Demonstration of Pectic Polysaccharides in Cork Cell Wall from *Quercus suber* L.

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Scanning electron microscopy (SEM) and chemical analysis were used to observe the cell wall changes that occur in cork with "mancha amarela", when compared to a standard cork. To mimic the microbial attack exhibited in cork with mancha amarela, the standard cork was treated enzymatically with commercial pectinase and hemicellulase preparations. The tissues treated with pectinase were comparable with those attacked with mancha amarela. Both were composed by deformed and wrinkly cells and exhibited cell wall separation at the middle lamella level, which suggests solubilization/removal of the pectic polysaccharides. The cork cell wall material, prepared as alcohol-insoluble residue, was fractionated by hot water ($Pect_{H_2O}$) and hot dilute acid ($Pect_{acid}$). The relatively large amount of hexuronic acid and the occurrence of Ara in the $SPect_{H_2O}$ and $SPect_{acid}$ allow to confirm, as far as we know, for the first time the presence of pectic polysaccharides in the cell walls of cork from *Quercus suber* L. They accounted for ca. 1.5% of the cork and may consist of polymers with long side chains of arabinosyl residues. These polymers have to be taken into account in any realistic model of the cork cell wall. Cork with mancha amarela contained a smaller amount of pectic polysaccharides (ca. 0.5%), which confirms that the cellular separation observed by SEM is related to the degradation/removal of the middle lamella pectic polysaccharides.

Keywords: *Cork; Quercus suber L.; cell wall; pectic polysaccharides; yellow spot; SEM*

INTRODUCTION

The cork is the premium raw material used to produce wine-bottling stoppers. The information on cork cell is crucial for a better understanding of the biochemical changes that occur during cork formation and manipulation. The presence of pectic substances has not been reported in the studies of chemical composition of cork from *Quercus suber* L. (Asencio, 1987a,b; Asencio and Seone, 1987; Pereira, 1988), although it has been shown to occur in lignified tissues, such as *Pinus* (Minor, 1991), mature runner bean pods (Selvendran and King, 1989), and olive seed hulls (Coimbra et al., 1995). Studies by scanning electron microscopy (SEM) carried out on healthy cork and cork attacked by the defect known in the industry as "mancha amarela" (MA) showed that the cellular structures of the infected and the healthy tissues were different and the attacked tissues were composed by deformed and wrinkly cells with cell wall separation at the middle lamella level (Rocha et al., 1996). These changes were related to the degradation of lignin and of pectin, as could be inferred by the deposition of calcium in the intercellular space of the attacked cells (Rocha et al., 1996). Furthermore, microscopic studies carried out on the defected wetcork ("verde") showed the occurrence of empty spaces in the middle lamella, which are considered to be caused apparently by lack of wall substances, especially pectin, lignin, and perhaps phenolics (Parameswaran et al., 1981).

As the cork stopper production, from the forest to the industry, involves a large number of steps susceptible to microbial attack (Moreau, 1978; Lefebvre et al., 1983; Daly et al., 1984), it is important to know in which extension the polysaccharides from the cell wall are affected by the infection, specifically those that may originate the observed degradation of the middle lamella. Microbiological studies carried out on sterilized agar−cork powder media demonstrated that the fungi (recovered from industrial cork slabs) grow better on the media prepared with cork powder from mancha amarela than from standard cork (S. Rocha and I. Delgadillo, unpublished results).

The aim of our paper is to demonstrate the occurrence of pectic polysaccharides in cork cell walls and to compare the pectic polysaccharide composition of standard and fungal-attacked cork. The present study reports the isolation and a partial characterization of the pectic polysaccharides of a standard cork (S) and of a cork with mancha amarela (MA) (which means "yellow spot" and is considered to be caused by the basidiomycete *Armillaria mellea*). To mimic the microbial attack exhibited in MA, the standard cork was treated enzymatically with commercial pectinase and hemicellulase preparations. The cell wall material was prepared as an alcohol-insoluble residue (AIR), which was then fractionated by hot water and hot dilute acid. The preparation of AIR is a relatively easy and quick method for obtaining the cell wall polymers and has been used in previous studies of cell walls (Selvendran and O'Neill, 1987).

