

Flooding tolerance and cell wall alterations in maize mesocotyl during hypoxia⁽¹⁾

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Abstract – This research aimed to characterize the tolerance to flooding and alterations in pectic and hemicellulose fractions from mesocotyl of maize tolerant to flooding when submitted to hypoxia. In order to characterize tolerance seeds from maize cultivars Saracura BRS-4154 and BR 107 tolerant and sensitive to low oxygen levels, respectively, were set to germinate. Plantlet survival was evaluated during five days after having been submitted to hypoxia. After fractionation with ammonium oxalate 0.5% (w/v) and KOH 2M and 4M, Saracura BRS-4154 cell wall was obtained from mesocotyl segments with different damage intensities caused by oxygen deficiency exposure. The cell wall fractions were analyzed by gel filtration and gas chromatography, and also by Infrared Spectrum with Fourier Transformation (FTIR). The hypoxia period lasting three days or longer caused cell lysis and in advanced stages plant death. The gelic profile from pectic, hemicellulose 2M and 4M fractions from samples with translucent and constriction zone showed the appearance of low molecular weight compounds, similar to glucose. The main neutral sugars in pectic and hemicellulose fractions were arabinose, xilose and mannose. The FTIR spectrum showed a gradual decrease in pectic substances from mesocotyl with normal to translucent and constriction appearance respectively.

Index terms: pectic substances, hemicellulose.

Tolerância ao alagamento e alterações de parede celular em mesocótilo de milho durante hipoxia

Resumo – Objetivou-se caracterizar a tolerância ao alagamento e alterações nas frações pécicas e hemicelulósicas de mesocótilos de milho submetidos à hipoxia. Sementes de milho cultivar Saracura BRS-4154 e BR 107, tolerante e sensível à hipoxia, respectivamente, foram submetidas à germinação e a sobrevivência das plântulas em condições de hipoxia, foi avaliada durante cinco dias. O material de parede celular, obtido de segmentos de mesocótilos da cultivar Saracura BRS-4154 com diferentes intensidades de danos causados por hipoxia, foi submetido a fracionamento com oxalato de amônio 0,5% e KOH 2M e 4M. As frações de parede celular obtidas foram analisadas por cromatografia em gel, cromatografia gasosa e espectro de infravermelho com transformação dos dados pela série de Fourier (FTIR). Períodos de hipoxia superiores a três dias causaram a lise celular (aparência translúcida), e, em estádios mais avançados, a morte das plantas. O perfil géllico das frações pécicas, hemicelulose 2M e 4M das amostras de mesocótilos translúcidos e com constrição apresentaram compostos de baixos pesos moleculares semelhantes à glicose. Os principais açúcares neutros nas frações pécicas e hemicelulósicas foram arabinose, xilose e manose. O espectro de FTIR mostrou um decréscimo gradual nas substâncias pécicas do mesocótilo com aparência normal, para translúcido e constrição respectivamente.

Termos para indexação: substâncias pécicas, hemicelulose.

⁽¹⁾Accepted for publication on September 20, 2000.

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Introduction

In Brazil, there are approximately 28 million hectares of swamp land with great agricultural potential. The low oxygen level in this environment has decreased the productivity in this type of area and consequently is a limiting factor for the maize crop expansion (Silva, 1986).

These areas, which have high fertility, are submitted to intermittent periods of soil flooding by pluvial system or inundation of the waterway by lack of drainage (Lopes et al., 1988). In this environment, where the diffusive resistance of most gases is 10,000 higher than the air, the plant and microorganism respiration using the remaining oxygen create a hypoxic and anoxic environment highly unfavorable to crop growth (Armstrong et al., 1994).

