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Changes in acidity of plant growth media during heat sterilisation

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INTRODUCTION

Tissue culture media provide ideal conditions for growth of plant cells, but also bacteria and fungi. It is therefore necessary to sterilize media to remove these microbes prior to incubation of explants. Growth media are commonly sterilised by autoclaving at 121°C and pressure of 105 kPa for 15 minutes, or longer for larger volumes (Beyl, 2000). Some components of the growth media such as gibberellins (GA_3) and ca-panthothenate are heat-labile and would become inactive when autoclaved (Nissen & Sutter, 1990). Such heat sensitive components are sterilised by filtering through bacteria-proof membrane (0.22µm pores) and added to the sterilised medium after it has cooled down to at least 60°C. Autoclaving the growth media at 121°C and pressure of 105 kPa for 15 - 20 minutes also breaks down sucrose into D-glucose and D-fructose, resulting in alteration in the osmotic potential of the growth media. Thus, it is important to consider these changes when performing osmotic-sensitive procedures such as protoplast culture. Moreover, the simple sugars resulting from sucrose degradation apparently have inhibitory effects on *in vitro* regeneration of some plant tissues (Dodds & Roberts, 1990).

In our laboratory, autoclaving the commonly used Murashige and Skoog (MS) growth medium at 120°C with pressure automatically set at 120 kPa for 20 minutes consistently caused a decrease in pH from 5.8 to 4.2. The low pH reduced the gelling potential of the agar, thus necessitating use of more agar which increases costs. Moreover, banana explants incubated in this growth media failed to regenerate properly. High acidity (pH below 4.5) inhibits *in vitro* plant growth through destabilisation of growth regulators and precipitation of phosphate and ion salts (Dodds & Roberts, 1990). The challenge of reduced pH made us seek the assistance of others with experiences in similar operations. Thus, the problem was presented and shared online with ProMusa members (http://www.promusa.org/) seeking their opinion and assistance. This article documents the numerous valuable comments that were received from and exchanged between proMusa members.

MATERIALS AND METHODS

Medium preparation procedure in our laboratory: The Plant Tissue Culture Laboratory at Sokoine University of Agriculture in Tanzania micropropagates various vegetatively propagated crops, including banana. In this laboratory, we prepare our own stock solutions and formulate the MS growth media using prescribed ingredients (Mushirage & Skoog, 1962) with slight modification in amounts of growth regulators depending on banana genomic groups and growth behaviour. The medium consists of MS salts, vitamins, growth regulators, brown cane sugar and is solidified using agar (Maerere *et al.*, 2003). The pH of medium is adjusted to 5.8 using a pH meter (Hanna Instruments) that is calibrated to take into account temperature

variations. The medium is first warmed in a microwave oven (Bosch) to dissolve the agar, and then stirred to mix the ingredients thoroughly. Thereafter, the medium is sterilised using an autoclave (SLLI AVX 90.E) at 120°C and 120 kPa for 20 minutes. While autoclaving temperature and time is adjustable, the chamber pressure in this autoclave is automatically set. After autoclaving, the pH of the growth medium (as assessed at 50°C) was routinely observed to drop from 5.8 to 4.2.

ProMusa members' response: The problem of increased acidity of the MS growth medium after autoclaving was presented to ProMusa members to solicit their opinion and ideas on the possible causes and management. ProMusa is a community of scientists working on bananas and plantains (*Musa* spp.) to meet the needs of resource-poor smallholder banana farmers in developing countries. The exchange occurred entirely by email with six members commenting on the issue. Responding members were F. Bakry (CIRAD, France), K.W. Khawar (University of Ankara, Turkey), S. Hamill (Department of Emerging Technologies, Australia), B. Becker (Queensland University Technology, Australia), A. Kiggundu (National Agricultural Biotechnology, Uganda) and A. James (Biotechnology Unit, Centro de Investigacion Cientifica de Yucatan, Mexico). Additional comments were obtained from one non-ProMusa member, J. Boccon-Gibod (Institut National d'Horticulture, France). We documented all the comments and experimented on some of the suggestions in our laboratory.

RESULTS

Based on the comments from ProMusa members, the factors that could be responsible for the variations noted in media pH are summarised into four major categories, i.e. (a) faulty instruments or equipment, (b) improper mixing of growth media, (c) improper autoclaving conditions and (d) hydrolysis of medium components. We experimented on some of the effects, e.g. gelling agents, method of mixing growth media and autoclaving conditions, and the results are shown in Table 1.

Table 1: Effect of gelling agents, autoclave conditions and mixing methods on pH of MS growth medium.