MATERIALS AND METHODS

Plant Material. Reproduction cork from *Q. suber* L. was used, from which all dried phelloderm and phloem tissues from the outer surface of the slab were removed. Two kinds of samples were chosen as follows: good quality slabs (low porosity and absence of defects) were labeled S (standard), and slabs which showed pale yellow spots were named MA (mancha amarela).

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10.1021/jf991147g CCC: $19.00
Published on Web 06/02/2000

Enzymatic Treatment. The cork cubes (ca. 2.0 × 2.0 × 2.0 cm) were treated with *Rhizopus* sp. pectinase and *Aspergillus niger* hemicellulase extracts, which contain also cellulase activity. The enzyme extracts were obtained from Sigma Chemical Co. (St. Louis, MO). The conditions of operation were chosen on the basis of the work of Pacchiano et al. (1993). Due to the high impermeability of cork cell walls, the enzyme concentration and reaction time were increased by a factor of 2 for each treatment. For hemicellulase and pectinase treatments, respectively, the following conditions were used: substrate concentration, 120 and 140 mg/mL; enzyme concentration, 80 and 6.0 mg/mL; buffer, 50 mM sodium acetate, pH 5.5 and 4.0; temperature, 45 and 31 °C; reaction time, 204 and 16 h.

SEM and EDS/SEM Analyses. The SEM and EDS/SEM analyses were performed according to the methodology described by Rocha et al. (1996). Strips with ca. 0.8 cm thickness were cut with razor blades, and the surface was coated with a gold film of approximate 200 Å thickness, with the edges parallel to the radial, tangential, and axial directions, in relation to the tree. Similar effects were observed from the three sections. Measurements of cell wall thickness were made directly on the scanning electron microscopy (SEM) photographs.

For calcium-relative estimations, cork samples were examined in a field emission SEM HITACHI S4100, equipped with a EDS (energy dispersive spectroscopy) KEVEX detector with a polymeric window (detect B-U). The EDS/SEM technique presents some limitations due to the thickness of the cork cell walls. Relatively low spatial resolution results from the fact that the region analyzed is larger than the cell wall morphological region. These effects can be ignored because relative comparisons were made with the affected and unaffected regions in the same sample. The peak of gold was used as the peak reference in each sample. EDS analysis in a restricted morphological region was preferred to a point analysis.

Preparation of the Cell Wall Material. Cell wall material was prepared as alcohol-insoluble residue (AIR), as a powder (60 mesh), obtained from standard cork (S) and from the yellow spots dissected out of the affected slabs (MA). The AIR was obtained by immersing the powder (18 g) in boiling 95% ethanol (100 mL) for 1 h, under stirring as adapted from Coimbra et al. (1995). The AIR (4 g) was then extracted with hot water (100 mL) for 1 h at 80 °C, followed by an extraction with nitric acid, 0.02 M (100 mL), for 1 h at 80 °C, to give fractions $Pect_{H_2O}$ and $Pect_{acid}$, respectively. The extracts were concentrated under reduced pressure by rotary evaporation at 37 °C and freeze-dried; $Pect_{acid}$ was previously neutralized and dialyzed before being freeze-dried.