One of the problems with maize cultivated in soils with intermittent period of water excess is that this crop is sensitive to flooding conditions due to the lack of resistance mechanisms (Drew, 1997). In Brazil, the Embrapa-Centro Nacional de Pesquisa de Milho e Sorgo (CNPMS) developed the "Saracura BRS-4154", which is a maize variety with a broad genetic basis, obtained after nine cycles of massal selection under high moisture conditions in the soil. This genetic material showed to be one of the best to be used in areas susceptible to flooding. The commercial production of this variety in areas submitted to flooding started in the summer of 1997/98. Although this variety has been recommended to flooding areas, the mechanism that confers the high degree of tolerance to oxygen deficient level in the soil haven't been studied yet.

Saracura BRS-4154 has similar tolerance aspects that were observed in BH73Ht, like the softening in mesocotyl region when submitted to prolonged hypoxia (Saab & Sachs, 1996). This alteration appeared earlier and was more pronounced in sensitive variety BR 107 (Vitorino, 1999). After three days of hypoxia these changes in tissue consistency and appearance became irreversible culminating with plant death. Probably the cause of this loosening could be due to cell wall alterations like in fruit ripening (Huysamer et al., 1997). Although the characterization of maize cell wall has been studied (Kim & Carpita, 1992; Inouhe & Nevins, 1997), the direct effect of hypoxia on cell wall composition remains unknown.

The aims of this study were to characterize the flooding tolerance and some cell wall fractions alterations from a mesocotyl region of maize cultivar Saracura BRS-4154 submitted to hypoxia.

Material and Methods

Characterization of tolerance

Maize caryopsis from Saracura BRS-4154 and BR 107 cultivars tolerant and susceptible, respectively, to oxygen deficiency were washed with 0.5% clorox (v/v) by 10 min, then put to germinate in rolled paper previously treated with 0.5% Captan during four days, at 27°C in the dark. After this, four roller papers, containing 25 plantlets each cultivar, were fully submerged, in a vertical position, in a recipient with 1 L of flooding 5 mM Tris HCl buffer, pH 8.0, 100 mg/L ampicillin according to Saab & Sachs (1996). After N₂ (1 L/min) bubbling during three min in order to obtain a low oxygen atmosphere (0.1% O₂), the recipients were sealed and stored at 28°C in the dark, for different periods of time up to five days. Four roller paper containing 25 plantlets stayed in germinator as a control. After one day intervals, the plantlets were transferred to boxes with vermiculite and maintained at 28°C, with relative humidity at least 85% under 16 hours photoperiod. The survival was evaluated by the appearance, greening of leaves and plantlet dry matter after five days related to control. The experimental design was in random blocks with four replicates from eight rolls each one.

Cell wall analysis

Mesocotyl segments of Saracura BRS-4154 with distinct appearance and consistency (normal, translucent and constriction) obtained from proximal (basal) and distal (apical) caryopsis region were separated, frozen in liquid N₂ and kept at -20°C until further analysis.

The cell wall was extracted according to Mitchan & McDonald (1992) with some modifications. Around 15 g from each mesocotyl region were triturated with 15 mL of 0.1M phosphate buffer, pH 7.5 containing 0.02% sodium azide, 1% dodecil sodium sulphate and 1% 2 mercaptoethanol (w/w) and kept for two hours at 40°C. After homogenization, filtration and washing, the residue containing the cell wall material (CWM) was submitted to KI/I₂ test (Johansen, 1940) to verify the starch presence. The mixture was resuspended in 50 mL of 1:1 (v/v) chloroform methanol, vacuum filtered and washed with acetone, dried at room temperature, resulting in isolated CWM.

For the pectic substances extraction, 25 mL of 0.5% ammonium oxalate was added in CWM and kept during one hour in water-bath at 60°C under agitation. After centrifugation at 2,000 g by 20 min, the residue was extracted two more times. The combined supernatant was dialyzed during 24 hours, followed by lyophilization to obtain finally the cell wall pectic fraction.

In the precipitate resulting from oxalate extraction 40 mL of 2M KOH were added, staying this mixture in nitrogen atmosphere containing 100 mM NaBH₄ for 12 hours. After vacuum filtration, the pH was adjusted to 7.0 in ice bath, followed by dialysis during 24 hours, lyophilization for 72 hours, originating the 2M hemicellulose fraction. The filtration remaining residue was submitted to 4M KOH solution, in order to obtain the fraction 4M hemicellulose.

