Significance of autoclaving-induced toxicity from and hydrolysis of carbohydrates in *in vitro* studies of pollen germination and tube growth

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Autoclaving of various carbohydrates resulted in decreased *in vitro* pollen germination and/or pollen tube growth in species belonging to different families. This toxicity was eliminated by treatment of autoclaved sucrose and glucose solutions with activated charcoal. Autoclaving-induced hydrolysis of sucrose at pH 6 resulted in stimulated *Agapanthus praecox* pollen growth (in detoxified media) probably due to the formation of glucose which was found to be a superior carbohydrate source for *in vitro* pollen growth in this species. Considering these results as well as previous reports on anther culture studies, autoclaving-induced toxicity from and hydrolysis of carbohydrates should be taken into account in all pollen and anther culture studies as well as *in vitro* pollination of ovules.

Outoklavering van verskeie koolhidrate het aanleiding gegee tot verminderde *in vitro*-stuifmeelontkieming en/of stuifmeelbuisgroei in spesies behorende tot verskillende families. Outoklavering-geïnduseerde hidrolise van sukrose by pH 6 het verbeterde *Agapanthus praecox*-stuifmeelgroei tot gevolg gehad (in gedetoksifeerde mediums) waarskynlik a.g.v. die vrystelling van glukose wat 'n hoogs-effektiewe koolhidraatbron vir *in vitro*-stuifmeelgroei in die betrokke spesie is. Op grond van hierdie resultate sowel as dié van vroeëre ondersoeke van helmknopkulture, behoort outoklavering-geïnduseerde toksisiteit en hidrolise van koolhidrate in ag geneem te word by alle stuifmeel- en helmknopkulture en by *in vitro*-bestuiwing van saadknoppe.

Keywords: Activated charcoal, autoclaving, carbohydrates, pollen, toxins

Introduction

The importance of pollen viability in breeding projects and studies on pollination requirements of different species and cultivars necessitates routine determinations of *in vitro* pollen germination. Pollen is usually germinated in a liquid or on a semi-solid sucrose-containing medium. Pollen grains germinate within a few hours and within 12 h the length of pollen tubes in some species may reach up to 3 mm. As the first visual signs of fungal growth are evident only at the end of the first day in culture, this *in vitro* test is being performed under non-sterile conditions, thereby facilitating easy handling of the material.

In one of our routine determinations of Clementine mandarin (*Citrus reticulata* Blanco) pollen viability, the medium was autoclaved prior to pollen viability tests. The unexpected suppression of pollen tube growth observed when using this medium gave rise to the present study.

Materials and Methods

The studies on *Citrus maxima* were performed in Nelspruit during the citrus blossoming period in Spring (August/September). The studies on pollen germination of two different indigenous species [*Agapanthus praecox* Willd and *Eucomis autumnalis* (Mill.) Chitt.] were done during late summer (February) in Cape Town from plants growing in the Kirstenbosch National Botanic Garden.

Composition of growth media

Citrus

In preliminary unpublished studies, the optimum sucrose concentration and pH value were determined to be 20% and 7 respectively. However, for the purpose of comparing different carbohydrates at equimolarity and because of the relatively low solubility of some carbohydrates in aqueous media, a lower concentration had to be employed. A molarity of 0,365 was chosen which, in the case of sucrose, is equivalent to approximately 12,5% (m/v). These preliminary studies have also shown a relatively simple medium to be superior to that of Brewbaker (Brewbaker & Kwack 1963). The final medium consisted of 0,365 M carbohydrate in 0,6% Difco Bacto agar. The pH was adjusted to 7,0 using diluted HCl and KOH solutions.

Indigenous species

Optimal conditions for *in vitro* pollen growth were not determined in these species. The inorganic salts of the Brewbaker medium (Brewbaker & Kwack 1963) were incorporated into the medium. All carbohydrates involved were used at 0,365 M in 0,6% Difco Bacto agar. The final pH was adjusted to 6,0.