Carbohydrate Analysis. Neutral sugars were released by Saeman hydrolysis (Selvendran et al., 1979) and analyzed as their alditol acetates by GLC according to Blakeney et al. (1983) and Harris et al. (1988). The residues (3−5 mg) were treated with 1 M H_2SO_4 at 100 °C for 2.5 h (after 1 h, 0.5 mL of the hydrolysate was removed for uronic acid determination). The acid hydrolysate was cooled, and an internal standard (2-deoxyglucose, 1 mg/mL, 200 μL) was added. This solution (1 mL) was then neutralized with 200 μL of a solution of 25% NH_3, and the sugars were reduced with 100 μL of 15% $NaBH_4$ in 3 M NH_3 (30 °C, 30 min); the reaction was stopped by two additions of 50 μL of acetic acid. An aliquot (0.3 mL) of this solution was treated with 1-methylimidazole (0.45 mL) and acetic anhydride (3 mL) for 30 min at 30 °C. This solution was then treated with water (3 mL) to decompose the excess acetic anhydride, and the acetylated sugars were extracted with dichloromethane (3−5 mL). The dichloromethane phase was washed three times with water and evaporated to dryness. The alditol acetates were dissolved in dichloromethane (70 μL) and analyzed by GLC using a Hewlett-Packard 5890 equipped with split injector (split ratio 1:60) and FID detector. A 25 m column, CP-Sil-43 CB, with an i.d. of 0.15 μm and 0.20 μm film thickness was used. With the injector and detector operating at 220 °C, the following temperature program was used: 180 °C for 5 min and 200 °C for 20 min, with a rate of 0.5 °C/min. The linear velocity of the carrier gas (H_2) was set at 50 cm/s at 200 °C.

Hexuronic acids were determined colorimetrically by a modification (Coimbra et al., 1996) of the method of Blumenkrantz and Asboe-Hansen (1973). An aliquot of 0.5 mL of hydrolysate previously removed from the neutral sugar determination was filtered through a GF/C filter and diluted four times with water. Then, 0.5 mL of this solution was added to 3 mL of ice-cold concentrated sulfuric acid containing 50 mM boric acid, which was mixed on the vortex and boiled in a water bath for 10 min. After cooling, 100 μL of *m*-phenylphenol (0.15% in 0.5% NaOH) was added, and the solution was allowed to stand in the dark for 30 min to develop the pink-red color; the absorbance was read at 520 nm. Standards were made with galacturonic acid, with a linear correlation from 0 to 40 μg of hexuronic acid in the tube.

RESULTS AND DISCUSSION

SEM and EDS/SEM Analyses. The standard cork shows a regular structure, with closed cells of approximately prismatic form, stacked in rows in the radial direction of the tree, connected base to base, as reported previously (Pereira et al., 1987; Rocha et al., 1996). In the tangential section (Figure 1A) the cork cells are seen as polygons, with five, six, or seven faces, in a "honeycomb"-type arrangement. The cell wall thickness is 1.1−1.4 μm (Figure 1C).

Macroscopically, it may be observed by visual inspection that cork suffering of mancha amarela (MA) presents pale yellow spots. The observation of these spots at the cellular level with the SEM (Figure 1B,D) shows an abrupt transition between the spots and the surrounding unaffected cells. Comparison of Figure 1 panels A with B and C with D shows that the cells of the yellow spots were corrugated and thinner than those of the standard cork. These cells lost their well-defined prismatic form, exhibiting pleats in the cell walls (Figure 1D). Degradation of the cell wall occurs in all of the region of attack. It is also observed that the attack in MA cork occurs randomly through the cork slab, crosses in the radial direction, and is restricted to the particular area showing the spots. By careful selection of the various areas of degradation, it is possible to observe the thinning of the cell wall (average dimensions 0.6−0.9 μm) and cellular separation at the middle lamella level (Figure 1D).

The EDS/SEM analysis shows the occurrence of a significant increase (four to six times higher; see Figure 2A,B) in the amounts of calcium in the region where it was observed, the cellular separation at the middle lamella level, compared to an affected region where there is no rupture of middle lamella or to an unaffected region in the same sample. This could be attributed to a solubilization and removal of pectic polysaccharides since galacturonic acid units have the capacity to bind certain cations such as calcium (Alonso et al., 1995). These results may indicate that the fungi solubilize and remove the middle lamella pectic substances leading to a relative accumulation of calcium in this region. This effect seems to be complementary to the lignin degradation previously reported to occur in the cork affected by mancha amarela (Rocha, 1997). As a consequence of the degradation/removal of the cell wall material, the wrinkle of the cells was observed.