Sephadex G-200 chromatography

A Sephadex G-200 column (72 X 1.8 cm) was previously calibrated with dextrose (MW = 70,000) and glucose (MW = 180.6), with 23 mL/h flux. The blue dextran (2,000 kDa) was used to calculate void volume (V₀). For sample application, 3 mg of lyophilized cell wall fraction was resuspended in 2 mL of 0.1M sodium phosphate buffer, pH 7.5. In a fraction collector Bio Rad 2110 model each 2 mL aliquot was collected and the pectin was analyzed by carbazol colorimetric method (Bitter & Muir, 1962). After each sample application the column was washed with buffer at least two times its total volume.

Gas chromatography

For neutral sugars determination, previously isolated fractions from pectic substance, hemicellulose 2M and 4M were derived according to Albersheim et al. (1967). After the hydrolysis with trifluoroacetic acid at 121°C, the samples were reduced with a mixture of 1N ammonium hydroxide and 10 mg/mL sodium borohydride. The acetylation was made during three hours at 121°C using acetic acid anhydrous. The derived samples were diluted in 200 mL of acetone and injected in a gas chromatograph Varian (series 3000) model 3300 with an integrator Intralab model 4290. A 1 g/L sugar standard mixture, containing rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and mioinositol (internal) was used. The capillary column OV-DB 225 with 25 mm diameter and 25 m of length was the stationary phase, and as mobile phase, nitrogen and hydrogen. The sensitivity and alternation were 10⁻¹¹ and 8, respectively. The pressure and flux used were 21 psi, 3.0 mL/min and the burst gas 30 mL/min. The column temperature was 210°C, injector, 250°C and detector, 300°C.

Infrared spectrum with Fourier transformation

The mesocotyl segments from basal and apical region and with appearance normal, translucent, and with constriction were frozen, lyophilized and triturated. Around 10 mg from each sample were mixed with 100 mg of KBr. After

trituration and homogenization, a reading was made, in infrared range to obtain the infrared spectrum with Fourier Transformation (FTIR). The qualitative interpretation for different carbohydrate groups was realized according to McCann et al. (1997).

Results and Discussion

Analysing the results from low oxygen availability in the environment, it was observed that Saracura BRS-4154 showed survival percentage levels of 15, 43 and 42% higher than BR 107 after 1, 2 and 3 days of hypoxia, respectively (Figure 1). After four days, the BR 107 plantlets showed their sensitivity to low oxygen levels, with the survival percentage near to zero. In the same period, the Saracura survival level was 80%. In relation to BR 107 variety, the Saracura showed a high tolerance to hypoxia. Another group of researchers (Lemke-Keyes & Sachs, 1989), working with anaerobic stress in maize, observed that B73Ht showed better performance, reaching survival levels of 61 and 14%, three and four days after anaerobic stress, respectively. For the same period, under oxygen deficiency in the environment, the Saracura survival percentages were 85 and 73% and after five days of hypoxia, this variety still had a 30% survival rate (Figure 1). This value was twice higher than the American variety B73Ht (Lemke-Keyes & Sachs, 1989).

In this research, plantlets that could not survive post-hypoxia, showed, during this stress, in the mesocotyl region, a translucent aspect, with apparent cell lysis (Figure 2). During the stress, the plant-

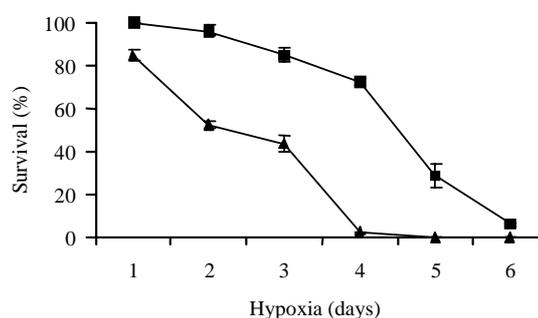


Figure 1. Maize plantlets survival of Saracura BRS-4154 (■) and BR 107 (▲) cultivars submitted to hypoxia up to five days.

let became fully soft, appearing the translucent region with strong constriction, responsible for the falling off and plants death. These results indicate that the formation of this translucent region is the first signal to indicate the irreversibility of the damages caused by the oxygen deficit. The BR 107 variety when compared with the Saracura BRS-4154 showed early formation of this phenomenon.

