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Mannitol and oligosaccharides as new criteria for determining cold tolerance in sugarcane varieties

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Abstract

Sugarcane can be very susceptible to damage by freezes. Freeze-deteriorated cane can cause problems in processing and sometimes leads to a factory shut-down. This study was undertaken during the 2000/2001 harvest season to assess the cold tolerance performance of six commercial sugarcane varieties and to establish new and more sensitive criteria to measure cold tolerance. Two varieties CP 70-321 and CP 79-318, with known cold tolerance, were planted in the study as controls. The other varieties included LHo 83-153, LCP 85-384, HoCP 85-845 and HoCP 91-555. Freezing temperatures occurred on 20 December 2000 when the min. field temperature was −4.4 °C, and again on 21 December, 30 December through 5 January 2001, 9–10 January and 20–21 January. The lowest field temperature recorded was −5.6 °C on 4 January. Freezing conditions prevailed for 8–15 h during each freeze incident. Stalks of all varieties were frozen to the ground following the initial freeze, with freeze cracks evident only after the 4 January freeze. For this study, samples were taken on the date of the first freeze, 20 December, and subsequently again at 7, 14, 22 and 30 days after the first freeze. Criteria used to measure overall stalk cold-tolerance included changes in pH, Brix, dextran (ASI-II method), sucrose, glucose, and fructose concentrations. Mannitol, ethanol and the oligosaccharides, palatinose, leucrose, isomaltotriose and 1-kestose, were simultaneuously measured using IC-IPAD. Marked differences were observed in most criteria for all varieties, particularly 22 and 30 days after the first freeze. Mannitol was strongly correlated ($r^2 = 0.84$) with dextran, confirming its use as an indicator for cane dextran deterioration. In comparison, ethanol was only weakly correlated ($r^2 = 0.55$) with dextran and did not always predict cane dextran deterioration. Iso maltotriose was the most sensitive oligosaccharide indicator of freeze deterioration, although both leucrose and palatinose could be used to confirm whether severe dextran formation (>1500 ppm/Brix) has occurred in cane. Isomaltotriose was strongly correlated ($r^2 = 0.89$) with dextran and pH ($r^2 = -0.83$); pH was also a strong indicator of both dextran ($r^2 = -0.85$) and mannitol ($r^2 = -0.92$) formation. Four of the varieties, CP 79-318, LCP 85-384, HoCP 85-845 and HoCP 91-555, were shown to be susceptible to other sources of microbial and enzymic deterioration as well as dextran deterioration from *Leuconostoc* bacteria, especially 30 days after the first freeze. This was indicated by increased glucose/fructose ratios, ethanol formation, changes in 1-kestose concentration, and further sucrose losses.
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Keywords: Sugarcane freeze deterioration; Sugarcane cold tolerance; Dextran; Mannitol; Ethanol; Oligosaccharides; Dextransucrase; *Leuconostoc* bacteria; 1-Kestose

1. Introduction

It is well known that sugarcane can be very susceptible to damage by freezes and freeze-deteriorated cane can cause problems in processing and sometimes leads to a factory shut-down. The exposure of sugarcane to damaging frosts occurs in over 20 of the 70 sugarcane-producing countries, but is most frequent on the mainland of the United States. The frequent winter freezes of Louisiana force the industry to adapt to a short growing season (7–9 months) and a short milling season (approx. 3 months). The nature and extent of damage to cane depends on the type and number of periods of the frost, which may be light, mild, or severe (Naqvi & Alam, 1976). Damage from a freeze can be even more severe when warm, wet weather, which is ideal for microbial

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growth, follows the freeze. Generally, cane damaged by a severe freeze produces juices of lower purity, higher acidity and abnormal amounts of polysaccharides, especially dextran (see Legendre, Tsang, & Clarke, 1985). Following freeze injury, dead and moribund cells become vulnerable to the invasion of microbes, particularly *Leuconostoc* bacteria which are mostly responsible for the formation of dextran by dextransucrase enzyme. The entry of microbes into cane tissue is faciliated by dead lateral buds (−4.4 °C) and by freeze cracks (−5.6 °C). Irvine and Legendre (1985) proposed two mechanisms for deterioration: (1) susceptibility of tissue to freezing, and (2) susceptibility to microbe invasion and subsequent polysaccharide formation after the freeze.

Because of the prevalence of damaging frosts in Louisiana, great emphasis has historically been placed on breeding for "cold tolerant" or frost resistant cane varieties. Legendre et al. (1985) showed that there was a varietal effect on level of dextrans and total polysaccharides in cane left in the field after freeze-damage. Such breeding programs are highly reliant on quality criteria/indicators to allow proper selection and development of cold-tolerant varieties. Sugarcane agronomists and technologists have reported a variety of physico-chemical criteria to measure cane deterioration after a freeze. Changes in juice pol, titratable acidity and dextran content were reported by Legendre et al. (1985) to be the most useful criteria. However, not all criteria (which are mostly formed deterioration products) have an impact on future factory processing, and some are not as direct or sensitive as required, while others are difficult or too time-consuming to measure. New and more sensitive criteria for levels of freeze-deterioration need to be established in order to aid the breeding programs, and better predict the quality of the cane to be processed as well as the effect on process conditions.

In the last decade, oligosaccharides have been reported as indicators of deterioration in stored cane, particularly burnt billeted cane (Eggleston, Legendre, & Richard, 2001a, 2001b; Morel du Boil, 1995; Ravelo, Ramos, & Tones, 1991). Like the dextran polysaccharide which has a negative impact in processing, mostly associated with increased viscosity effects, certain oligosaccharides directly and negatively affect the efficiency of factory processing as they can interfere with crystallization (Morel du Boil, 1995), deforming the crystal shape (crystal elongation). Recently, Eggleston (2002) also showed that mannitol could be a very sensitive indicator of dextran deterioration in stored, deteriorated cane juice and Steinmetz, Buczys, and Bucholz (1998) previously observed that mannitol was strongly correlated with the quality of frost-damaged sugarbeets. Furthermore, mannitol has been known to increase the viscosity of sugar syrups, making sucrose recovery from them difficult (Bliss, 1975). Like dextran, mannitol is also formed by the lactic acid *Leuconostoc* bacteria (Soetart, 1991) but, unlike dextran, which is formed by dextransucrase, mannitol is formed by the enzyme mannitol dehydrogenase, which is illustrated in the following theoretical fermentation equation (derived by Vandamme, Raemaekers, Vehemans, & Soetart, 1996):

$$2\ \text{Fructose} + \text{Glucose} \rightarrow 2\ \text{Mannitol} + \text{D(}-\text{)-Lactic Acid} + \text{Acetic Acid} + CO_2$$

Fructose can also be simply reduced to mannitol by mannitol dehydrogenase NADH linked activity in *Leuconostoc* bacteria (Grobben et al., 2001).