Figure 3 shows the SEM photographs of cork cubes submitted to hemicellulase (panels A and C) and pectinase treatments (panels B and D). The cork cubes treated with hemicellulase extracts, which contain also cellulase activity, become darker brown, and no cellular separation and/or no thinning of the cell walls were

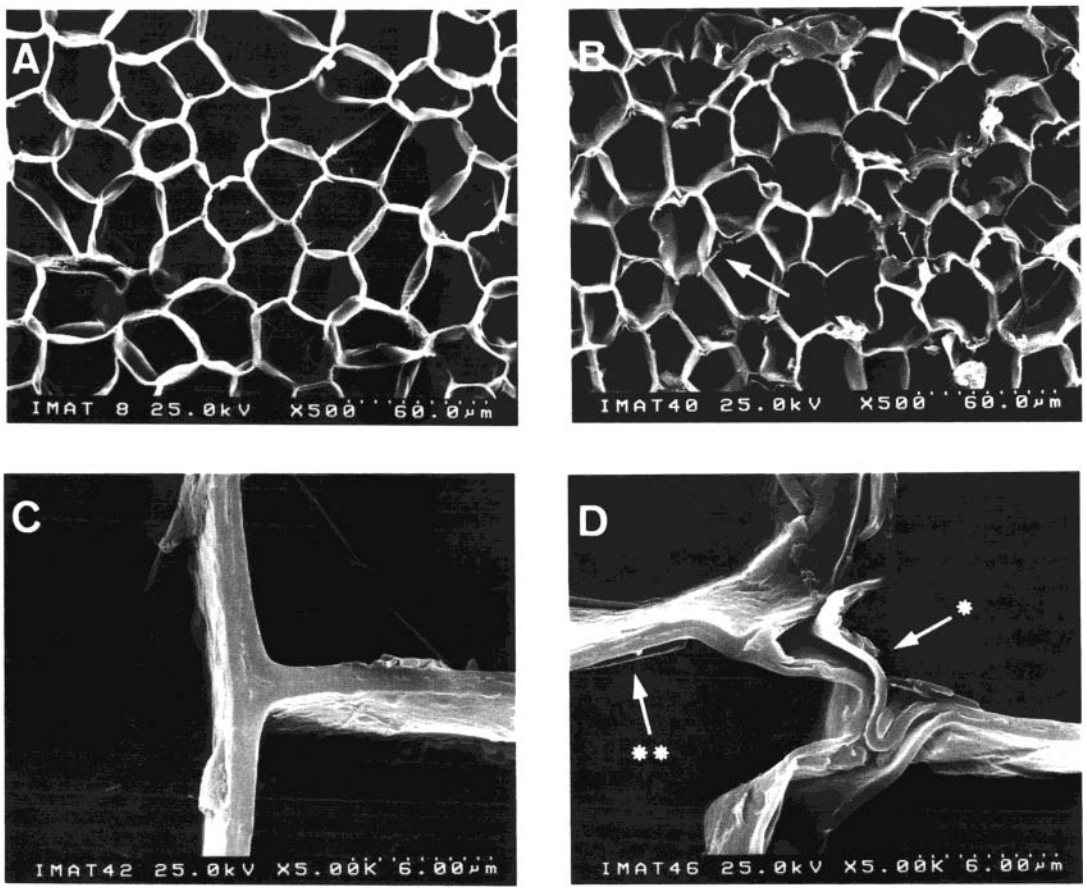

Figure 1. SEM photographs of the tangential section of reproduction cork showing a honeycomb-type arrangement of cells of standard cork (A) and of cork with mancha amarela, showing the cellular separation (B). Cell wall of standard cork (C) and of cork with mancha amarela, showing the cellular separation (*) and thinning of the middle lamella (**) (D).

