengths were affected by rosmarinic acid, after 24 h of cultivation (Table 1). In comparison to the controls, root length decreased by 22.8% and 24.8% in plants treated with 0.75 and 1.5 mM rosmarinic acid, respectively. By contrast, fresh and dry weights increased by 14.5% and 21.4% after 1.5 mM rosmarinic acid exposure.

Rosmarinic acid-affected enzyme activities compared to the respective controls (Fig. 2). Roots exposed to 0.25, 0.75 and 1.5 mM rosmarinic acid had significantly decreased PAL activity (18%, 23% and 21%, respectively) and TAL activity (30%, 45% and 70%, respectively). By contrast, C4H activities increased by 58% and 75% in seedlings treated with 0.75 and 1.5 mM rosmarinic acid, respectively. Activities of COMT, 4CL and CAD were not affected by rosmarinic acid treatment.

The contents of total lignin and its monomer G were not affected by rosmarinic acid at any concentration (Fig. 3). However, H monomer content decreased from 32% (0.25 mM treatment) to 49% (1.5 mM treatment), and S monomer decreased 37% with the 1.5 mM treatment, in comparison to the respective controls. Consequently, the sum of monomers H + G + S, a value which could be referred to as equivalent lignin content, was reduced by 27% after 1.5 M treatment. Finally, 1.5 mM rosmarinic acid slightly increased saccharification, by 9% in comparison to the control (Fig. 4).

3.2. Effects of rosmarinic acid after a longer-term exposure

After 14 d of cultivation, fresh and dry weights and lengths of stems and leaves were not affected by any concentration of exogenously applied rosmarinic acid, in comparison to the respective controls (Table 2). We also did not observe changes in PAL and TAL activities under this experimental condition (data not shown); therefore, other enzymes were not tested. In addition, variations in total lignin, monomer composition and the sum of H + G + S in stems were not observed (Fig. 5). As a result, leaves were not assayed.

Saccharification of leaves and stems was not altered by rosmarinic acid before the pretreatment procedure (data not shown). After alkaline pretreatment, saccharification was not changed in leaves (Fig. 6A); however, it was significantly increased in maize stems (Fig. 6B). In fact, 0.25 0.75 and 1.5 mM, rosmarinic acid increased stem saccharification by 60%, 55% and 38%, respectively, in comparison to the control; an average of 51%, regardless of the concentration of rosmarinic acid.

4. Discussion

Currently, a promising plant genetic engineering target to improve biomass saccharification is based on the biomimetic incorporation into the cell wall lignin of alternative monomers, including rosmarinic acid, to facilitate alkaline depolymerization (Grabber et al., 2019). Hypothesizing that rosmarinic acid could induce saccharification without disturbing plant growth, we evaluate the effects of its application on maize. Rosmarinic acid was found to increase saccharification from stems tissues under the longer-term exposure, without affecting growth and lignin formation.

4.1. In a short-term exposure, rosmarinic acid affected maize growth and lignin

Artificially incorporated into the lignin structure (Tobimatsu et al., 2012), rosmarinic acid improved maize cell wall saccharification *in vitro*; however, possible undesirable physiological effects on plant growth were not assessed. Because plant roots are susceptible to many chemical compounds, we monitored the effects of rosmarinic acid on earlier maize growth. Our findings revealed that 0.75–1.5 mM rosmarinic acid reduced maize growth (Table 1), indicating that it was highly phytotoxic, although its mechanism of action was unknown. While rosmarinic acid exhibits a wide spectrum of pharmacological and biological activities (Petersen, 2013), its effect on plant growth have been uniquely reported by Kusano et al. (1998). In the dicots, sesame (*Sesamun indicum*) and mustard (*Brassica rapa*), and monocots, onion (*Allium tuberosum*) and barnyard millet (*Echinochloa utilus*), root growth was strongly inhibited by 0.1–0.3 mM rosmarinic acid.