Consequently, in this study various known and new quality parameters, including oligosaccharides and mannitol, were studied to find more reliable predictors of freeze-deteriorated cane in six commercial sugarcane varieties. This information could then be used by breeders to aid cold tolerance breeding programmes, and by processors to assess the quality of cane being processed.

2. Materials and methods

2.1. General sampling

Field experiments consisting of 3-row plots are routinely planted at the Ardoyne Farm of the USDA-ARS-SRRC at Houma, Louisiana for the estimation of stalk cold tolerance of commercial and candidate varieties. In this study, commercial varieties of known cold-tolerance were grown as controls and included CP 70-321 for good cold-tolerance (Irvine & Legendre, 1985) and CP 79-318 which is known for poor cold-tolerance. Six commercial varieties were planted, which included the two control varieties; the other varieties were LHo 83-153, LCP 85-384, HoCP 85-845 and HoCP 91-555. Planting took place on 27 August 1999, and was done on raised ridges 6ft apart. Variety plots were approx. 50ft long and 3 rows wide. The experimental design was a randomized complete block with four replications. Plots were cultivated and fertilized according to recommended plantation practices; insecticides were applied as required, according to the economic threshold (Legendre, 2001). The cane was allowed to remain in the field until 18 December 2000 which was just before the first freeze of the harvest season in the year following planting. Just prior to or immediately following the freeze, five samples were removed serially along the centre row of each plot. Each sample consisted of 10 stalks cut at the ground by hand but not stripped or topped of leaves. The 10-stalk sample was passed once

though a 3-roller sample mill. A sub-sample mill juice was then immediately taken for dextran (ASI-II method) analysis. The rest of the juice was frozen and then transported to the analytical laboratory. Composite juices of each variety for each sampling date were made by physically combining 15 g of each of the four replicates. The biocide sodium azide (0.02%) was added immediately to each composite to prevent any further microbial deterioration from occurring. Juices were stored in a −40 °C laboratory freezer until analyzed.

2.2. Freezes during the 2000/2001 cane harvest and sampling dates

Freezing temperatures that affected the Louisiana sugar industry during the 2000/2001 harvest occurred on 20–21 and 30–31 December 2000 and 1–5, 9–10, 20–21 and 23 January 2001. The official temperatures recorded at the Houma research station near to where the Ardoyne farm is located are illustrated in Fig. 1. On 20 December subfreezing temperatures were reported for more than 12 h with a minimum field temperature of −4.4 °C reported at Houma (Fig. 1). No freeze cracks were apparent, although most stalk tissue showed signs of being frozen and the top portion of most stalks (18–24 inches) had begun to soften and wilt. The coldest field temperatures (field temperatures were usually lower than the official temperatures recorded at nearby Houma research station shown in Fig. 1) occurred during the period 4–11 January and the temperature dipped to −5.6 °C at Houma on 4 January (Fig. 1). On 10 January it was observed that freeze cracks had occurred in all varieties with juice leakage from the cracks as well as from the axillary buds. It was evident, from field investigations, that serious tissue damage occurred within the stalks following the 4 January freeze. Sampling dates for this study are indicated in Fig. 1. Samples were collected on 18 December and processed on 20 December and 27 December 2000, 3, 11 and 19 January 2001.

2.3. Ion chromatography with pulsed amperometric detection (IC-IPAD) analysis of carbohydrates, sugar alcohols and ethanol

See Eggleston (2002) for method. All samples were °Brix-adjusted to the lowest sample °Brix before diluting (1 g/25 ml) in order to compare chromatograms directly. Oligosaccharides, mannitol, and ethanol were quantified in reference to raffinose, mannitol, and ethanol standards, respectively.

