IN TISSUE CULTURE OF *LILIUM* EXPLANTS MAY BECOME HEAVILY CONTAMINATED BY THE STANDARD INITIATION PROCEDURE

Naser Askari[1,2], YouGuo Wang[1,3], and Geert-Jan de Klerk[1]*

[1]Wageningen UR Plant Breeding, P. O. Box 16, 6700 AA Wageningen,
The Netherlands, *Fax:+ 31 317 418094, *E-mail: geertjan.deklerk@wur.nl
[2]Department of Plant Sciences, University of Jiroft, P. O. Box 364 , Jiroft, Iran
[3]College of Landscape and Horticulture, Yunnan Agricultural University,
Bei ShiQu, Kunming 650201, Yunnan Province, China

Abstract

In tissue culture of *Lilium*, the standard initiation procedure brought about substantial contamination in two ways. (1) When scales were detached from the mother bulb, microorganisms could enter via the wound. This source of contamination was strongly enhanced by the negative hydrostatic pressure within the scales by which nonsterile fluid was sucked up at detachment. Contamination decreased strongly when the scales were detached from bulbs submerged in 0.03% NaClO. Evidence is presented that this type of contamination was endogenous, *i.e.*, localized in the interior of the explant. (2) During the rinsing of scales after surface-sterilization, the rinsing water became contaminated with microorganisms associated with the scales that had not been killed during surface-sterilization. This caused cross-contamination. This type of additional contamination was controlled by rinsing in 0.03% NaClO instead of 'sterile' water. In our conditions, these initiation-related sources of contamination led to ca. 20% and ca. 25% contamination, respectively, of otherwise uninfected scales.

Key words: contamination, chlorine, initiation, lily, *Lilium*, micropropagation, negative hydrostatic pressure

INTRODUCTION

Several microorganisms have been identified as contaminants in plant tissue culture, in particular fungi, yeast and bacteria. Bacterial contamination is most common (Leifert et al. 1991). With respect to their topographical localization, contaminants may inhabit the surface of the tissue (epiphytic) or live within the tissue (endophytic). The former are for the greater part removed by adequate surface-sterilization but for the latter there is no easy treatment. The main obstacle in controling internal contaminants is that within the tissue, antibiotics etc. added via the medium do not reach a concentration sufficiently high to be effective. This is caused by general difficulties in uptake and transport of medium ingredients in tissue-cultured plants (De Klerk 2010). In spite of this, many researchers and companies add antibiotics to the nutrient medium. When the antibiotics are omitted after a number of subcultures, the contaminants always "return". Addition of antibiotics is, however, helpful because they prevent overgrowing of the nutrient medium. Endophytic microorganisms may be beneficial to some extent (Hallmann et al. 1997) but they may also be inhibitory (for example, Pirttilä et al. 2008).

At the time of collecting explants, contaminants are present at the surface of the tissue and within the tissue. Apart from improper handling by operators, for example, carelessness or inadequate flaming (Kunneman and Faaij-Groenen 1988), there are during the initiation procedure two possible ways of infection that are as yet not or only little recognized.

(1) When the explant is excised from the stock plant, open vascular tissue is exposed to the nonsterile environment. Since the xylem has a negative hydrostatic pressure brought about by transpiration (Taiz and Zeiger 2002), neighbouring fluids containing contaminants are sucked up after detachment (*cf.* Van Meeteren 1989). (2) Since it is not feasible to sterilize explants individually, they are processed in batches of 5 to 50 or more. Cross-contamination may occur after the surface-sterilization with concentrated NaClO during the rinsing of explants with sterile water. Usually, the explants are rinsed three times with sterile water (see, e.g., Pierik 1997 and George 1993). Rinsing is done rigorously probably because researchers are afraid that NaClO

affects plant tissues also at low concentration. This is, however, unlikely. Some researchers even add low levels of NaClO during tissue culture to avoid flourishing of microorganisms (Sawant and Tawar 2011, Teixeira et al. 2006, Yanagawa et al. 2007). Researchers ignore the possibility of cross-contamination during rinsing because there seems to be no feasible alternative procedure and because it is believed that the period during which cross-contamination may occur is too short to cause serious problems.

Organs growing underground like bulbs are notorious for contamination (Ziv and Lilien-Kipnis 2000). The aims of the present study are to determine whether contaminants are introduced during the initiation step and if so, to reduce this contamination by using a low concentration of NaClO instead of water.