Preparation of media

Citrus

Unautoclaved carbohydrates

Distilled water was adjusted to pH 7,0. Agar was added to 1,2% (w/v) and the medium was autoclaved for 15 min

at 122°C and 1,2 kg cm^{-2}. After autoclaving, the agar media were mixed with equal volumes of a double strength carbohydrate solution (0,73 M, pH 7,0) to yield media with carbohydrates at final concentration of 0,365 M and agar at 0,6%.

Autoclaved carbohydrates

The same procedures were followed as described above except that the carbohydrates were added to the media before autoclaving.

Indigenous species

The following procedures were employed:

Unautoclaved carbohydrates

Double strength inorg. salts, pH 6; 1,2% agar, autoclaved	+ equal vol. 0,73 M CHO, pH 6	mixed after autoclaving solution at 40°C	→	Final strength inorg. salts, 0,365 M CHO, 0,6% agar

Autoclaved carbohydrates

0,73 M CHO, pH 6, autoclaved	+ equal vol. double strength inorganic salts, pH 6; 1,2% agar, autoclaved	→	Final strength inorg. salts, 0,365 M CHO, 0,6% agar

Or

0,73 M CHO, pH 6, autoclaved, cooled to room temp. + 2% activated charoal, stirred 30 min, filtered.	+ equal vol. double strenth inorg. salts, pH 6, 1,2% agar, autoclaved	→	Final strength inorg. salts, 0,365 M CHO, 0,6% agar

In vitro culture

In all the experiments anthers were collected just before anthesis and kept in the laboratory. Pollen from freshly dehisced anthers was used.

Citrus pollen was dusted onto agar media in 4-ml watch glasses to densities of approximately 50 grains mm^{-2} and pollen from the indigenous species onto agar in 10-ml transparent plastic containers at densities of approximately 20 grains mm^{-2}. The latter lower density was necessitated by prolific pollen tube growth, in some cases complicating measurements of individual pollen tubes. After incubation (*Citrus* 17 h; indigenous spp. 12 h) at 26°C, 100 pollen grains were scored for germination and 10 randomly selected pollen tubes were measured micoscopically using a drawing tube. Individual pollen grains were considered germinated when the length of a pollen tube exceeded the diameter of the pollen grain.

Statistical analysis

All the experiments were statistically designed (completely randomized designs) and the appropriate analyses of variance were performed. The Student-Newman-Keuls' multiple range test (Miller 1961) was applied in the *Citrus* pollen experiment and in all the other experiments the t-test was used. In all the experiments the treatments were replicated three times except in one (Figure 1) where five replicates were done.

Results

Citrus maxima

The effect of 10 different carbohydrates, each unautoclaved and autoclaved, on germination of pollen and growth of pollen tubes, was studied (Table 1). In the case of two of the disaccharides, trehalose and maltose, no pollen germination could be observed.

A comparison between control and autoclaved media indicates that autoclaving on glucose, galactose, cellobiose and fructose resulted in drastically reduced pollen germination. Autoclaving of sucrose and raffinose had no effect on pollen germination, whereas autoclaving of melezitose stimulated pollen germination. Although differences in pollen tube length were not in all cases statistically significant, autoclaving generally resulted in decreased pollen tube growth.

Effect of autoclaving of sucrose and glucose on pollen germination and pollen tube growth of Agapanthus praecox and Eucomis autumnalis

The results are shown in Table 2. Germination of pollen and growth of pollen tubes in *E. autumnalis* were drastically reduced in both autoclaved carbohydrate treatments. In *A. praecox*, autoclaving of glucose also resulted in decreased germination and pollen tube growth. Although autoclaving of sucrose led to reduced *A. praecox* pollen tube growth, pollen germination was stimulated. This apparent discrepancy, as well as the possibility of toxicity being induced by autoclaving, were investigated in the following experiments.

