**in lily tissue culture, explants may become heavily contaminated by the standard initiation procedure**

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**Abstract**

We report here that in tissue culture of lily the standard initiation procedure caused substantial contamination in two ways. (1) When scales were detached from the mother bulb, microorganisms were able to enter via the wound. This type of contamination is probably strongly enhanced by the negative hydrostatic pressure within the scales by which nonsterile fluid is sucked up at detachment. Contamination decreased strongly when the scales were detached in the presence of 0.03% NaClO. Evidence is presented that this type of contamination is endogenous, *i.e*., localized in the interior of the explant. (2) During 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, micropropagation

# 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 the 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, fungicides and disinfectants 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; De Klerk and Askari 2012). In spite of this, many researchers and companies add antibiotics to the nutrient medium but 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. The present article is about additional explant-related contamination that is caused by the first step in the tissue culture procedure, initiation. Apart from improper handling by operators, for example, 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 motherplant, open vascular tissue is exposed to a 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 together in batches of 5 to 50 or more explants. 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, p. 89 and George 1993, Fig. 56). Researchers ignore this possibility of cross-contamination because there seems to be no feasible alternative procedure and because it is believed that the period in 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. We examined whether a low concentration of NaClO could control these contaminants. It has been reported that NaClO is not toxic at low concentration. 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).

# 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 (on average for 1-2 h) in sterile water. 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 µE.m-2.sec-1 (Philips TL 33) for 16h per day. Contamination was scored at time intervals of 2-4 days for 6 weeks. After 11 weeksof culture, the regeneration percentage, bulblet number and fresh weight per bulblet were determined.

## Minimal concentration of NaClO for decontamination of fluids

To determine the minimal effective concentration of NaClO in fluids, increasing quantities of NaClO were added to heavily contaminated rinsing 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 LB fluid medium (Duchefa, Netherlands) was added to 2 ml from each NaClO concentration and incubated at 37 °C for 3 d. 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*-*2h) 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:

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## 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:

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***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 d 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 the 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 statisticalsigniﬁcances of differences in percentagesand means were evaluated *by* the *χ2*and bythe Student*t*-test, respectively.

# Results

## Determination of the effective concentration of NaClO

Bacteria did grow at 0% and 0.01% NaClO (Table 1). The lowest NaClO concentration that fully inhibited bacterial growth was 0.03%. We used this concentration in the following experiments because a higher concentration might damage the scale tissue.

## Rinsing scales after surface-sterilization with 0.03% NaClO instead of water

After a standard surface-sterilization treatment with 1% NaClO, scales were rinsed with water or with diluted (0.03%) NaClO. The presence of contaminants was examined both in the rinsing fluids and in the scale tissues. The rinsing fluids were examined with solid and liquid LB. Table 2 shows that there were no contaminants in rinsing NaClO solutions but that they did occur in rinsing water. Bacteria were present in the 3rd rinsing water and in the storage water as shown with both solid and liquid LB and in the 2nd rinsing water as shown in liquid LB. The bacterial contaminants in the rinsing and storage water are expected to bring about cross-contamination. Cross-contamination could 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.

After rinsing, explants (7 x 7 mm, two per scale) were cut and cultured on standard lily medium. Contamination was monitored during 6 weeks. 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 (highly 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 (see formula in Materials and Methods). 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).

## Effect of collecting excised scales in 0.03% NaClO or water

As mentioned in the Introduction, the xylem in shoots is under negative hydrostatic pressure caused by transpiration. To the best of our knowledge, the rate of transpiration by bulbs is not known. Figure 3 shows that per scale, transpiration is 20 µl per hour or 5 µl.cm-2.h-1. 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 8h).

In order to inhibit contamination by sucking nonsterile water into the vascular tissues, scales were detached from the mother bulb in streaming 0.03% NaClO or as a control in water and stored in 0.03% NaClO and water, respectively. The actual surface-sterilization (30 min in 1% NaClO) was carried out 60 min later. After that, the scales were rinsed in 0.03% NaClO to remove the excess of NaClO remaining from surface-sterilization. Explants (7 x 7 mm, two per scale) were cut and transferred to standard lily medium. Contamination was monitored for 6 weeks. Detachment in diluted NaClO instead of water reduced contamination from 41% to 25% and from 55 to 37% respectively in two consecutive experiments (Fig. 4; both *P*<0.05). Contamination by sucking nonsterile water into the vascular tissues occurred in about 20% of the previously uninfected inner scales (calculated with the formula in Materials and Methods).