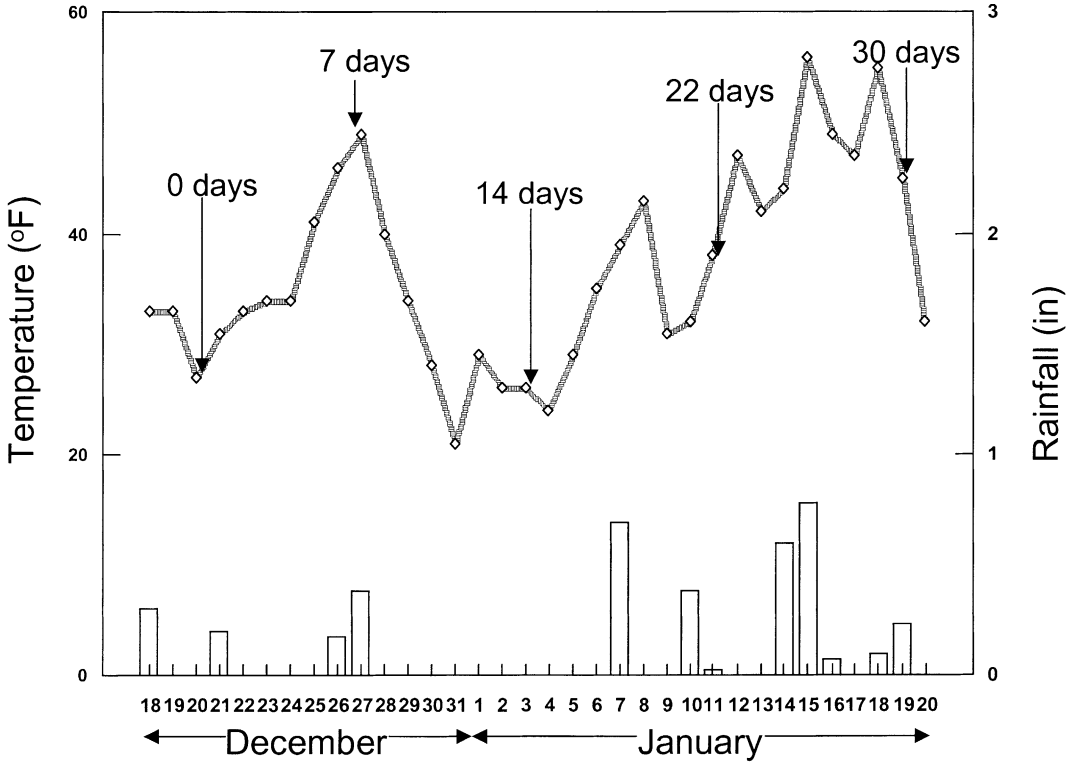

Fig. 1. 2000/2001 weather data and sampling dates for USDA-ARS Research Station, Houma, LA, USA.[a,b]

2.4. Sucrose, glucose and fructose by gas chromatography (GC)

See Eggleston (2002) for method.

2.5. Dextran

Duplicate samples on each replicate were analyzed for dextran using the ASI (Audubon Sugar Insitute) II method (Sarker & Day, 1986) which uses dextranase enzymes. Average results are reported.

2.6. °Brix

The mean °Brix of triplicate samples was measured using an Index Instruments TCR 15-30 temperature-controlled refractometer accurate to ±0.01 °Brix.

2.7. pH

Measurements of pH were at room temperature (−25 °C), using an Ingold™ combination pH electrode calibrated at room temperature, with two different pH buffers (pH 7 and 10). The electrode was connected to a Metrohm 716 DMS pH meter.

2.8. Statistical correlations

Pearson correlation coefficients were calculated to investigate relationships among the various deterioration criteria ($N = 30$) using PC-SAS 6.12 (SAS Institute, Cary, NC).

3. Results and discussion

3.1. Changes in pH

Changes in pH amongst the varieties are listed in Table 1. Organic acids, particularly lactic acid, are produced during cane deterioration from the degradation of sugars and cause a reduction in pH. However, pH is not usually considered a sensitive measure for deterioration because the buffering capacity of the juice reduces this pH change on deterioration. Nevertheless, there was a marked difference in pH decrease with post-freeze dates, particularly 22 and 30 days after the initial freeze.

Table 1
Effect of days after initial freeze on the pH of the cane juices

Cane variety	pH				
	0 days	7 days	14 days	22 days	30 days
CP 70-321	5.38	5.36	5.41	5.34	4.32[b]
CP 79-318	5.26	5.35	5.35	4.60[a]	3.99[b]
LHo 83-153	5.36	5.28	5.32	4.90[a]	4.08[b]
LCP 85-384	5.29	5.30	5.30	5.12	4.32[c]
HoCP 85-845	5.30	5.32	5.34	4.72[a]	4.18[b]
HoCP 91-555	5.40	5.37	5.47	5.24	4.18[b]

[a] The natural brown juice colour had turned to yellow.
[b] Yellow and smells of alcohol.
[c] Yellow with slight smell of alcohol.

3.2. Dextran formation

The ASI-II method used to determine dextran in this study measures both high and low molecular weight dextran and results are illustrated in Fig. 2. Marked differences in dextran formation were observed amongst the varieties, particularly 22 and 30 days after the initial freeze when freeze cracks were visible. Variety LCP 85-384 was susceptible to dextran formation, even after the first freeze (at 0 days), and HoCP 91-555 was susceptible 7 days later. Varieties CP 70-321 and LHo 83-153 were the least susceptible to *Leuconostoc* invasion and subsequent dextran formation, even after 22 days. Results suggest that the final freeze which caused cracks to appear on the cane stalks, accelerated the invasion of the wounded cane by *Leuconstoc* bacteria and the utilization of sucrose for dextran formation.

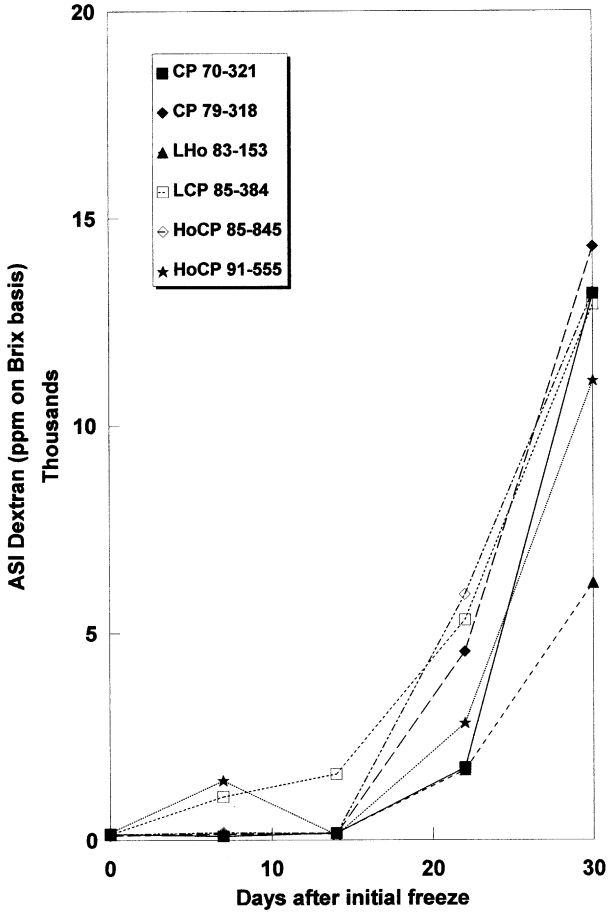

Fig. 2. Dextran changes with time after freezes.