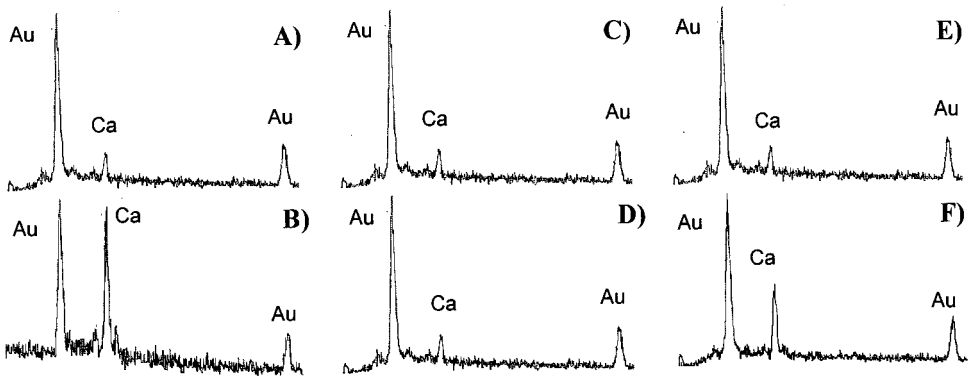

Figure 2. Energy dispersive spectra (EDS) of an unaffected region of cork with mancha amarela (A) and of an attacked region, in the same sample, where the cellular separation at the middle lamella level was observed (B), of an inner, not attacked, region of cork cubes treated with pectinase (C) and of a superficial area of attack, in the same sample (D), and of an inner, not attacked, region of cork cubes treated with hemicellulase (E) and of a superficial area of attack, in the same sample (F).

(was) observed (Figure 3A,C). Although the extent of hydrolysis promoted by the enzymatic treatment seemed to be low, the degradation of the hemicellulosic and cellulosic polysaccharides, even in small amounts, only promotes the cell wall corrugation. The level of lignification of the walls can be a restricting factor to the attack by the enzymes (Ruel, 1984), and this might be the case in this experiment, as cork contains ca. 20% lignin (Pereira, 1988). The EDS/SEM analysis showed that no significant modifications were detected in the calcium level in the attacked regions when compared with the inner, not attacked, regions (see Figure 2C,D).

When the cork cubes were treated with pectinase (Figure 3B,D), the 12−14 superficial cell layers were wrinkled, and some of them collapsed. It is also possible to detect regions where cellular separation occurred, possibly as a result of enzymatic attack to the middle lamella pectic polymers. The EDS/SEM analysis showed an increased concentration of calcium in these regions (four times higher; see Figure 2E,F), when compared to the inner, not attacked, regions. These two facts suggest that the middle lamella pectic polysaccharides were attacked by the pectinase treatment and that these polymers are determinant to the maintenance of the tissue and cell wall integrity. Comparison of Figures 1D and 3D shows that the pectinase treatment and the attack by yellow spot promoted a similar way of degradation of the cork cell wall. This observation allows