Deleterious effects of secondary metabolites on plant root growth are related to changes of phenylpropanoid pathway enzymes. In

Table 1
Rooth length and fresh and dry weights of maize seedlings exposed to the rosmarinic acid for 24 h.

Rosmarinic acid (mM)	Root length (cm)	%	Fresh weight (g)	%	Dry weight (g)	%
0	2.50 ± 0.05		2.00 ± 0.03		0.14 ± 0.001	
0.25	2.24 ± 0.07		2.06 ± 0.06		0.15 ± 0.005	
0.75	1.93 ± 0.10[a]	−22.8	1.93 ± 0.07		0.14 ± 0.005	
1.50	1.88 ± 0.08[a]	−24.8	2.29 ± 0.06[a]	+14.5	0.17 ± 0.003[a]	+21.4

[a] Values (n = 5 ± SE) differ statistically (ANOVA with Dunnett's multiple comparison test) from the corresponding control (P ≤ 0.05). The symbol % represents activation (+) or inhibition (−) of statistically significant means in comparison to the respective controls.

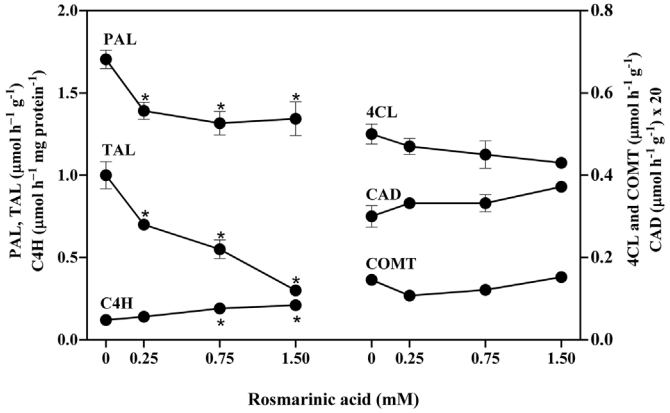

Fig. 2. Activities of phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD), and caffeate 3-*O*-methyltransferase (COMT) in roots of maize seedlings exposed to 0.25–1.5 mM rosmarinic acid for 24 h. Values ± standard error (SEM; n = 4) marked with * are different from the control according to Dunnett's test with P ≤ 0.05.

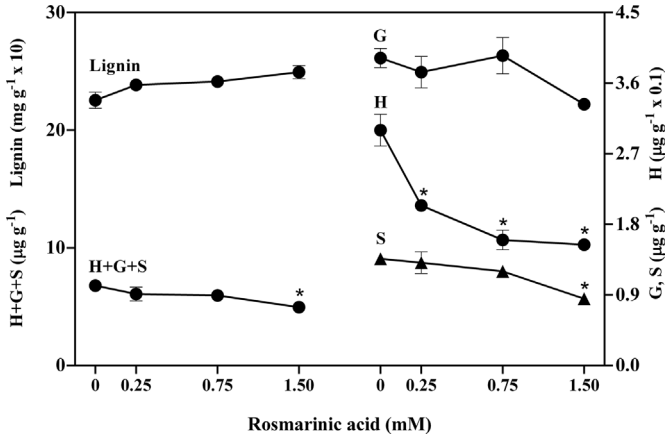

Fig. 3. Lignin content and monomer composition in roots of maize seedlings exposed to 0.25–1.5 mM rosmarinic acid for 24 h. H, *p*-hydroxyphenyl; G, guaiacyl; and S, syringyl monomers. Values ± standard error (SEM; n = 5) marked with * are different from the control according to Dunnett's test with P ≤ 0.05.

monocots, the first steps of the phenylpropanoid pathway are catalyzed by PAL, TAL and C4H resulting in the formation of *p*-coumarate (Boerjan et al., 2003; Vanholme et al., 2019). While L-phenylalanine is converted to *p*-coumarate by two subsequent reactions catalyzed by PAL and C4H (Fig. 1A), TAL is highly selective for L-tyrosine, which is directly converted to *p*-coumarate (Ferrer et al., 2008). In the current study, rosmarinic acid decreased PAL and TAL activities and increased C4H activity (Fig. 2). Low activities of key enzymes, such as PAL and TAL, are associated with changes in plant growth, the lignin content and its monomeric composition (Bubna et al., 2011; Zanardo et al., 2009). A negative effect of 0.75 and 1.5 mM rosmarinic acid on TAL