3.3. Effect of freezes on sucrose, glucose and fructose concentrations

Freezes are known to kill and rupture cells which causes sucrose inversion to occur, because of higher acidity levels and increased activity of invertase enzymes. The latter could be due to increased mobilization of cell invertases, the possible synthesis of a wound-induced invertase, and/or because of decreased activity in sucrose synthesis enzymes induced by the pH changes (personal communication, S. Lingle). Furthermore, the wound sites and associated cell rupture allow microbes to invade and utilize sugars such as sucrose. The effects of freezes on sucrose, glucose and fructose concentrations are listed in Table 2.

Initially (0 days) there were slight differences in sugar concentrations amongst the six varieties. CP 79-3–18 and HoCP 91-555 had the lowest sucrose and highest glucose and fructose. Minor increases in glucose and fructose, 7 days after the initial freeze, indicated that most varieties had been susceptible to slight deterioration after the first freeze, with HoCP 91-555 being particularly susceptible, as indicated by the concomitant increase in dextran after 7 days (Fig. 2). Subsequently, marked degradation of sucrose, with formation of glucose and fructose, occurred, particularly 22 and 30 days after the initial freeze (Table 2). This is further confirmation that the freeze of 4 January (equivalent to day 15 after the initial freeze) when freeze cracks were visible, caused dramatic deterioration to occur in all cane varieties, although varietal differences were still apparent.

Table 2
"True Purity" concentrations

Cane variety	0 days	7 days	14 days	22 days	30 days
(a) % Sucrose (on Brix Basis)					
CP 70–321	92.40	92.89	92.44	87.69	75.60
CP 79–318	88.42	88.91	88.76	78.12	54.34
LHo 83–153	91.26	92.0	88.56	88.04	77.32
LCP 85–384	94.09	92.38	92.60	85.79	78.02
HoCP 85–845	92.23	92.1	91.17	88.75	73.23
HoCP 91–555	89.62	85.04	88.88	80.16	65.22
(b) *Fructose (on Brix Basis)*					
CP 70–321	0.80	0.86	0.86	1.17	4.05
CP 79–318	1.40	1.66	1.57	2.75	4.01
LHo 83–153	0.81	0.88	0.79	1.12	3.11
LCP 85–384	0.80	0.91	0.87	1.41	2.64
HoCP 85–845	0.93	0.91	0.97	1.92	3.66
HoCP 91–555	1.47	2.20	1.56	2.54	3.90
(c) *% Glucose (on Brix Basis)*					
CP 70–321	0.77	0.83	0.84	1.11	2.93
CP 79–318	1.34	1.66	1.49	2.72	5.64
LHo 83–153	0.84	0.88	0.76	1.00	2.47
LCP 85–384	0.83	0.80	0.73	1.10	2.26
HoCP 85–845	0.97	0.92	0.99	1.22	3.22
HoCP 91–555	1.55	2.12	1.61	2.37	3.90

Glucose/fructose (G/F) ratios were also calculated and are listed in Table 3. Lowering of G/F ratios usually indicates dextran formation because dextransucrase, an enzyme secreted extracellularly by *Leuconostoc* bacteria, hydrolyzes glucose from the sucrose molecule to form dextran, leaving fructose (from the sucrose molecule) as a secondary product (Legendre et al., 1985), which is illustrated in the following basic equation:

$$ {}_{n}(\underset{\underline{\text{Sucrose}}}{\text{Glucose} - \text{Fructose}}) + H_2O \rightarrow \underset{\underline{\text{Dextran}}}{H - (\text{Glucosyl})_n - OH} + {}_{n}\,D - \text{Fructose} $$

However, for the four varieties CP 7-318, LCP 85-384, HoCP 85-845, and HoCP *91-555,* 30 days after the initial freeze, the G/F ratio actually increased and, in the case of CP 79-318, there was a dramatic increase (Table 3). This strongly suggests that dextran deterioration reactions were not solely responsible for the total freeze-deterioration around this date, and that other, simultaneous microbial infections and enzymic/chemical reactions were most likely occurring. These four varieties were obviously more susceptible to other microbial infections and deterioration reactions as well as infection from *Leuconostoc* bacteria. Furthermore, no significant correlations were found between G/F ratios and other deterioration criteria studied, which is most likely because G/F ratios are a reflection of multiple types of freeze-induced cane deterioration.

3.4. Formation of oligosaccharides, mannitol and ethanol in the freeze-deteriorated cane varieties

Using factory cane juices which were allowed to deteriorate under different controlled conditions, Eggleston (2002) showed that the cane deterioration products, oligosaccharides, mannitol and ethanol, could be simultaneously analyzed using an IC-IPAD method with a sodium hydroxide/sodium acetate gradient. This method was similarly applied to the samples in this study. Typical chromatograms across the freeze dates for varieties CP 70-321 and HoCP 85-845 are shown in Figs. 3 and 4, respectively.

Table 3
Variations in glucose/fructose ratios

Variety	Glucose/fructose ratios				
	0 days	7 days	14 days	22 days	30 days
CP 70-321	0.96	0.97	0.98	0.95	0.72
CP 79-318	0.96	1.00	0.95	0.99	1.41
LHo 83-153	1.04	1.00	0.96	0.89	0.79
LCP 85-384	1.04	0.88	0.84	0.78	0.86
HoCP 85-845	1.04	1.01	1.02	0.64	0.88
HoCP 91-555	1.05	0.96	1.03	0.93	1.00