MATERIALS AND METHODS

Standard tissue culture conditions

Field-grown bulbs (circumference 18-20 cm) of *Lilium* cv. Santander were harvested, cold-treated to break dormancy and stored at -1.0°C until use. The procedure was according to Aguettaz et al. (1990). Scales were surface-sterilized for 30 min in 1% (w/v) NaClO, rinsed for 1, 3, and 10 min with sterile water and after that stored until use in sterile water (on average for 1-2 h). Two explants of 7 x 7 mm were cut from the scales and placed with the abaxial side on 15 ml medium in plastic culture tubes (3.5 cm diameter). The medium was composed of macro- and microelements (Murashige and Skoog 1962), 30 g l^{-1} sucrose, 0.4 mg l^{-1} thiamin, 100 mg l^{-1} myo-inositol, 7 g l^{-1} agar (Microagar), and 0.05 mg l^{-1} NAA (α-naphthaleneacetic acid). The explants were cultured at 25°C and 30 μmol.m^{-2} sec^{-1} (Philips TL 33) for 16 h per day. Contamination was scored at time intervals of 2-4 days for 6 weeks. After 11 weeks of culture, the regeneration percentage, bulblet number and fresh weight per bulblet were determined.

Minimal concentration of NaClO for decontamination of fluids

Twenty nonsurface-sterilized scales were kept for 3 h in 300 ml water to obtain contaminated water. To determine the minimal effective concentration of NaClO in fluids, increasing quantities of NaClO were added to this contaminated water to obtain increasing concentrations (0, 0.01, 0.03, 0.06, 0.1, and 1.5%, w/v) and the solutions were stored for 24 h at room temperature. After that, 2 ml of fluid LB medium (Lysogeny Broth medium, a nutritionally rich medium used for growth of bacteria; Duchefa, Netherlands) was added to 2 ml from each NaClO concentration and incubated at 37°C for 3 days. After that, bacterial growth was evaluated by visual inspection.

Estimation of cross-contamination

Sixty outer scales and 30 inner scales were sterilized for 30 min in one beaker with 1% NaClO solution plus a few drops Tween 20. Then the scales were divided into two groups (30 outer scales and 15 inner scales), distributed over two beakers (so per beaker 45 scales), rinsed three times (1, 3, and 10 min; the first group with sterile water and the second group with 0.03% NaClO), and then stored until use (1-2 h) in water or 0.03% NaClO, respectively. The rinsing fluids were stored at 4°C to examine contamination. Explants were prepared and cultured as indicated above. We monitored contamination of the scales during 6 weeks of culture. The percentage contamination due to cross-contamination was calculated with the following formula:

$$\text{cross contamination \%} = \frac{(\text{contamination\% after water–rinse}) - (\text{contamination\% after NaClO–rinse})}{100(\text{contamination\% after NaClO–rinse})} \cdot 100$$

Estimation of hydrostatic-pressure related contamination

Scales were detached from the mother bulb under streaming water or under streaming 0.03% NaClO and stored in water or 0.03% NaClO, respectively. They were surface-sterilized in the usual way (30 min in 1% NaClO), and rinsed three times with 0.03% NaClO. Explants were prepared and cultured as indicated above. Contamination was monitored for 6 weeks and the hydrostatic-pressure related contamination that occurs when scales are detached from the mother bulb was calculated according the following formula:

$$\text{Hydrostatic pressure related contamination \%} = \frac{(\text{contamination\% after water–rinse}) - (\text{contamination\% after NaClO–rinse})}{100(\text{contamination\% after NaClO–rinse})} \cdot 100$$

Determination of contamination in the rinsing fluids

The rinsing fluids (water and NaClO solutions) were inoculated on LB solid medium in a 9-cm Petri dish and 30 ml LB liquid medium in a plastic test tubes (Duchefa, Netherlands). On the solid medium 25 μl was inoculated and on the liquid medium 30 ml. Bacterial growth was determined after 3 days in dark at 37°C.

Determination of transpiration by scales

Scales were detached from the bulbs, transferred to plastic culture tubes (3.5 cm diameter) with 10 ml water solidified with 0.7% agar and kept standing upright with their basal part on the medium. A layer of 1.5 mm paraffin oil was carefully added to prevent evaporation from the solidified medium. The weight of container + medium + scale + paraffin oil was determined every 60 min and the weight loss was taken as transpiration by the scale. There was negligible weight loss when no scales were present (less than 0.1 μl per hour) showing that almost all weight loss occurred via the scale.