The gel filtration on Sephadex G-200, from pectic substances, hemicellulose 2M and 4M fractions isolated from apical and basal mesocotyl segments with normal appearance, translucent, and constriction zone suggest that oxygen stress evolution caused cell wall degradation. It is possible to note in pectic substances from mesocotyl apical sections with translucent and constriction zones, the appearance of a peak around fraction 31 and another related to sugars with low molecular weight since fraction 61 is not present in samples from normal tissue (Figure 3A). The appearance of low molecular weight polymers, present in various fractions from mesocotyl basal region, affected by low oxygen concentration in the buffer, resulted in cell wall degradation in that region (Figure 3B).

In relation to profile obtained from hemicellulose 2M extracted from constriction and translucent zones

from apical and basal portion (Figure 3C and 3D), it is possible to observe some intermediary molecular weight polymers close to fractions 25 and 35, and the presence of low molecular weight polymers around fraction 55, suggesting a cell wall degradation absent in normal tissue. Similar results were observed in hemicellulose 4M fractions in apical and basal portion (Figure 3E and 3F).

In the same way, the samples from mesocotyl with translucent and constriction appearance, from pectic substances, hemicellulose 2M and 4M fractions, showed higher absorbance values than the normal tissue (Figure 3). This fact suggest that cell wall degradation in affected tissue by oxygen deficiency, exposed the polymers extremities to attack of chromogen agent, according to damage intensity (normal < translucent < constriction). This sequence of damage intensity was probably caused by coordinated action of cell wall hydrolases (Peschke & Sachs, 1994; Saab & Sachs, 1996) like cellulase, pectin methylesterases, and xiloglucanendotransglycosylase (Fisher & Bennett, 1991; Fry, 1995) that act depolymerizing its components, originating compound with low molecular weight.

Recent studies of cell wall enzymes modifications associated with oxygen deficit in maize showed an



Figure 2. Effect of hypoxia duration on the normal (0 day), translucent (3 days) and constriction (5 days) appearances of maize Saracura BRS-4154 mesocotyl segments.