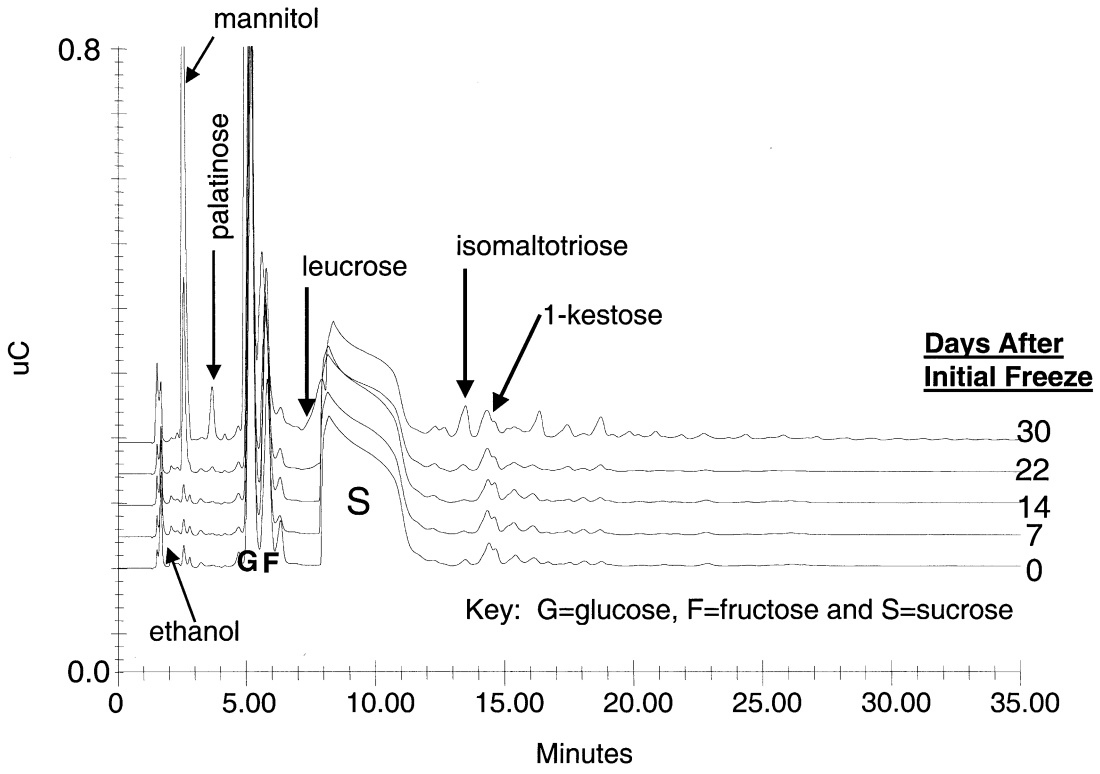

Fig. 3. IC-IPAD chromatograms for cane variety CP 70-321 (Brix standardised to 16.7).

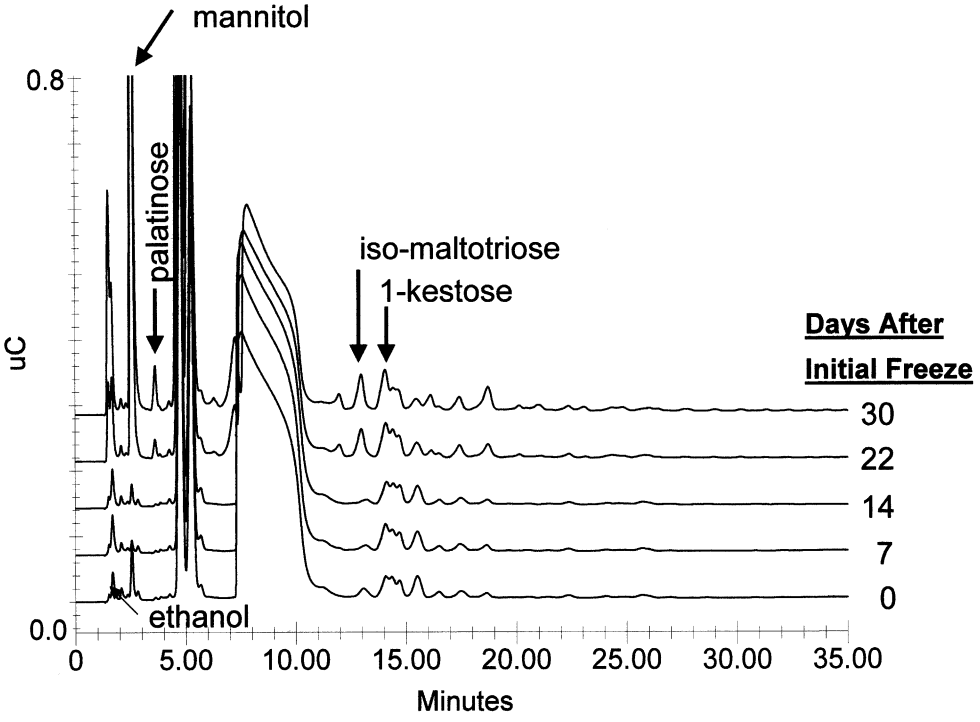

Fig. 4. IC-IPAD chromatograms for cane variety HoCP 85-845 (Brix standardised to 16.1).

3.5. Oligosaccharides

Isomaltotriose, leucrose, palatinose (isomaltulose) and 1-kestose oliogosaccharides were analyzed in this study and marked varietal differences in formation of oligosaccharides were observed (see Figs. 3–5).

Dextransucrase enzyme, secreted mostly by *Leuconostoc* bacteria, not only catalyzes the synthesis of dextran and dextran branch linkages, using sucrose as the substrate, but, in the presence of other carbohydrates, named "acceptors", such as glucose, fructose, maltose also transfer glucose from the sucrose molecule to the carbohydrate. These are known as acceptor reactions and the products formed are acceptor products (Robyt, 1995; Robyt & Eklund, 1982). Isomaltotriose, leucrose, and palatinose are all such acceptor products (Robyt & Eklund, 1982) and, therefore, could be potential criteria for cane dextran deterioration. As illustrated in Figs. 5a and b, leucrose and palatinose were only measurable using the IC-IPAD method in this study, approximately 14 days after the first freeze, although leucrose was apparent in LCP 85-384 after 7 days. The detection of leucrose before palatinose is not surprising. Both leucrose and palatinose are acceptor products formed when fructose is the acceptor, and their synthesis depends on the ring form of the fructose (Robyt, 1995). The major product is always leucrose, because it is formed from D-fructopyranose, the most abundant ring form of fructose, whereas the minor product palatinose is formed when D-fructofuranose is the acceptor. Because of their relatively slow rate of formation by dextransucrase, both leucrose and palatinose only appear to be sensitive indicators of cane dextran deterioration when severe dextran formation has occurred, i.e. $> \sim 1500$ ppm/Brix of dextran. This is further supported by the fact that, although 1058 ppm/Brix dextran formed after 7 days in LCP 85-384, neither leucrose nor palatinose were detected. Steinmetz et al. (1998), studying frost-damaged sugar beet found that the leucrose content was a direct indicator of dextran, although they found that mannitol was more strongly correlated with the quality of the damaged beets. In this study, the

