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STERILIZATION OF NERINES USING THE TWIN SCALING TECHNIQUE

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INTRODUCTION

Nerine, a genus belonging to the Amaryllidaceae family, is becoming an important ornamental bulbous plant in New Zealand. Already there have been large plantings of *Nerine* in New Zealand. *Nerine* species have a high potential as export cut flowers. However the natural multiplication rate of *Nerines* is fairly low. Although seed propagation can increase plantlet production by 1,000-fold, such a method does not maintain hybrid traits important for commercial crop production (5).

Large bulbs may only produce only a few daughter bulbs each year. To raise this multiplication rate growers have used a method known as "twin-scaling". This technique involves dividing the bulb into small portions, each consisting of a section of the basal plate. Grootaarts, et al. (2), showed that *Nerine bowdenii* bulblet regeneration always occurred at places where scales contained basal-plate tissue. This technique can be used *in vitro*, for most bulbous species including *Nerine* and *Narcissus* (1). Pierik and Ippel (4) developed the first *in vitro* twin-scaling technique for *Nerine bowdenii* and *Nerine sarniensis*. Bulblet regeneration from twin-scales *in vitro* is dependent on the size and age of the explant material as well as the position in relation to inner and outer regions of the bulb. Twin-scales taken from the outer region of *Nerine* bulbs, seem to regenerate bulblets better than those from the inner bulb (2).

Tissue culture techniques have been used commercially for *Nerine*. Various explant sources such as flower stems, twin-scales,

and axillary shoots have been used (1). At the Department of Scientific and Industrial Research (DSIR), Palmerston North, New Zealand some *Nerine sarniensis* hybrids have been successfully micropropagated using flower stems (Balasingam, pers comm). Micropropagation of *Nerine* using twin-scale explants to form bulblets has not been attempted at DSIR. More recently, several commercial laboratories including Multiflora in Auckland, New Zealand, and Twyfords in England, have been successful using micro-twin-scaling *in vitro*. However, contamination by micro-organisms, especially fungi, is a problem in commercial laboratories. Contamination rates of up to 70% can occur. (Beynon, pers. comm.).

The objective of these experiments was to reduce the rate of contamination of *Nerine* twin scales by effective sterilization procedures and the use of systemic fungicides.

Three strategies were investigated:

1. Standard treatment of whole bulb with hypochlorite.
2. Treatment of bulbs with systemic fungicides and hypochlorite treatment.
3. Treatment of bulbs with fungicide and hypochlorite followed by dissection of twin-scales and resterilization with fungicide and hypochlorite.

MATERIALS AND METHODS

Plant material. Flowering size bulbs of *Nerine sarniensis* hybrids were collected from plants cultured under glass. Bulbs were collected from May 1987 to June 1987.

Experiment 1.

Step 1. The upper third of the bulb was removed together with the lower brownish part of the basal plate and roots. All soil was removed by washing. Bulbs were then left in a beaker of running tap water for 30 min. This procedure was used to reduce the possibility of contamination. Any visible necrotic tissue was also removed (Figure 1).