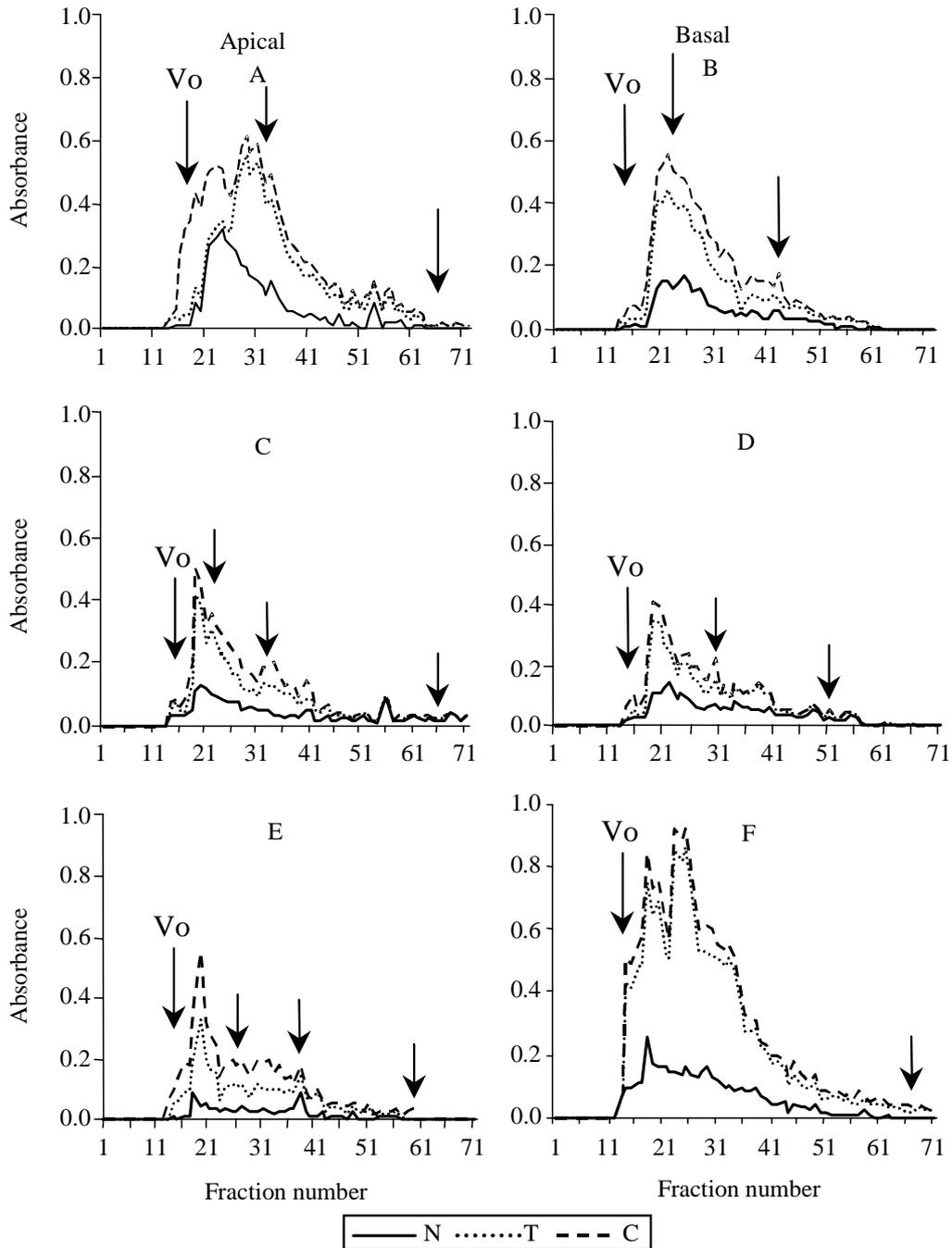


Figure 3. Gelic profile of pectic substances (A and B), hemicellulose 2M fraction (C and D) and hemicellulose 4M fraction (E and F) from apical and basal portion of Saracura BRS-4154 maize mesocotyl segments with normal (N), translucid (T) and constriction (C) appearances caused by hypoxia.

expressive increase in cellulase activity from the second day until the fifth day when constriction zone appeared (Dantas, 1999). These results suggest that with the increase of hypoxia, the cellulase activity cause the appearance of translucent and constriction zone in the mesocotyl tissue. In the same way, He et al. (1994) observed a gradual increment in cellulase activity in maize under hypoxia concomitant with stress, which decreased only when the cell wall was disassembled.

The neutral sugar composition in pectic fraction showed a predominancy of xylose, arabinose and mannose. In apical portion of mesocotyl, the concentration of these sugars decrease during stress development (Table 1). Possibly, after initial stages of cell wall degradation by oxygen stress, these sugars could be translocated to mesocotyl basal region, and this fact can be confirmed by high sugar level in translucent tissue.

In relation to hemicellulose 2M fraction, the arabinose, xylose and mannose predominance was ob-

served in the three tissue types in addition to glucose and fucose (Table 1). The presence of these low molecular weight sugars, was also showed by gelic chromatography for apical hemicellulose 2M fraction, where it is possible to visualize peaks around fractions 70. For hemicellulose 4M, fraction mainly the xylose, fucose, arabinose and mannose were observed.