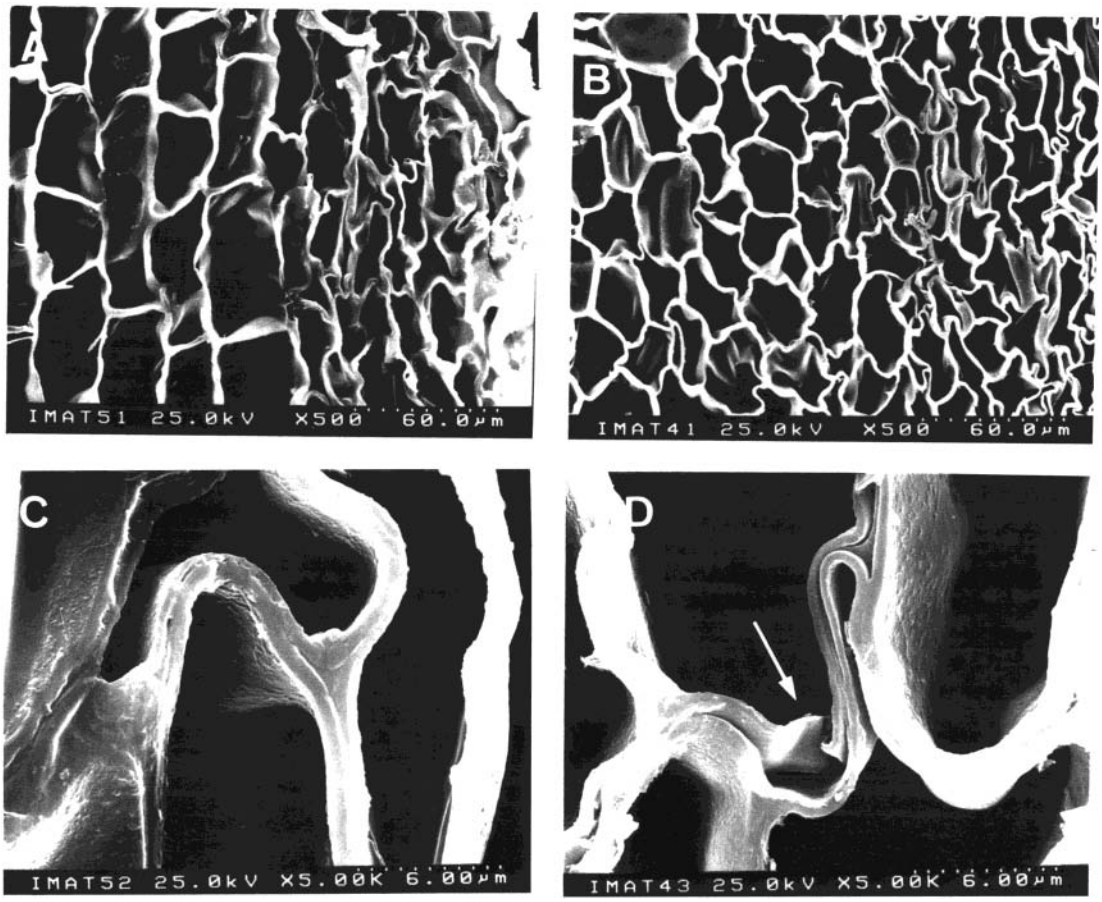

Figure 3. SEM photographs of the radial section of cork cubes treated with hemicellulase (A) and of the tangential section of cork treated with pectinase, showing cellular separation (B). Both photographs show the transition between the superficial area of attack and the inner, not attacked, region. Cell wall of cork treated with hemicellulase (C) and of cork treated with pectinase, showing the cellular separation (D).

Table 1. Sugar Composition of $SPect_{H_2O}$ and $SPect_{acid}$ Fractions Obtained by Ethanol Precipitation Followed by Extraction with Hot Water and Hot Dilute Nitric Acid, Respectively

fraction	yield[a] (mg/g)	cell wall sugars (mol %)								total sugar[b] (mg/g)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	HexA[d]	
$SPect_{H_2O}$	9 (11)	t[c]	t	5	2	t	1	2	90	121.1 (7)
$SPect_{acid}$	21 (8)	t	t	6	t	t	t	t	94	298.7 (4)

[a] Yield is expressed in mg/g of cork powder; numbers in parentheses correspond to the coefficient of variation (%). [b] Values are expressed as mg of anhydrous sugar/g of dry fraction; numbers in parentheses correspond to the coefficient of variation (%). [c] Trace. [d] Hexuronic acid.