Table 1 Effect of different carbohydrates at 0,365 M, unautoclaved and autoclaved, on *in vitro* germination of *Citrus maxima* pollen and pollen tube growth. Mean values followed by common letters within the 'pollen germination' and 'pollen tube length' columns do not differ significantly (P=0,05)

	Germination of pollen (%)		Average length of pollen tubes (μm)	
Carbohydrate	Unautoclaved	Autoclaved	Unautoclaved	Autoclaved
Glucose	70,7 ab	1,7 f	296 d	81 fgh
Sucrose	69,3 ab	64,0 b	523 b	258 de
Raffinose	69,3 ab	69,0 ab	330 c	226 def
Melezitose	66,3 b	77,0 a	616 a	301 d
Galactose	52,7 c	2,0 f	165 defgh	115 efgh
Cellobiose	41,3 d	0,3 f	189 defg	45 gh
Fructose	22,7 e	0 f	159 defgh	–
Lactose	8,7 f	1,3 f	219 def	127 efgh
Trehalose	0 f	0 f	–	–
Maltose	0 f	0 f	–	–

Table 2 The effect of autoclaving of sucrose and glucose on *in vitro* germination of *Agapanthus praecox* and *Eucomis autumnalis* pollen and growth of pollen tubes

		Germination of pollen (%)		Average length of pollen tubes (μm)	
Species	Carbohydrate	Unautoclaved	Autoclaved	Unautoclaved	Autoclaved
A. praecox	Sucrose	82,6	95,2	490	282
	Glucose	96,0	4,4	2816	124
E. autumnalis	Sucrose	92,0	65,5	801	121
	Glucose	59,6	3,4	615	65

LSD P=0,05: 4,4% & 124,5 μm
P=0,01: 5,9% & 167,8 μm

Effect of activated charcoal treatment of autoclaved carbohydrate media on toxicity

The effect of autoclaved-induced toxicity from glucose-containing media on both pollen germination and pollen tube growth of *A. praecox* is shown in Table 3. This toxicity was completely neutralized by treatment with charcoal. Results of the sucrose treatments are seemingly not in agreement with those in the glucose treatments. Autoclaved sucrose treated with activated charcoal resulted in a significant increase in pollen tube length when compared to unautoclaved sucrose. Autoclaving of sucrose also resulted in a statistically significant increase in pollen germination. A further increase in germination of pollen was evident when autoclaved sucrose was treated with activated charcoal.

Activity of the presumed toxin(s)

Varying quantities of autoclaved glucose were incorporated into the media. Dilution of autoclaved glucose down to 1% of the total glucose concentration resulted in a statistically significant reduction in the length of *A. praecox* pollen tubes (Figure 1). The average lengths of pollen tubes in the 0%; 1%; 10% and 25% autoclaved glucose treatments (% of the total glucose concentration) were 2 858 μm, 2 479 μm, 1 407 μm and 565 μm respectively, with only 119 μm growth in the 100% autoclaved glucose treatment.

The inclusion of 25% or more autoclaved glucose in the media resulted in decreased pollen germination.

Table 3 The effect of different autoclaving and activated charcoal treatments of sucrose and glucose on *in vitro* germination of *A. praecox* pollen and growth of pollen tubes

	Germination of pollen (%)			Average length of pollen tubes (μm)		
Carbohydrate	Unautoclaved	Autoclaved	Autoclaved and AC*-treated	Unautoclaved	Autoclaved	Autoclaved and AC*-treated
Sucrose	80,7	92,0	98,0	535	567	2022
Glucose	94,6	14,7	92,0	2280	192	2060

*AC: Activated charcoal
LSD P=0,05: 5,4% & 266 μm

Effect of different sucrose: glucose ratios on growth of A. praecox pollen tubes

This experiment was performed to investigate the possibility that partial hydrolysis of sucrose during autoclaving could have accounted for the apparent discrepancies shown in Table 3. The results are shown in Figure 2. It was clearly shown that the incorporation of a quantity of glucose as low as 1% relative to the total sucrose + glucose concentration increased average pollen tube lengths from 471 μm (sucrose only) to 1 872 μm. A statistically significant levelling off of pollen tube growth could be detected in the 50% to 100% glucose: sucrose + glucose media.