Factor	Treatment	n	Initial pH	Final pH ±SE
Type of gelling agent	Without gelling agent	25	6.8	$6.31^{c} \pm 0.03$
	Agar (Conda- Spain)	25	6.8	$6.15^{b} \pm 0.03$
	Gelrite (Sigma)	25	6.8	$5.85^{a} \pm 0.03$
	Agarose (Sigma)	25	6.8	$5.80^{a} \pm 0.03$
Autoclave temperature and duration	120°C, 120kP for 20 min	20	6.8	$5.17^{a} \pm 0.01$
	115°C, 120kP for 15 min	20	6.6	$5.84^{b} \pm 0.01$
Mixing of media	Use of magnetic stirrer only	20	6.8	$5.61^{a} \pm 0.01$
	Use of magnetic stirrer and hand shaking	20	6.8	$5.60^{a} \pm 0.01$

a, b, c: Means bearing the same superscript letter within the row for each factor are insignificantly ($P < 0.05$) different according to Tukey-HSD test. SE: standard error of the mean. n = sample size.

Autoclaving conditions: The most important autoclaving conditions are temperature, chamber pressure and duration. Adjusting the autoclaving temperature to 118°C and I.0 bar for 15 to 20 minutes is sufficient to sterilise regardless of the volume of the growth medium in the culture vessels (*D. Becker, personal communication*). At a temperature of 120°C and pressure of 120 kPa, the actual maximum temperature in the autoclave chamber is theoretically 125°C (*J. Boccon-Gibod, personal communication*). In our laboratory, autoclaving at 120°C for 20 minutes reduced the pH of MS medium from 6.80 to 5.17 whereas autoclaving at 115°C for 15 minutes caused a drop to pH of 5.84. As the ProMusa members suggested earlier, these data provide evidence that the reduction in pH of the growth media in our laboratory was partly due to higher autoclave chamber temperatures and longer autoclaving duration.

Medium composition: Agar, which is added to media for gelling property, contains acids and undergoes hydrolysis during autoclaving, resulting in substantial

reduction in pH of the growth media. In our laboratory, the pH of liquid MS medium (initially at pH 6.4) dropped to 6.31 after autoclaving. However, the pH of the same medium dropped to 6.15, 5.85 and 5.80 when the same medium was solidified with agar (Conda, Spain), gelrite (Sigma) and agarose (Sigma), respectively. This suggests that the routinely observed medium pH drop in our laboratory was largely due to agar hydrolysis as also reported by the ProMusa members.

Faulty working instruments/equipment: Most members suggested that the autoclave and pH meter could be faulty. For instance, manometers lose their reliability as autoclaves age. Therefore, to verify the actual maximum autoclaving temperature, it is recommended to place a maximum/minimum thermometer in the autoclave chamber. Similarly, the autoclaving duration should be verified using a timing device. In correctly working autoclaves, the programmed duration should be similar to that indicated by the timer. Additionally, the time taken for the temperature to decrease from 120°C to the level at which the autoclave can be opened safely should be monitored. In most autoclaves, this should not exceed one hour. Alternatively, a faulty autoclave can be detected by comparing the pH of the growth medium when another autoclave is used.

Faulty pH meters and variations in the temperature at which pH is measured, can also be causes of false or inconsistent readings. Malfunctioning pH meters can be verified using other pH meters. Since pH is sensitive to temperature, it should be measured at the same temperature always. Alternatively, a pH meter which takes into account temperature variations should be used. It is recommended that the pH of autoclaved media should be measured when the temperature falls below 60°C.

Improper mixing of medium: Large volumes of growth medium are sometimes difficult to mix, especially when small magnetic stirrers are used. Before adjustment, the pH of well mixed growth medium is usually below 5.8, but improper mixing can result in much lower pH. For instance, a pH drop from 5.8 to 5.4 has been reported when medium is improperly mixed up, though it might not be noticed since at this pH, plant cultures still grow well. In our laboratory, we have observed insignificant difference in pH when two methods of mixing media were used. For instance, the pH is around 5.60 when the medium is mixed using magnetic stirrer and 5.61 when mixing is done using both magnetic stirring and shaking the bottle upside down.

Conclusion: Several factors could account for the variations noted in pH during media preparation. These could be faulty equipment, inappropriate autoclaving conditions, improper mixing, and hydrolysis of media ingredients. In our laboratory, we have concluded that the low pH observed after autoclaving media is due to improper autoclaving conditions and hydrolysis of gelling agents during autoclaving. These factors should be regularly monitored during preparation of plant growth media.

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