Statistics

In the figures, the means are shown ± SE. The statistical significances of differences in percentages and means were evaluated by the χ^2 and by the Student t-test, respectively. For each treatment, at least 50 explants were taken. Each experiment was carried out at least twice.

RESULTS

Determination of the minimal concentration of NaClO for decontamination of fluids

Bacteria did grow at 0% and 0.01% NaClO (Table 1). The lowest NaClO concentration that fully inhibited bacterial growth was 0.03%. Thus, a 0.03% solution of NaClO is suitabe to prevent contamination after the surface-sterilization provided the tissues are not being damaged.

Estimation of cross-contamination caused by rinsing with water

NaClO remaining from surface-sterilization was remuved by rinsing with water or with diluted NaClO (0.03%). The presence of microorganisms was examined both in the rinsing fluids (Table 2) and in the scale tissues (Fig. 1). The rinsing fluids were examined with solid and liquid LB. No contaminants occured in NaClO rinsing solutions but they were present in rinsing water (Table 2). Bacteria were present in the 3rd rinsing water and in the storage water as shown with both solid and
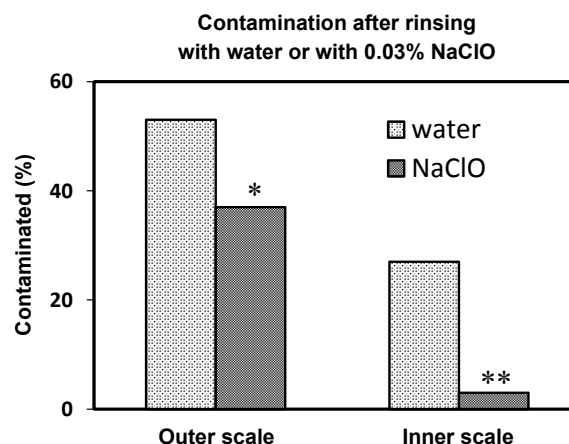

Fig. 1. Contamination of explants cut from inner and outer scales. After surface-sterilization with 1% NaClO, the lily scales were rinsed with water or with 0.03% NaClO. Contamination was monitored for 6 weeks. The significance of the reduction by NaClO-rinsing is indicated by * ($p < 0.05$) or ** ($p < 0.01$).

liquid LB and in the 2nd rinsing water as shown with liquid LB. The bacterial contaminants in the rinsing and storage water expectedly bring about cross-contamination. Cross-contamination can not occur in 0.03% NaClO since at this concentration all contaminants are killed. The 1st rinsing water contained no contamination probably because of a low concentration of NaClO due to carry-over from the surface-sterilization.

Table 1. Minimum concentration of NaClO for disinfection of fluids.

		NaClO concentration (%)					
		0	0.01	0.03	0.06	0.1	1.5
LB liquid medium	1	+++	++	-	-	-	-
	2	+++	++	-	-	-	-
	3	+++	++	-	-	-	-

To contaminated fluid, a concentrated solution of NaClO was added. After 2 days at 25°C, liquid LB was and after another 3 days at 37°C, bacterial incidence was scored. (- not contaminated, ++ medium contaminated, +++ highly contaminated).

Table 2. Contamination of rinsing fluids as detected with LB solid (SM) and liquid (LM) medium.

		Water				NaClO			
		1st rinse (1 min)	2nd rinse (3 min)	3rd rinse (10 min)	Storage (120min)	1st rinse (1 min)	2nd rinse (3 min)	3rd rinse (10 min)	Storage (120min)
Test SM	1	-	-	+	++	-	-	-	-
	2	-	-	+	++	-	-	-	-
	3	-	-	+	++	-	-	-	-
Test LM	1	-	+	++	+++	-	-	-	-
	2	-	+	++	+++	-	-	-	-