Table 2. Sugar Composition of $MAPect_{H_2O}$ and $MAPect_{acid}$ Fractions Obtained by Ethanol Precipitation Followed by Extraction with Hot Water and Hot Dilute Nitric Acid, Respectively

fraction	yield[a] (mg/g)	cell wall sugars (mol %)								total sugar[b] (mg/g)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	HexA[d]	
$MAPect_{H_2O}$	10 (13)	t[c]	t	5	3	t	1	3	88	59.6 (11)
$MAPect_{acid}$	17 (10)	t	t	6	t	t	t	t	94	119.1 (8)

[a] Yield is expressed in mg/g of cork powder; numbers in parentheses correspond to the coefficient of variation (%). [b] Values are expressed as mg of anhydrous sugar/g of dry fraction; numbers in parentheses correspond to the coefficient of variation (%). [c] Trace. [d] Hexuronic acid.

to infer that pectin degradation is involved in yellow spot attack of the cork, possibly by the production of pectin-degrading enzymes.

Chemical Analysis. Tables 1 and 2 show the sugar composition of the polymers solubilized from standard cork and from cork affected by mancha amarela, respectively. For the standard cork, the total amount of cell wall polymers extracted, based on carbohydrate content (neutral sugars + uronic acid) of the $SPect_{H_2O}$ and $SPect_{acid}$, was 12% and 30% of the dry weight of the fraction, respectively, which corresponds to ca. 1.5% of the dry weight of cork. This low content of carbohydrate and the fact that these fractions exhibited a significant amount of UV-absorbing material (280 nm) suggest a mixture of phenolic material together with the polysaccharides. Both $SPect_{H_2O}$ and $SPect_{acid}$ extracts exhibited a similar sugar composition when expressed as molar percent; these fractions are composed by significant amounts of hexuronic acid (HexA) and low contents of Gal, Xyl, and Glc. The presence of pectic polysaccharides was inferred from the relatively large amount of HexA and the occurrence of Ara and

Rha. Rha, although present in small amounts, is diagnostic of pectic substances (Waldron and Selvendran, 1990). Also, unambiguous identification of the occurrence of galacturonic acid was achieved by FT-IR spectroscopy (results not shown) (Coimbra et al., 1998, 1999). The fact that the ratio of Ara/HexA is very low (0.006), as well as the amount of Rha, allows to infer that the pectic polymers are only slightly branched, which is characteristic of middle lamella polysaccharides (Selvendran, 1985).

The pectic polysaccharides of $MAPect_{H_2O}$ and $MAPect_{acid}$ accounted for 6% and 12% of the total material removed, respectively, which corresponds to ca. 0.5% of the dry weight of MA cork. $Pect_{H_2O}$ and $Pect_{acid}$ of standard cork and of cork with mancha amarela showed a similar sugar composition expressed in molar percent, although the former contained a larger amount of pectic polysaccharides. These results confirm that the cellular separation observed by scanning electron microscopy is related to the degradation of the middle lamella pectic polysaccharides.

CONCLUSIONS

The relatively large amount of hexuronic acid and the occurrence of Ara in the $SPect_{H_2O}$ and $SPect_{acid}$ allow to confirm, as far as we know, for the first time the presence of pectic polysaccharides in the cell wall of cork from *Q. suber* L., which have to be taken into account in any realistic model of the cork cell wall. The pectic polysaccharides accounted for ca. 1.5% of the cork and may consist of a slightly branched polymer, exhibiting relatively long side chains of arabinosyl residues. Interestingly, the relative proportions of the neutral sugars and hexuronic acids in all the fractions examined are highly comparable (water- and acid-soluble fractions in standard and MA-attacked cork). It seems clear that although the cell wall of both qualities of corks is quite different, the overall pectic polysaccharide composition of S and MA, when expressed as molar percent, was similar. The fungal degradation of the cork pectic polysaccharides may contribute to the availability of a nutrient source in the cork that can be used by subsequent/simultaneous attack of other microorganisms, with possible consequences in the cork stopper aroma quality.

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Received for review October 20, 1999. Revised manuscript received March 16, 2000. Accepted March 23, 2000. S.M.R. was supported by a grant from the JNICT (BD/1218/91-IF).

JF991147G