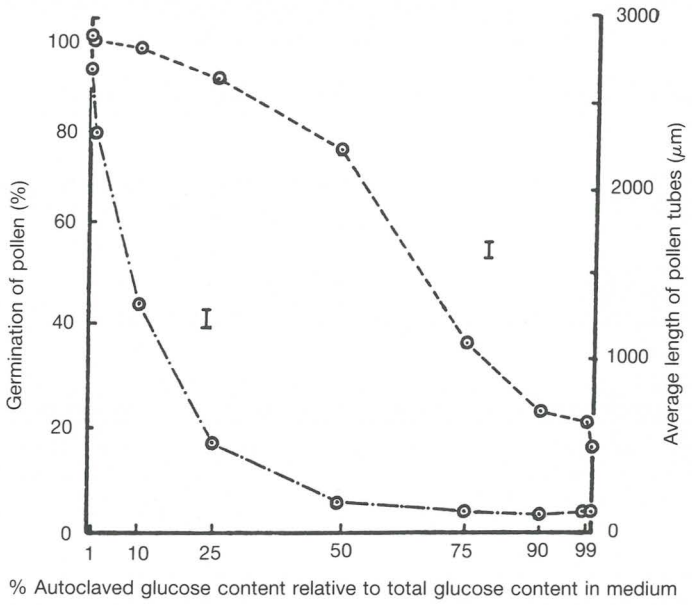

Figure 1 The effect of different autoclaved:total glucose ratios on *in vitro* germination of *A. praecox* pollen (Θ-----Θ) and growth of pollen tubes (Θ–·–·–·–Θ) (vertical bars *LSD* P=0,05).

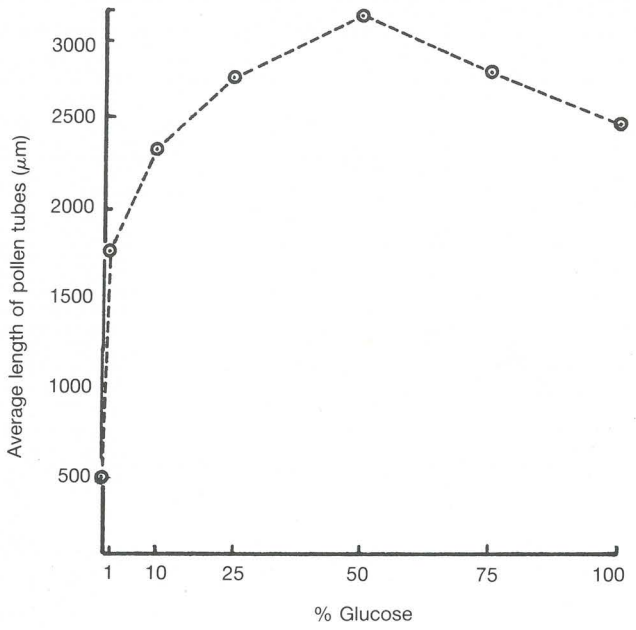

Figure 2 The effect of different molar ratios of glucose and sucrose on *in vitro* pollen tube growth of *A. praecox* (each medium at a total of 0,365 M) (vertical bar *LSD P*=0,05).

Discussion

Autoclaving-induced toxicity

Autoclaving-induced toxicity in *in vitro* germination of pollen and/or pollen tube growth was found to be a general phenomenon in various carboydrates as well as in species of several families. Sucrose and glucose were involved in most of the studies. Autoclaved glucose generally exerted more toxicity than autoclaved sucrose. Even media containing only 1% autoclaved glucose relative to total glucose content, still exerted reductions in pollen tube growth (Figure 1). Treatment of autoclaved sucrose and glucose with activated charcoal effectively eliminated the autoclaving-induced toxicity.