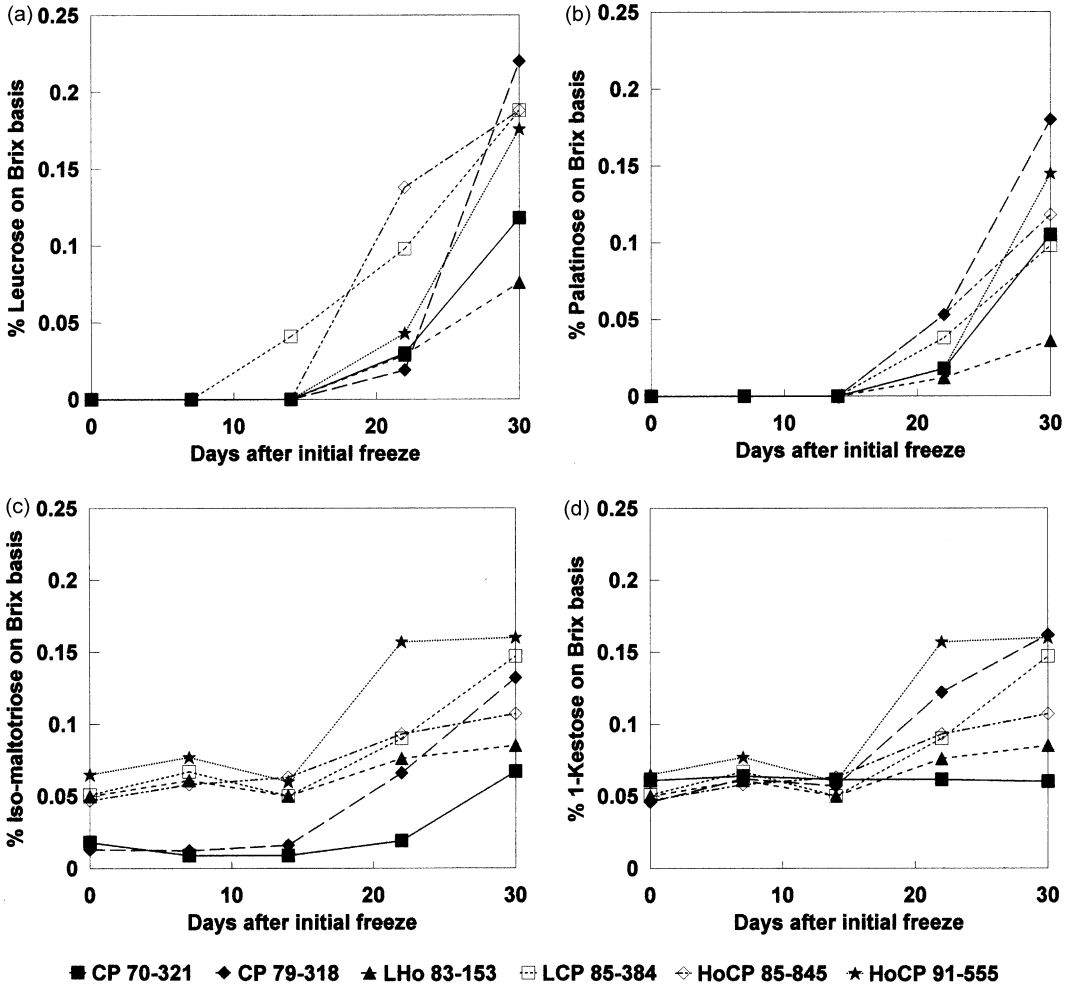

Fig. 5. Changes in (a) leucrose, (b) palatinose, (c) isomaltrotriose, (d) 1-kestose.

correlation between palatinose and dextran ($r^2 = 0.89$, $P < 0.0001$) was actually slightly greater than that for leucrose and dextran ($r^2 = 0.78$, $P < 0.0001$) although N values were only 12 because, in general, both of these oligosaccharides were not detected from 0–14 days. In comparison to leucrose and palatinose, the oligosaccharide isomaltotriose was detected at earlier dates. This may be because isomaltotriose is an acceptor product which is formed earlier than leucrose and palatinose by dextransucrase (Robyt & Eklund, 1982). Furthermore, there was not only a very strong correlation of isomaltotriose with dextran ($r^2 = 0.89$, $P < 0.0001$) but also with mannitol ($r^2 = 0.91$, $P < 0.0001$) and pH ($r^2 = -0.83$, $P < 0.0001$) which strongly indicates that isomaltotriose is the most sensitive oligosaccharide indicator for dextran cane deterioration.

The oligosaccharide 1-kestose is not associated with the formation of dextran by dextransucrase. It is formed from the action of other enzymes, including invertases (β-fructofuranosidases). Invertases not only hydrolze sucrose into its constituent monosaccharides, glucose and fructose but, if the sucrose concentrations are high enough (>0.29 M sucrose), are capable of catalyzing transfructosylating reactions with sucrose to produce various kestose (fructosyl sucrose) oligosaccharides of which 1-kestose is the most predominant (Pollack & Cairns, 1991; Farine et al., 2001). Invertases occur in several isoforms in cane and are also produced by yeasts. It may also be possible that kestoses are also catalyzed by transfructosylating enzymes in the cane plant. A slight amount of kestoses can be formed in very acidic conditions by the acid degradation of sucrose (Eggleston, 2002) but this usually occurs at elevated temperatures. Except for CP 70-321, there were slight increases in 1-kestose concentrations for the varieties at 7 days after the initial freeze (see Fig. 5d). This is in contrast to the other cane deterioration criteria studied, including dextran, which, 7 days after the initial freeze, increased in only two varieties: LCP 85-384 and HoCP 91-555 (Fig. 2), although slight increases in isomaltotriose concentrations were seen in two other varieties (Fig. 5c). This strongly suggests that, although little microbial deterioration occurred after the first freeze, some enzymic cane deterioration occurred in all varieties except CP 70-321. Further evidence of this is the apparent increases in glucose and fructose concentrations (Table 2) for most varieties. For all varieties, 1-kestose concentrations became stable between days 7 and 14 days after the initial freeze but, except for CP 70-321, increased rapidly thereafter (Fig. 5d). The very stable concentrations of 1-kestose, from 0 to 30 days for CP 70-321, strongly indicate that CP 70-321 was very resistant to enzymic cane deterioration. This resistance may be because of a biochemical factor or because of a physical factor which protects the integrity of cells and their enzyme contents from freezes.