We noted that in explants excised from water-collected scales the period during which contaminants started to grow outside the explant was much longer than in NaClO-collected scales. Therefore, we scored contamination flourishing on the medium in the first week and contamination that appeared after seven days. 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 speed at which contaminants start to grow outside the explant reflects the topographical localization of the contaminants. Thus, the contaminants that become visible after one week of culture are located more towards the interior of the explants.

Possibly the early exposure to 0.03% NaClO inhibited 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).

## Correlation between right and left explant contamination

We were interested whether explants excised from the same scale would have a similar extent of contamination. To this end, we excised from one scale two explants, 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 (=explants excised from the same scale but from the right side) were contaminated. When the left explant was not contaminated, from the corresponding right explants only 14% was contaminated. 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. 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 endogenous and therefore not reachable by disinfectants because diffusion of solutes is very slow (chapter 3 in 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 with only a few reports demonstrating intracellular colonization (Hallmann, et al. 1997). In the present paper, we show that explant-related contamination can also be caused to a large extent by inadequate procedures during initiation. This may lead to both surface-localized and endogenous contaminants.

In the experiments, we used a low concentration (0.03%) of NaClO for additional disinfection. This concentration was effective (Table 1) and is reported to be adequate in medical practice (Heling 2001). It should be noted that the target bacteria are moving freely in liquid and are therefore vulnerable. Therefore, 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 (p. 89 in Pierik 1997; Fig. 56 in George 1993) and the storage up to processing cross-contamination may occur. We showed that when rinsing with sterile water for the 2nd and 3rd time, the rinsing water became heavily contaminated with bacteria. 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. The xylem has a negative hydrostatic pressure because of transpiration of water from the leaves. Bulbs are underground so they may not display transpiration. On the other hand, bulbs are modified leaves, in the case of lily swollen petioles, so they likely have stomata. Microscopic examination demonstrated the presence of stomata at both the abaxial and adaxial side (N. Askari and G.J. de Klerk, unp. observations) and 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 into the interior of the scale. 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. As a result, contaminants are moved 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. Moreover, flagellated bacteria may move actively reaching a speed of over 1 m per hour (Schneider and Doetsch 1974).

We used again 0.03% NaClO to control these contaminants. Indeed, when the scales were during detaching submerged in 0.03% NaClO, contamination decreased in outer and inner scale explants by more than 10-15% (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 leave 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 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 decomposed.

At present, we are carrying out additional experimentation to show the xylem localization of contaminants. 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). By sterilizing bamboo, tea and rose shoots of 30-50 cm instead of a few cm, the contamination percentage dropped from 50-60% to 20-25%. Apparently, the distance was too large for the penetrating microorganism to reach the section of the shoot that was transferred to tissue culture.

# Conclusion

In tissue culture of lily, substantial contamination may be caused during 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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**Table 1. Minimum concentration of NaClO for sterilization of fluids.**

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

A highly contaminated rinsing solution was prepared with lily scales. A concentrated solution of NaClO was added to obtain the indicated concentrations. After 2 d at 25 ºC, 2 ml liquid LB was added to 2 ml of the solution. After another 3 d at 37 ºC, 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, the incidence of microorganisms was examined with LB solid and LB liquid medium. The tests were done 3 and 2 times, respectively. Bacterial incidence was scored after 3 d at 37 ºC. (- not contaminated, ++ medium contaminated, +++highly contaminated)

**Legends to Figures**

**Figure 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).

**Figure 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 w in tissue culture. 0.03% NaClO did not have a statistically significant effect on the three parameters that were scored.

**Figure 3.** Transpiration from lily scales at ambient humidity (48%) and temperature (21 ºC).

**Figure 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).

**Figure.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 144h) 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).

**Figure 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 w of tissue culture. The significance of the effect of the additional 0.03% NaClO-treatment is indicated by \*\* (*p*<0.01).

**Figure 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).