The conversion of sucrose into 5-hydroxymethyl-furfuraldehyde (HMF) upon treatment with 0,25% oxalic acid has previously been demonstrated (Haworth & Jones 1944). Acidic treatment of fructose also resulted in the formation of HMF as well as a number of other unidentified substances (Shaw *et al.* 1967). HMF was shown to be toxic in *Nicotiana tabacum* anther culture (Weatherhead *et al.* 1978). These researchers also analysed media on which anthers had been cultured and recovered *inter alia* HMF. There is a strong possibility that the toxicity involved in the present study could also have been mediated by HMF and other components produced from the carbohydrates during autoclaving.

Hydrolysis of sucrose

Hydrolysis of sucrose upon autoclaving to its component monosaccharides, glucose and fructose, has been reported before. Ball (1953), by using paper chromatography found 0,7 to 0,9% glucose or fructose following autoclaving of Knops' medium (pH 5,5) which originally contained 3,0% sucrose. He also found differences in texture and growth of callus of *Sequoia sempervirens* in autoclaved and filter-sterilized media; callus in the former medium reaching a larger size.

In an experiment not described here, using visual inspection of thin layer chromatograms as a semi-quantitative test, it was shown that, upon autoclaving, sucrose at pH 2 hydrolysed completely or nearly so to glucose and fructose. Considerable hydrolysis still occurred at pH 3 and 4. At pH values 5 to 7, hydrolysis was still evident although to a lesser extent. Tissue culture media generally contain sucrose, are autoclaved and have pH values ranging from 5 to 6. It should always be borne in mind that final media after autoclaving would also contain varying quantities of glucose and fructose.

The performance of *Agapanthus praecox* pollen in *in vitro* germination and pollen growth tests proved to be an excellent demonstration of the importance of considering autoclaving-induced carbohydrate hydrolysis. Compared to sucrose, glucose is a superior carbohydrate in this system. However, autoclaved sucrose solutions detoxified by treatment with activated charcoal proved to be equally effective (Table 2). The results shown in Figure 2 clearly demonstrate that partial hydrolysis of sucrose must have accounted for the apparent discrepancies in Tables 2 and 3.

Some autoclaving-induced toxicity might have resulted from glucose and fructose as hydrolytic by-products of autoclaved sucrose solutions. The limited toxicity produced by relatively low concentrations of these two monosaccharides after autoclaving could have been offset by the beneficial effects of glucose.

In four conparable experiments, *A. praecox* pollen germination was stimulated in each case by autoclaving of sucrose whereas the effect on pollen tube growth was variable:

Table 2	83→95%;	490→282 μm
Table 3	81→92%;	535→567 μm
*	61→96%;	399→491 μm
*	45→65%;	340→308 μm

*Experiments not described in this paper

This phenomenon might have resulted from different ratios of toxin production and hydrolysis in different experiments due to slight differential pH drifts before or during autoclaving. The more consistent stimulation of pollen germination, when compared to the effect on pollen growth, can be explained considering results in Figure 1: pollen tube growth is much more drastically reduced than pollen germination, by small quantities of the toxin(s).

Improved growth in *in vitro* culture of plant tissues and organs by incorporation of activated charcoal in media has been reported by various workers (Anagnostakis 1974; Irikura 1975; Wang & Huang 1976; Wernicke & Kohlenbach 1976; Weatherhead *et al.* 1978; Johansson 1983). This phenomenon has been ascribed to various possible reasons such as darkening of the medium or absorption of toxic substances exuding from plant tissues or produced by autoclaving of the media.

Considering the results in the present study it might be worthwhile in anther and pollen cultures as well as in

studies on *in vitro* pollination of ovules to consider the possibility of autoclaving-induced toxicity/hydrolysis of carbohydrates and to use filter-sterilized or activated charcoal detoxificated media, in addition to choosing the best carbohydrate for the species concerned.

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