3.6. Mannitol

Mannitol formation across all freeze dates is shown in Fig. 6. For all varieties, a small amount of mannitol was present, even after the first freeze at 0 days, whereas very little dextran polymer was detected (Fig. 2). This may suggest that mannitol is an even better indicator of *Leuconostoc* growth in cane than dextran. Seven days after the initial freeze, similar to the dextran results (Fig. 2), mannitol only formed in LCP 85-384 and HoCP 91-555, further indicating that these two varieties were the least resistant to *Leuconostoc* invasion even when no stalk cracks were visible. Dramatic varietal increases in mannitol occurred, particularly after 22 and 30 days, and at much higher concentrations than the oligosaccharides. Mannitol formation was least in CP 70-321 and highest in CP 79-318. Furthermore, there was a very strong correlation between mannitol and dextran ($r^2 = 0.84$, $P < 0.0001$) and between mannitol and isomaltotriose ($r^2 = 0.91$, $P < 0.0001$). However, the advantage that mannitol has over isomaltotriose as a cane deterioration indicator, is that much higher concentrations are formed which can be more readily detected by both IC-IPAD and GC techniques, and mannitol is known to directly affect cane processing (Bliss, 1975).

3.7. Ethanol

Ethanol has been used as a cane deterioration indicator, particularly in burnt whole-stalk cane in South Africa (Lionnet & Pillay, 1987). Ethanol is a metabolic by-product of numerous yeast, bacterial and fungal reactions, and the amount formed depends on the type of microbe, as well as microbial growth parameters. Ethanol is especially a major by-product of yeast fermentation reactions, with yeast converting sucrose into ethanol and carbon dioxide, especially under dry and anaerobic conditions. *Leuconostoc* bacteria, besides forming dextran, can also produce ethanol, although this is usually only the case if glucose, not sucrose, is the carbohydrate energy source (see Eggleston, 2002). Ethanol concentrations are illustrated in Fig. 7. Like isomaltotriose and mannitol, ethanol was present in the cane even after the first freeze at 0 days. This may be a reflection of the maturity of the cane, previous yeast growth on the cane surface, or previous animal- or pest-deterioration of the cane. For all varieties, ethanol concentrations were relatively stable from 0 to 14 days and even decreased in CP 70-321, which is most likely because of evaporation. Except for CP 70-321, after 14 days, ethanol increases were observed in all varieties, strongly indicating that considerable microbial cane deterioration had occurred. However, in comparison to mannitol, ethanol was only weakly correlated ($r^2 = 0.55$,

$P<0.001$) with dextran and did not always predict cane dextran deterioration, which was the case with CP 70-321 (compare Figs. 2 and 7). Eggleston (2002) similarly observed that ethanol was not always a direct indicator of cane dextran deterioration.

3.8. Significant correlations amongst cane freeze-deterioration criteria

Significant ($P<0.01$) Pearson r^2 correlation co-efficients greater than 0.50 are listed in Table 4. Although, as expected, sucrose was strongly correlated with its primary degradation products glucose and fructose, only moderate correlations were found with other deterioration criteria. This is because sucrose losses are due to a complex of deterioration reactions, mainly enzymic and microbial in nature, rather than one principal reaction mechanism. Fructose, palatinose, leucrose, and isomaltotriose are all by-products from dextransucrase activity and, therefore, as expected, were strongly correlated with dextran formation, particularly palatinose and isomaltotriose (Table 4). *Leuconostoc* bacteria, as well as producing dextransucrase enzyme, also produce mannitol dehydrogenase, an enzyme that catalyzes the formation of mannitol from fructose. Mannitol was observed to be strongly correlated with dextran ($r^2=0.84$, $P<0.0001$), isomaltotriose ($r^2=0.91$, $P<0.0001$) and pH ($r^2=-0.92$, $P<0.0001$) and confirmed previous observations by Eggleston (2002) that mannitol is a valid indicator of cane dextran deterioration. 1-Kestose was only moderately correlated with other freeze-deterioration criteria (Table 4). This confirms that enzymic deterioration is only a minor contributor to total freeze-deterioration. In previous studies, Irvine and Legendre (1985) reported that there was a close relationship between post-freeze deterioration of sugarcane varieties and apparent sucrose, pH, titratable acidity, and dextran content of juice following a "hard" freeze (minimum temperture −10.6 °C), where all stalk tissue is damaged. Juice pH is not usually considered a sensitive indicator of post-freeze deterioration following a freeze of less severity (temperature no lower than −5.7 °C); however, in this study, where all the stalk tissue was damaged, pH was strongly correlated with dextran, mannitol, and isomaltotriose (all indicators of cane dextran deterioration) and to a lesser extent with sucrose, glucose, fructose, palitinose, and leucrose. Therefore, pH may in fact, be, more useful than originally thought as a post-freeze indicator of deterioration, especially when all internal stalk tissue is damaged.