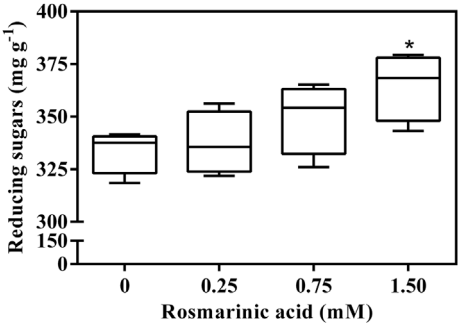

Fig. 4. Saccharification of roots of maize seedlings exposed to 0.25–1.5 mM rosmarinic acid for 24 h. Values ± standard error (SEM; n = 5) marked with * are different from the control according to Dunnett's test with P ≤ 0.05.

Table 2
Lenghts and fresh and dry weights of stems and leaves maize plants exposed to the rosmarinic acid for 14 d.

Rosmarinic acid (mM)	Stem		
	Length (cm)	Fresh weigth (g)	Dry weigth (g)
0	8.86 ± 0.56	1.04 ± 0.70	0.176 ± 0.023
0.25	9.30 ± 0.35	1.05 ± 0.66	0.172 ± 0.009
0.75	9.34 ± 0.24	0.99 ± 0.41	0.172 ± 0.015
1.50	8.84 ± 0.57	0.84 ± 0.95	0.138 ± 0.014
Rosmarinic acid (mM)	Leaf		
	Length (cm)	Fresh weigth (g)	Dry weigth (g)
0	3.01 ± 0.20	1.54 ± 0.11	0.38 ± 0.052
0.25	3.08 ± 0.07	1.74 ± 0.14	0.47 ± 0.038
0.75	3.24 ± 0.06	1.61 ± 0.07	0.40 ± 0.035
1.50	2.95 ± 0.24	1.39 ± 0.16	0.35 ± 0.042

Values (n = 5 ± SE) did not differ statistically (ANOVA with Dunnett's multiple comparison test) from the corresponding control (P ≤ 0.05).

was accompanied by increases in C4H activities (Fig. 2). Because both enzymes produced *p*-coumarate, it was possible that C4H was activated to compensate for low TAL activity, *i.e.*, low production of *p*-coumarate from L-tyrosine could induce C4H. Consistent with this hypothesis, 4CL was not affected by rosmarinic acid (Fig. 2), suggesting that the *p*-coumarate level could be deemed to have been insufficient for the catalytic action of C4H. Additionally, COMT and CAD activities were not affected by rosmarinic acid, and this agreed with the little or no change in the contents of lignin, G and S monomers, and H + G + S (Fig. 3). However, a notable reduction was noted in the content of the H monomer. This was certainly related to the decrease in PAL and TAL activities, since *p*-coumarate is an intermediate in one of the main paths towards H lignin (Fig. 1A).

Due to cross-linking with hemicellulose and cellulose, lignin interferes in the saccharification step, making access of enzymes to the cell wall polysaccharides difficult and inactivating hydrolytic enzymes by absorption (Guo et al., 2014; Souza et al., 2018). Exogenous application of 1.5 mM rosmarinic acid slightly increased saccharification from