Following the stress development, it is possible to observe in basal portion (Table 1), an increase in xylose, rhamnose and fucose. The rhamnose presence, that is a pectic component, in hemicellulose 4M fraction, basal portion, can be justified by the fact that some pectins are covalently bound, that difficult its remotion sometimes using a strong alkaline basis like KOH 2M (Gross, 1984). The FTIR profile showed a peak from 1,600 to 1,440 waves cm^{-1} corresponding to pectic substances (Perlin & Casu, 1995) that disappeared almost totally in constriction fraction (Figure 4E and 4F) when compared to normal (Figure 4A and 4B) and translucent (Figure 4C and 4D). These results suggest that oxygen deficiency

Table 1. Saracura BRS-4154 neutral sugar composition (%) associated with pectic substances, hemicellulose 2M fraction and hemicellulose 4M fraction from apical and basal maize mesocotyl segments with normal, translucent and constriction appearances caused by hypoxia.

Mesocotyl region	Mesocotyl appearance	Rhamnose	Fucose	Arabinose	Xilose	Manose	Galactose	Glucose
Pectic substances								
Apical	Normal	0.00	0.00	0.74	1.21	0.43	0.00	0.00
	Translucid	0.00	0.00	0.64	0.88	0.25	0.00	0.00
	Constriction	0.00	0.00	0.62	1.03	0.41	0.00	0.00
Basal	Normal	0.00	0.00	0.65	0.99	0.27	0.00	0.00
	Translucid	0.00	0.00	0.29	0.51	0.53	0.00	0.00
	Constriction	0.00	0.00	0.22	0.58	0.32	0.00	0.00
Hemicelulose 2M								
Apical	Normal	0.00	0.00	1.28	3.69	0.31	0.00	0.00
	Translucid	0.00	0.35	0.71	5.45	0.40	0.00	0.28
	Constriction	0.20	0.44	0.93	6.87	0.51	0.00	0.36
Basal	Normal	0.00	0.44	2.28	11.56	1.00	0.00	0.00
	Translucid	0.00	0.00	1.18	5.67	3.36	0.00	0.00
	Constriction	0.30	0.60	0.54	7.71	2.78	0.00	0.00
Hemicelulose 4M								
Apical	Normal	0.32	0.84	0.83	16.16	0.23	0.00	0.00
	Translucid	1.10	2.55	0.79	10.79	0.37	0.00	0.00
	Constriction	0.79	1.79	0.78	15.71	1.29	0.00	0.33
Basal	Normal	1.16	2.70	0.93	12.25	0.86	0.00	0.00
	Translucid	0.72	1.20	0.96	12.71	0.78	0.00	0.33
	Constriction	1.03	2.54	0.55	20.01	0.36	0.00	0.00