In rinsing and storage fluids, contamination was examined with solid and liquid LB. The tests were done 3 and 2 times, respectively. Bacterial incidence was scored after 3 d at 37°C. (- not contaminated, ++ medium contaminated, +++ highly contaminated).
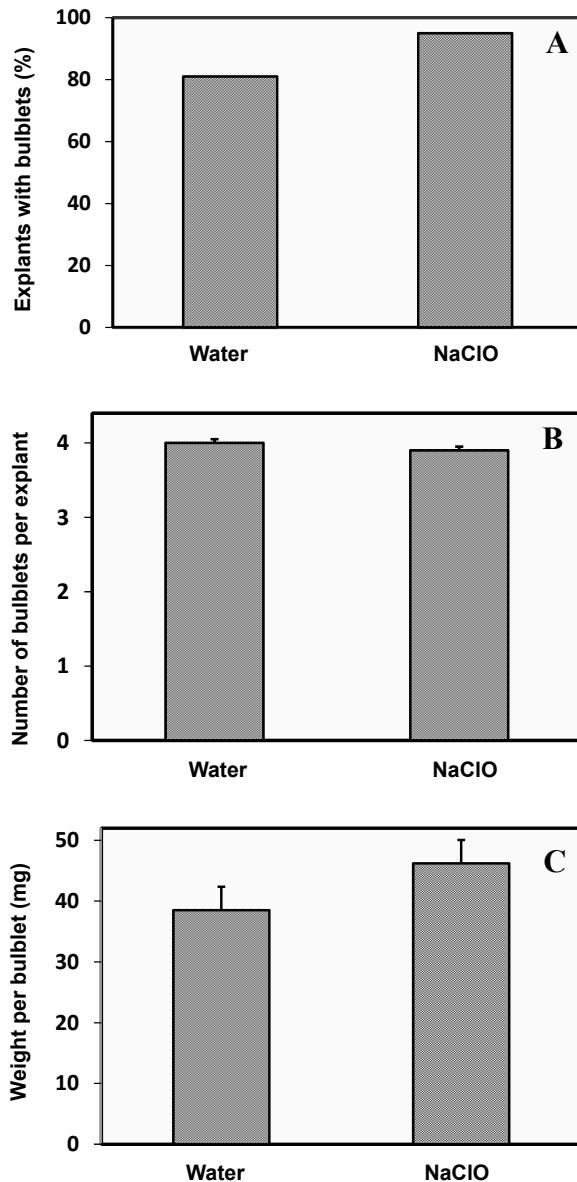
Fig. 2. Performance *in vitro* of explants cut from inner and outer scales. After surface-sterilization with 1% NaClO, the lily scales were rinsed with water or with 0.03% NaClO. The various parameters were determined after 11 weeks in tissue culture. Diluted NaClO (0.03%) did not have a statistically significant effect on the three parameters that had been scored.

Contamination after surface-sterilization can be attributed to incomplete surface-sterilization, endogenous contamination or cross-contamination during rinsing. We assumed that most cross-contamination occurs from outer scale explants (often endogenously contaminated) to inner scale explants (hardly endogenously contaminated). Evidently, there might also be cross-contamination from contaminated to noncontaminated outer scales. In inner scales, the percentage contamination decreased from 27% after rinsing with water to 3% after rinsing with NaClO (Fig. 1, $p < 0.05$). In this case,

most of the contamination in water-rinsed scales was due to cross-contamination during the rinsing. About 25% of the previously uninfected inner scales was cross-contaminated. Rinsing outer scales with 0.03% NaClO reduced the contamination from 53% to 37% (Fig. 1, $p < 0.01$). A similar calculation as done for inner scales showed that in this case cross-contamination also occurred in about 25% of the otherwise noncontaminated outer scales. Inner scale explants showed lower contamination than outer ones: when rinsed with water 27% *vs.* 53% and when rinsed with 0.03% NaClO 3% *vs.* 37%. Contamination in outer scales is high because these scales are often somewhat damaged and because they are much older.

After 11 weeks of culture, fresh weights of lily bulblets regenerated from scale explants rinsed with sterile water or NaClO solution were measured and showed no difference (Fig. 2A). In addition, there is no differences between bulblet numbers (Fig. 2B) and regeneration percentage (Fig. 2C).

Estimation of hydrostatic-pressure related contamination

The xylem in shoots has a negative hydrostatic pressure caused by transpiration. To the best of our knowledge, the rate of transpiration by subterranean organs like bulbs, if any, is not known. Transpiration is 20 µl per hour which equals 5 µl cm^{-2} h^{-1} (Fig. 3). In cacti, transpiration is 5-15 µl cm^{-2} h^{-1} (Larcher 1995). The negative hydrostatic pressure is also shown by water uptake when scales are submerged in water which is 40 µl per scale during the first hour becoming less after that (10 µl h^{-1} after 8 h).