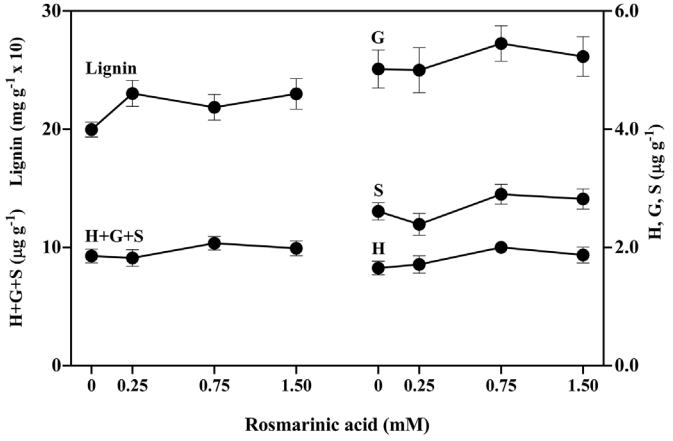

Fig. 5. Lignin content and monomer composition in stems of maize seedlings exposed to 0.25–1.5 mM rosmarinic acid for 14 d. H, *p*-hydroxyphenyl; G, guaiacyl; and S, syringyl monomers. Values ± standard error (SEM; n = 5) marked with * are different from the control according to Dunnett's test with $P \leq 0.05$.

maize roots (Fig. 4). Although total lignin content was not altered by 1.5 mM rosmarinic acid (Fig. 3), the increase in saccharification was consistent with the reduction in H + G + S, the equivalent lignin content. This observation was also in accordance with the widely held concept that the level of lignin needs to be reduced for efficient saccharification (Grabber et al., 2019). Specifically for rosmarinic acid, this is related to its incorporation into the polymer backbone. Even so, our findings need to be taken with some caution, as high concentrations of rosmarinic acid seemed to be phytotoxic for maize roots (Table 1).

In summary, rosmarinic acid decreased maize root growth, inhibited the first enzymes, PAL and TAL, of the phenylpropanoid pathway, altered lignin composition and slightly increased saccharification (only at 1.5 mM). These results suggested in the early stages of growth, at least, rosmarinic acid caused deleterious effects on maize roots.

4.2. In a longer-term exposure, rosmarinic acid did not affect maize growth and lignin, but increased saccharification

Lignin has a structural role in water transport, plant sustainability, and recalcitrance against pathogens and herbivores. Efforts to modify lignin structure and its monomeric composition without disturbing plant growth have included the use of tissue-specific promoters, genome editing, transgene regulation, and the redesign of lignins by the incorporation of novel monomers into the polymer, to facilitate its degradation (Eudes et al., 2014; Mottiar et al., 2016; Umezawa, 2018). Rosmarinic acid is a good candidate for redesigning lignin, since it presents an ester linkage prone to alkali hydrolysis. As such, sufficient incorporation of rosmarinic acid throughout lignin structure could make it easier to extract this polymer by saponification pretreatment. At least under the experimental conditions employed in this current study, treatment with rosmarinic acid did not interfere with maize growth (Table 2), lignin content and its monomeric composition (Fig. 5).

The efficiency of sugars release from plant biomass by hydrolytic enzymes is typically a measure of lignocellulose recalcitrance, which is partially due to polysaccharide complexity and the presence of lignin. Several studies have shown that lignin content and changes in its monomeric composition are negatively correlated with saccharification (Berthet et al., 2011; Goff et al., 2012; Van Acker et al., 2013; Zhang et al., 2013). Therefore, to increase the release of sugars from cell wall polysaccharides, the saccharification process is preceded by a standard alkaline pretreatment, as applied in the current study. Altogether, our results revealed that treatments with rosmarinic acid induced substantial increases in saccharification after alkali pretreatment of maize stems (Fig. 6B), with no changes in growth, lignin and its monomeric composition (Table 2, Fig. 5). Our findings of an improved sugar yield in combination with the alkaline pretreatment agreed with those for maize cell walls artificially lignified with rosmarinic acid (Tobimatsu et al., 2012).