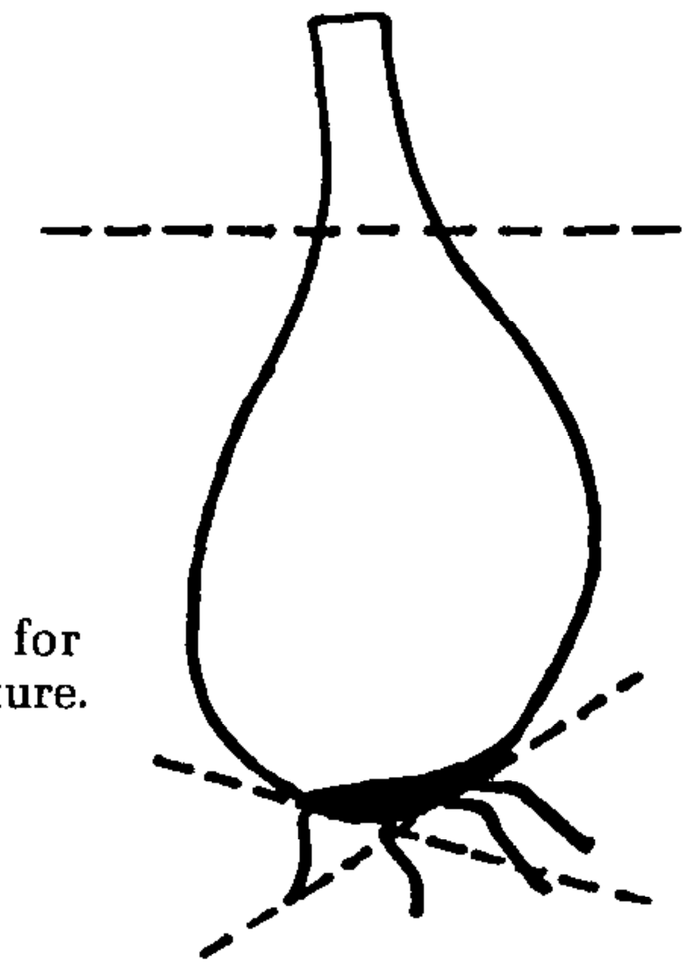
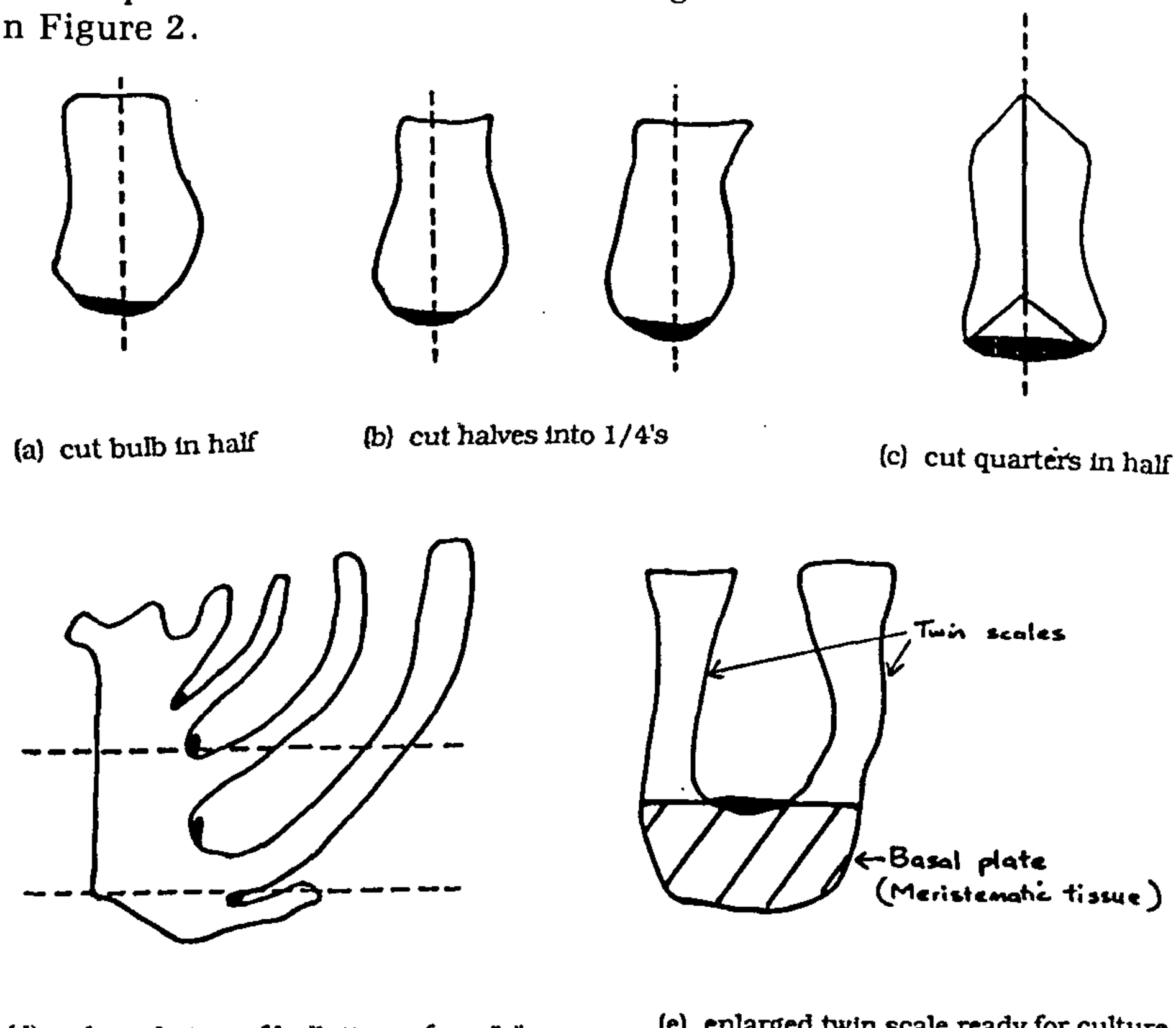