Detaching the scales from the buds in diluted NaClO instead of water reduced contamination from 41% to 25% and from 55 to 37% in two consecutive experiments respectively (Fig. 4; both $p < 0.05$). Contamination by sucking up nonsterile water into the vascular
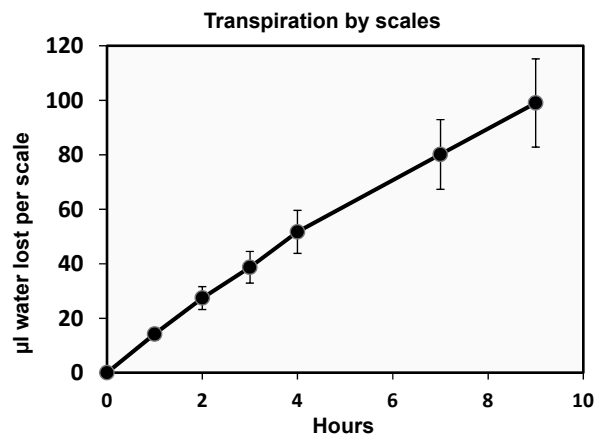
Fig. 3. Transpiration from lily scales at ambient humidity (48%) and temperature (21ºC).
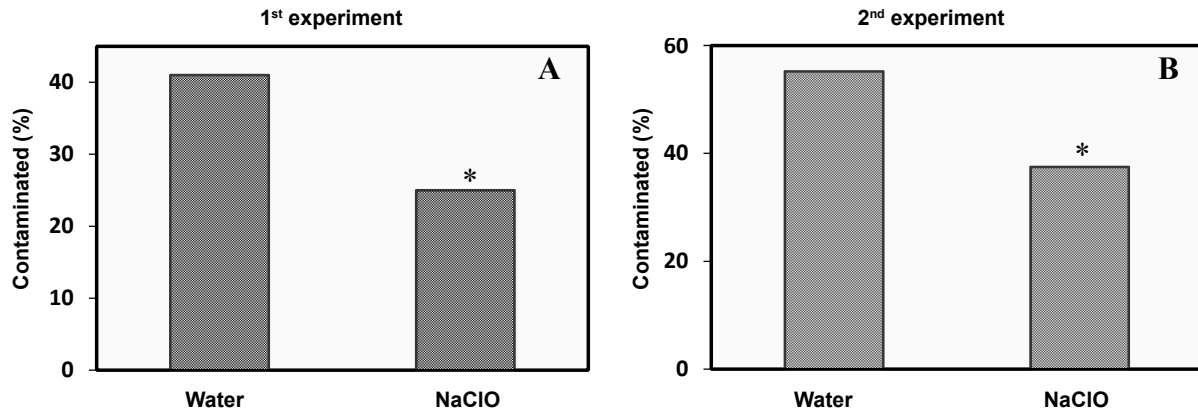
Fig. 4. Contamination of explants cut from inner and outer scales after detaching the scales in streaming water or streaming 0.03% NaClO. Contamination was monitored for 6 weeks. The results of two experiments are shown. The significance of the reduction of contamination by the additional 0.03% NaClO-treatment is indicated by * ($p < 0.05$).

tissues occurred in about 20% of the previously uninfected inner scales.

In explants excised from water-collected scales, the period during which contaminants started to grow outside the explant lasted much longer than in NaClO-collected scales. Therefore, we scored visible contamination occurring in the first week and contamination that appeared after the first week. During the first seven days, the contamination in NaClO-collected and water-collected scales was the same, but after that the water-collected scales showed higher contamination than the NaClO ones (Fig. 5, $p < 0.05$ in the 1st experiment

and $p < 0.001$ in the 2nd experiment). We assume that the period before contaminants started to grow outside the explant reflects the topographical localization of the contaminants. Thus, the contaminants that became visible after one week of culture were located more towards the interior of the explants.