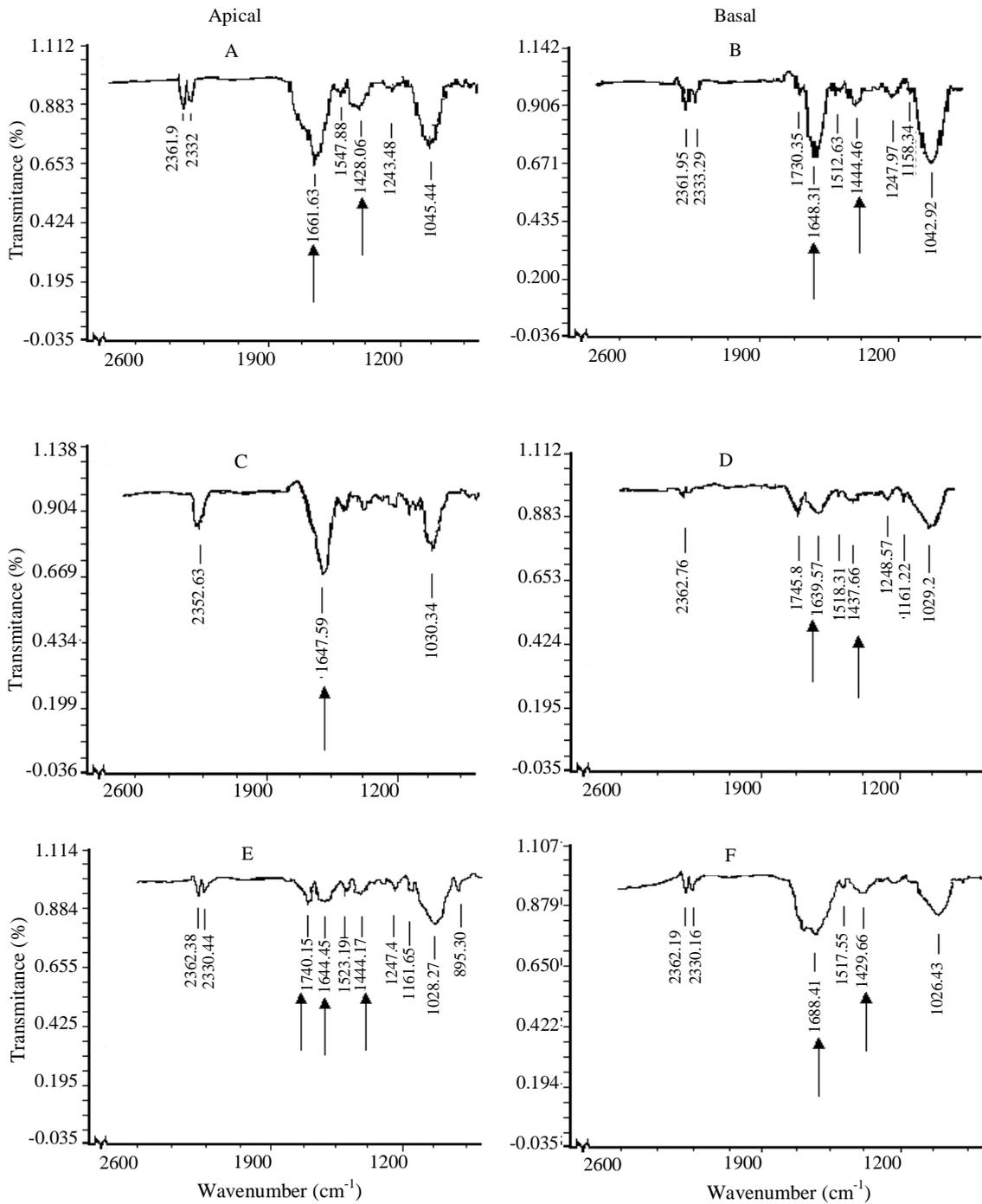


Figure 4. Infrared spectrum with Fourier transformation (FTIR) of apical and basal portions of Saracura BRS-4154 maize mesocotyl with normal (A and B), translucent (C and D) and constriction (E and F) appearances caused by hypoxia.

could induce cell wall hydrolase causing modifications in cell wall composition, confirmed by gelic, gaseous chromatography and FTIR.

The appearance of methyl esters in mesocotyl with constriction (Figure 4E) based by the peak presence in 1,740 waves cm^{-1} (Perlin & Casu, 1995) was detected, fact that did not occur when mesocotyl from normal tissue (Figure 4A) and translucid (Figure 4C) were analyzed. This fact was also noted by McCann et al. (1997), that studied tobacco cell walls during cellular division and expansion, and the methyl esterification of functional groups from carboxylic acid from pectic fraction. This alteration remove the negative charge used for cross linked with calcium. This fact suggests a higher enzyme activity that favours this process, allowing higher flexibility of cell wall. Therefore, the presence of these methyl esters peaks from tissue with constriction zone could be considered as a signal of degradation of cell wall.

Conclusions

1. Maize plantlet cultivar Saracura BRS-4154 has a high tolerance to oxygen deficit, confirming its good field performance under flooding conditions.

2. After four days of hypoxia it is possible to observe an intense lysis in mesocotyl region responsible for the decrease in the plantlet survival rate.

3. Sephadex G-200, gas chromatography and infrared spectrum with Fourier transformation analysis confirm the cell wall degradation during stress evolution.

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