Using *in vitro* peroxidase-catalyzed reactions, Tobimatsu et al. (2012) verified that rosmarinic acid and canonical monolignols generate quinone methide intermediates that are internally trapped, resulting in benzodioxane structures that are easily cleaved. Despite the enhanced saccharification due to the sole action of rosmarinic acid (Fig. 6B), there is no clear relationship between the *in vitro* mechanism of chemical incorporation of alternative monomers into the maize cell wall (Tobimatsu et al., 2012) and our *in planta* studies. While there is an extensive literature on the biosynthesis and biological functions of rosmarinic acid (Petersen, 2013; Petersen et al., 2009; Wang et al., 2019), its metabolization by plant cells has not been thoroughly elucidated. Because rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyl-lactic acid (Fig. 1B), it could be rapidly degraded to provide plant tissues with caffeate, an important precursor of lignin (Fig. 1A). Time course studies and the use of labeled rosmarinic acid revealed that it was not degraded, and no trace of the label was observed in lignin (Ellis and Towers, 1970). However, considering that caffeate is a moiety of rosmarinic acid, and given the plasticity of lignin (Grabber et al., 2019; Mottiar et al., 2016), direct esterification of rosmarinic acid into the polymer cannot be ruled out; however, this still requires evidence. If this was the case, and rosmarinic acid acted like a "weak brick" in the lignin polymer, it would explain the easier disassembling and removing of lignin by simple saponification, followed by increased saccharification (Fig. 6B). Clearly, the mechanism of action of exogenous rosmarinic acid, explaining its effect on saccharification, remains an open question.

5. Conclusions

This study indicated that on short-term exposure, rosmarinic acid decreased maize root growth, but did not affect total lignin content; however, it did alter monomeric composition and the enzyme activities of the phenylpropanoid pathway directed towards lignin biosynthesis.

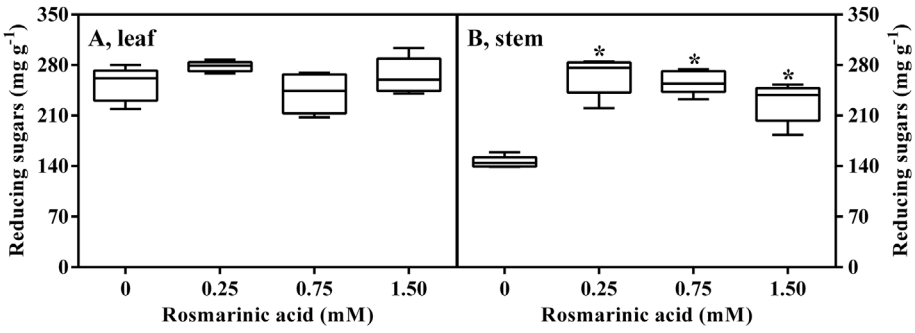

Fig. 6. Saccharification of leaves and stems of maize plants exposed to 0.25–1.5 mM rosmarinic acid for 14 d. A and B, saccharification before alkaline pretreatment; C and D, saccharification after alkaline pretreatment. Values ± standard error (SEM; n = 5) marked with * are different from the control according to Dunnett's test with $P \leq 0.05$.

These findings suggested potential phytotoxicity of rosmarinic acid on maize roots at an early stage of growth. Under long-term experimental conditions, enhanced saccharification was observed from maize stem tissues without any deleterious effects on plant growth, and no apparent disturbance of the phenylpropanoid pathway and lignin formation. These findings suggested that exogenous application of rosmarinic acid on maize plants had the potential to improve their saccharification. Nevertheless, experimental validation under field conditions is an important step in determining whether the increased saccharification from rosmarinic acid treatment could be reproduced on an agronomical scale, without negatively affecting productivity. Such studies would provide additional information in developing an agrochemical to induce saccharification, as well as data for lignin bioengineering approaches.

Conflicts of interest

The authors declare no conflicts of interest.

Authors contribution

OFF, RM and WDS planned the experiments, analyzed the results and wrote the manuscript. JMB performed all experiments with the help of AFT, APF, AVP and BMJ. DMO made Fig. 1.

Acknowledgements

JMB was a recipient of Coordination of Enhancement of Higher Education Personnel (CAPES) fellowship. RM and OFF are research fellows of National Council for Scientific and Technological Development (CNPq). AFT and APF were postdoctoral fellows of CNPq. AVP was a doctoral fellow of CAPES.

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