Possibly, the early penetration of 0.03% NaClO into the tissue might inhibit regeneration or the growth of the regenerated bulblets. This was not the case (Fig. 6). As a matter of fact, growth was somewhat (20%) enhanced by the NaClO treatment ($p < 0.01$).
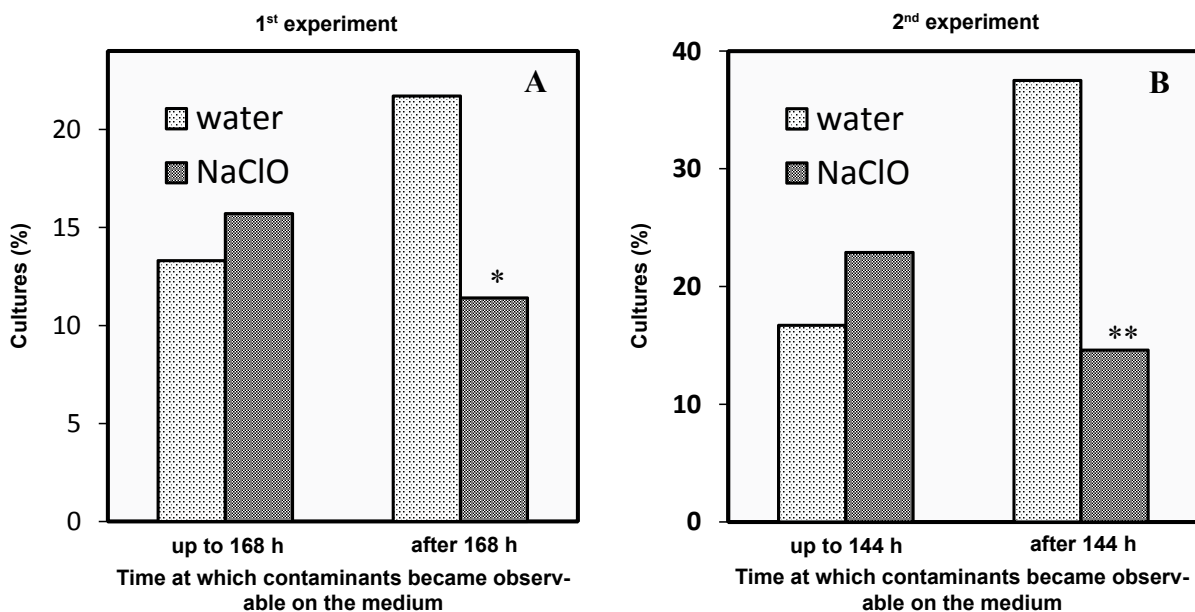

Fig. 5. First appearance of contamination with explants cut from inner and outer scales after detaching the scales in streaming water or streaming 0.03% NaClO. Contamination was monitored for 6 weeks and the appearance during the first week (up to 168 or 144 h) or after that are shown. The results of two experiments are shown. The significance of the reduction by the additional 0.03% NaClO-treatment is indicated by * ($p < 0.05$) and *** ($p < 0.001$).
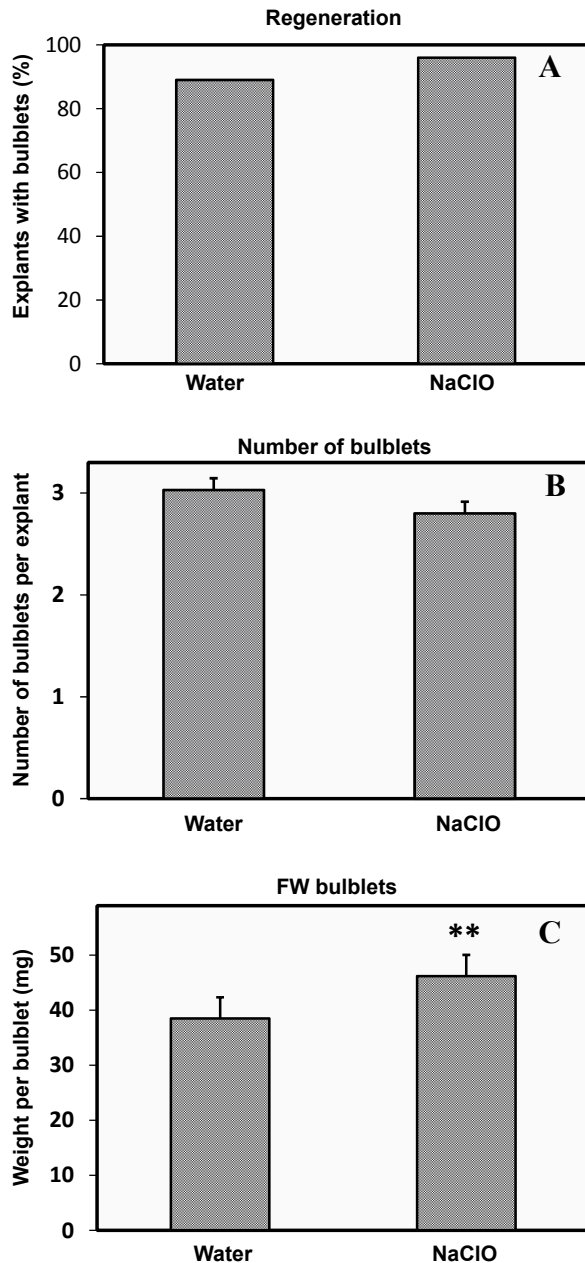
Regeneration