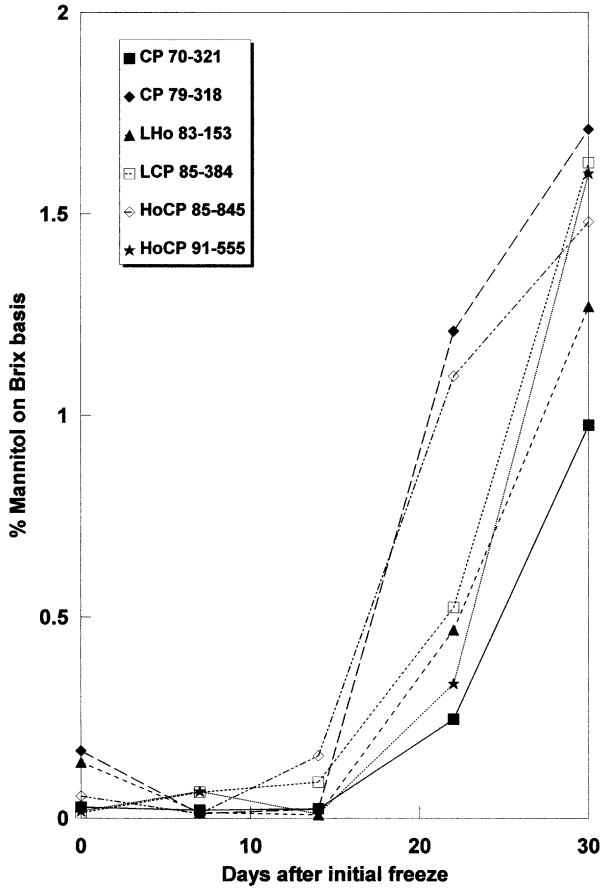

Fig. 6. Mannitol changes with time after freezes.

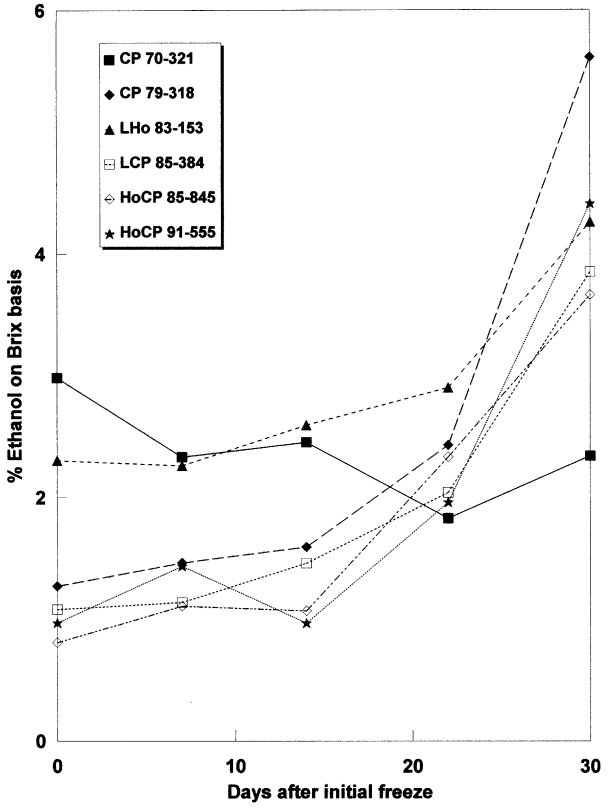

Fig. 7. Ethanol changes with time after freezes.

Table 4
Significant correlations of freeze-deterioration criteria[a]

	Sucrose	Glucose	Fructose	G/F	Dextran	Ethanol	Mannitol	pH	Brix	Palatinose	Leucrose	Isomaltotriose	1-Kestose
Sucrose	—												
Glucose	−0.95[b]	—											
	0.0001[c]												
Fructose	−0.85	0.86	—										
	0.0001	0.0001											
G/F				—									
Dextran	−0.76	0.67	0.79		—								
	0.0001	0.0001	0.0001										
Ethanol	−0.64				0.55	—							
	0.0001				0.0001								
Mannitol	−0.73	0.63	0.72		0.84	0.71	—						
	0.0001	0.0001	0.0001		0.0001	0.0001							
pH	0.75	−0.75	−0.78		−0.85	−0.67	−0.92	—					
	0.0001	0.0001	0.0001		0.0001	0.0001	0.0001						
Brix			−0.52						—				
			0.0001										
Palatinose	−0.72	0.67	0.61		0.89	0.57	0.73	−0.61		—			
	0.0005	0.0011	0.0027		0.0001	0.0045	0.0004	0.0027					
Leucrose					0.78	0.57	0.62	−0.53	0.79	—			
					0.0001	0.0042	0.0025	0.008	0.002				
Isonialtotriose	−0.78	0.68	0.70		0.89	0.62	0.91	−0.83	0.87	0.82	—		
	0.0001	0.0001	0.0001		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001			
1-Kestose	−0.67	0.60	0.54		0.52		0.62					0.67	—
	0.0001	0.0001	0.0001		0.0001		0.0001					0.0001	

[a] Only significant ($P < 0.05$) correlations greater than $r^2 = 0.50$ are listed.
[b] The r^2 value.
[c] The probability P level.

4. Main conclusions

Marked differences were observed for most criteria for all varieties, particularly 22 and 30 days after the first freeze. Mannitol was strongly correlated ($r^2 = 0.84$) with dextran, confirming its use as an indicator for cane dextran deterioration. In comparison, ethanol was only weakly correlated ($r^2 = 0.55$) with dextran and did not always predict cane dextran deterioration. Isomaltotriose was a more sensitive oiigosaccharide indicator of freeze-deterioration than either leucrose or palatinose and was strongly correlated ($r^2 = 0.89$) with dextran. pH was a strong indicator of dextran ($r^2 = -0.85$), mannitol ($r^2 = -0.92$) and isomaltotriose ($r^2 = -0.83$) formation. Four of the varieties, CP 79-318, LCP 85-384, HoCP 85-845 and HoCP 91-555, were shown to be susceptible to other sources of microbial and enzymic deterioration, as well as dextran deterioration from *Leuconostoc* bacteria, especially 30 days after the first freeze. This was indicated by increased glucose/fructose ratios, ethanol formation, changes in 1-kestose concentrations, and further sucrose losses. Legendre, Birkett, and Stein (2001), using traditional cold-tolerance criteria, such as theoretical recoverable sucrose (TRS), generally ranked the cane varieties, from best to worst, as follows: CP 70-321, LHo 83-153, LCP 85-384, HoCP 85-845, HoCP 91-555, and CP 79-318. However, with the increased sensitivity of mannitol and oligosaccharides, general ranking was different with, from best to worst: CP 70-321, LHo 83-153, HoCP 85-845, LCP 85-384, HoCP 91-555, and CP 79-318, with very little difference in the ranking of the latter three varieties.

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