A

Number of bulblets

B

FW bulblets

C

Fig. 6. Performance *in vitro* of scale-explants after detaching scales in streaming water or streaming 0.03% NaClO. The various parameters were determined after 11 weeks of tissue culture. The significance of the effect of the additional 0.03% NaClO-treatment is indicated by ** (*p* < 0.01).

Correlation between right and left explant contamination

We were interested whether explants excised from the same scale have a similar extent of contamination. To examie this, from each scale two explants were excised, one at the left side and the other at the right side. When in the inner scales the left explant was contaminated, 77% of the corresponding right explants were contaminated. When the left explant was not
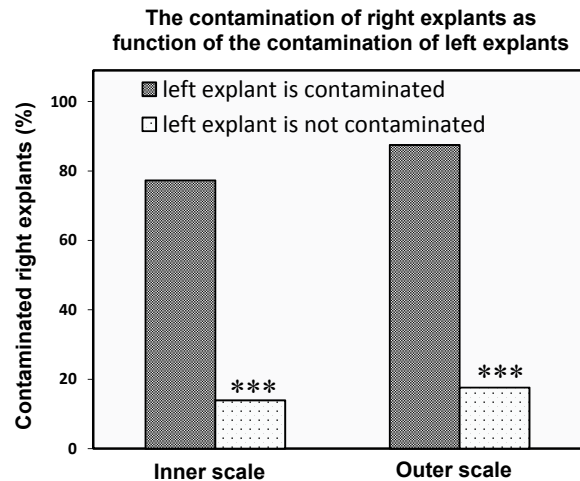

Fig. 7. Correlation of contamination between explants cut from one scale. From scales two explants, one at the right side and one at the left side, were cut. Contamination was monitored for 6 weeks. The diagram shows the contamination percentage of the right explant when the left explant was or was not contaminated. The significance of the difference in contamination of the 'right' explant depending of the contamination of the corresponding 'left' explant is indicated by *** (*p* < 0.001).

contaminated, from the corresponding right explants only 14% was contaminated (Fig. 7). The difference is highly significant. For the outer scales, these percentages were 88% and 15% respectively, also highly significant (*p* < 0.001).

DISCUSSION

Contamination is an everlasting problem in plant tissue culture. Apart from inadequate operating during manipulation in the laminar flow cabinet, poor equipment (*e.g.*, damaged filters in the laminar flow cabinet) and contamination by micro-arthropods (mites and thrips), the source of contamination is the explant that is transferred into tissue culture. In the present paper, we show in lily that explant-related contamination is caused to a substantial extent by faulty procedures during initiation. This may lead to both surface-localized and endogenous contaminants.

Explant-related contaminants may be surface-localized but difficult to kill by disinfectants probably because they are not adequately exposed to the disinfectant solution. Explant-related contaminants may also be internal and because of this not reachable by disinfectants. The latter is caused, among others, by the very slow long-distance translocation of solutes when driven only by diffusion (Taiz and Zeiger 2002). In this case, only a heat treatment may be successful (Langens-Gerrits et al. 1998). Endophytic bacteria are reported to colonize intercellular spaces and vascular tissues and only a few reports demonstrate intracellular

colonization (Hallmann et al. 1997).

In the experiments, we used a low concentration (0.03%) of NaClO for additional disinfection. This concentration was effective (Table 1) and is also reported to be adequate in medical practice (Heling 2001). It should be noted that the target bacteria are moving freely in fluid and are therefore vulnerable. Accordingly, we could use a much lower concentration of NaClO than the one used for surface-sterilization.

First, we examined cross-contamination during the sterilization/rinsing procedure. When tissues from field-grown plants are surface-sterilized, a batch of a few to tens of explants is processed in one beaker because it is unfeasible to process the explants individually in a large number of beakers. When NaClO is present (during the surface-sterilization itself), cross-contamination is not possible. However, during the three rinses with sterile water that are commonly used to remove the excess of NaClO (Pierik 1997, George 1993) and the storage up to processing, cross-contamination may occur. When the scales were rinsed with sterile water for the 2nd and 3rd time, the rinsing water became heavily contaminated with bacteria (Table 2). This resulted in considerable additional contamination of the explants. A simple way to reduce cross-contamination was rinsing with 0.03% NaClO instead of water. After rinsing in a diluted NaClO solution, the performance of the scale explants (regeneration and growth) was the same. The low toxicity (or the absence of toxicity) of a low concentration of NaClO corresponds to studies in which tissue culture was performed in the presence of a low concentration of NaClO (Sawant and Tawar 2011, Teixeira et al. 2006, Yanagawa et al. 2007). Rinsing in diluted NaClO may also be considered for other crops.

The second target of the present study is the open connection between the vascular tissues and the environment when the scales are detached from the mother bulbs. This allows movement of microorganisms into the vascular tissue. Entering is strongly enhanced by the negative hydrostatic pressure in the tissue which results in sucking up of fluids close to the xylem just after excision. These fluids likely contain microorganisms. The xylem has a negative hydrostatic pressure because of transpiration of water from the leaves. In the case of lily bulbs, it should first be considered whether bulbs do have such negative hydrostatic pressure. Bulbs are underground and therefore may not display transpiration. On the other hand, bulbs are modified leaves (in the case of lily swollen petioles) so they are likely to have stomata. Microscopic inspection demonstrated the presence of stomata at both the abaxial and adaxial side (N. Askari and G.J. de Klerk, unp. observations). Water was transpired at a similar rate as in cacti (Fig. 3). As a result, the xylem in lily scales will suck up liquid and contaminants may penetrate into the interior of the scale via this water flow. Provisional calculations showed that with a transpiration rate of 20 μl per scale per hour, water would penetrate in one hour more than 1 cm in addition to the intial penetration just after excision. As a result, contaminants penetrate so far into the interior of scales that disinfectants cannot reach them later on. It is important to note here that the diameter of xylem vessels is 10 to 100 μm and that the diameter of bacteria is a few μm. Thus there are no obstacles withh respect to sizes. Flagellated bacteria may move actively reaching a speed of over 1 m per hour (Schneider and Doetsch 1974) and penetrate the tissue in this way.

We used again 0.03% NaClO to control these contaminants. Indeed, when the scales were during detachment submerged in 0.03% NaClO, contamination decreased in outer and inner scale explants by 15-20% (Fig. 4). Interestingly, in the scales that had been transferred to water and NaClO, the contamination that became visible during the first week was the same but after that, the water-collected scales showed significantly higher additional contamination. Obviously, the more contaminants are located in the interior of the explant, the longer it will take them to reach the nutrient medium. This unexpected finding was reproduced in a second experiment (Fig. 5).

We conclude that after detachment bacteria invade the xylem. When scales are collected in water or in air, these bacteria are transferred to tissue culture. After that, they will gradually exit the tissue and flourish on the nutrient medium. However, after some days or weeks, the wound is repaired and a layer of periderm has been formed. This inhibits uptake of compounds from the medium (Smulders et al. 1990) but also release of microorganisms from the tissue into the medium. So the microorganisms become trapped in the tissue and because of their detrimental effect, growth of the regenerating bulblets may be reduced (Fig. 6). On the other hand, when NaClO has penetrated the xylem at detachment, it will kill the bacteria. NaClO may itself have a negative effect on growth but is presumably short-lived within the tissue. Various compounds promote the decomposition of NaClO, among others various metal ions that are administered with MS. Most notably, chemical interactions between chelating agents and NaClO result in a rapid loss of free available chlorine (Rossi-Fedele et al. 2012) and both plant tissues and MS contain chelating compounds. Thus, within a few days/weeks, NaClO is probably fully decomposed.

To our knowledge, the problem of exposure of xylem tubes in the cut ends to penetration of contaminants has only been dealt with by Thakur and Sood (2006). They report that when sterilizing bamboo, tea and rose shoots with a length of 30-50 cm instead of a few cm, the contamination percentage drops from 50-60% to 20-25%. Apparently, in the long shoots the distance is too large for the penetrating microorganism to reach the upper part of the shoot that is transferred to tissue

culture.

In tissue culture of lily, substantial contamination may be caused during the initiation, both by the entrance of microorganisms directly after detachment of the scales (ca. 20% extra contamination in our conditions) and during the rinsing after surface-sterilization (ca. 25% extra contamination in our conditions). Both are effectively prevented by a diluted solution of NaClO (0.03%). These effective measures in lily are most probably also suitable for other species.

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