Figure 1. Clean-up procedure for *Nerine* bulbs before culture.

Step 2. Bulbs were surface sterilized in a 20% bleach solution (1% available chlorine) in 250 ml sterile jars and then placed on a shaker for 25 minutes. The bulbs were then rinsed three times in sterile water.

Step 3. Bulbs were then cut into segments for culture as shown in Figure 2.



(a) cut bulb in half (b) cut halves into 1/4's (c) cut quarters in half

(d) enlarged piece of bulb tissue from "c" (e) enlarged twin scale ready for culture

Figure 2. Method used for excising twin-scales from *Nerine* bulb tissue (a–e).

When excising bulb twin-scales, care was taken not to damage the basal plate tissue (scales were 1 to 1.5 cm long × 0.5 cm in diameter).

Step 4. Explants were placed directly into 15cm by 2cm Pyrex tubes containing 10 mls of Murashige and Skoog (MS) medium (3) with no cytokinin or auxin. Difco-Bacto agar was used at 0.7%. The tube was sealed and kept at a constant temperature of 25°C.

Experiment 2.

The same procedure was followed as for Experiment 1, using surface sterilization of the bulb in 20% bleach for 25 min. An additional treatment of 0.2% Benlate fungicide solution soak of the bulbs in 250 ml jars for 30 min. on a shaker was given followed by rinsing three times in sterile water. Bulbs were then excised into small 1 to 1.5 cm scale segments as described in Experiment 1 Step 3

a–e. Explants were then cultured onto petri dishes containing 10 mls of MS media.

Experiment 3.

Bulbs were surface-sterilized with 20% bleach for 25 min. on a shaker, then rinsed three times with sterile water. Bulbs were further treated with a 0.2% solution of Benlate on a shaker for 30 min. and rinsed three times with sterile water. Twin-scale explants were dissected out and resterilized in 0.2% Benlate for 30 min., rinsed three times with sterile water and then given 20% bleach for 25 min. followed by three rinses of sterile water.

Experiment 4.

Bulbs were surface-sterilized with 20% bleach for 25 min., rinsed three times in sterile water, and then given 0.2% Benlate for 30 min. followed by three sterile water rinses. Twin scales, 1 to 1.5 cm, were excised from the bulbs and treated with 0.2% Benlate solution for 30 min. then 10% bleach and rinsed three times with sterile water. Jars were placed on a shaker through all treatments. Explants were cultured on petri dishes containing 10 mls of MS medium.

Experiment 5.

Bulbs were surface sterilized with 0.2% Benlate for 30 min., rinsed three times in sterile water and then given 20% bleach for 25 min. Twin-scale explants were then excised and resterilized with 0.2% Benlate for 30 min., rinsed three times in sterile water, and then given 20% bleach for 25 min. followed by three rinses in sterile water.

All experiments were kept at a constant temperature of 25°C.

RESULTS

Figure 3 gives a summary of the results. The following results were observed and recorded.

Experiment 1. After two weeks in culture, 97% contamination was recorded. The contamination was 80% fungal and 70% bacterial infection.

Experiment 2. After one week 90% contamination was recorded.

Experiment 3. After one week 15% contamination was recorded.

Experiment 4. After one week 10% contamination was recorded.

Experiment 5. After one week the contamination was 6%.

For identification of contaminated tissue, pure cultures were made of the fungi and bacteria, and identified by Massey University Pathology Department. The fungus *Cladosporium* spp., a common contaminant, was found not to be a pathogen in this case. Bacteria, also was not found to be a plant pathogen.

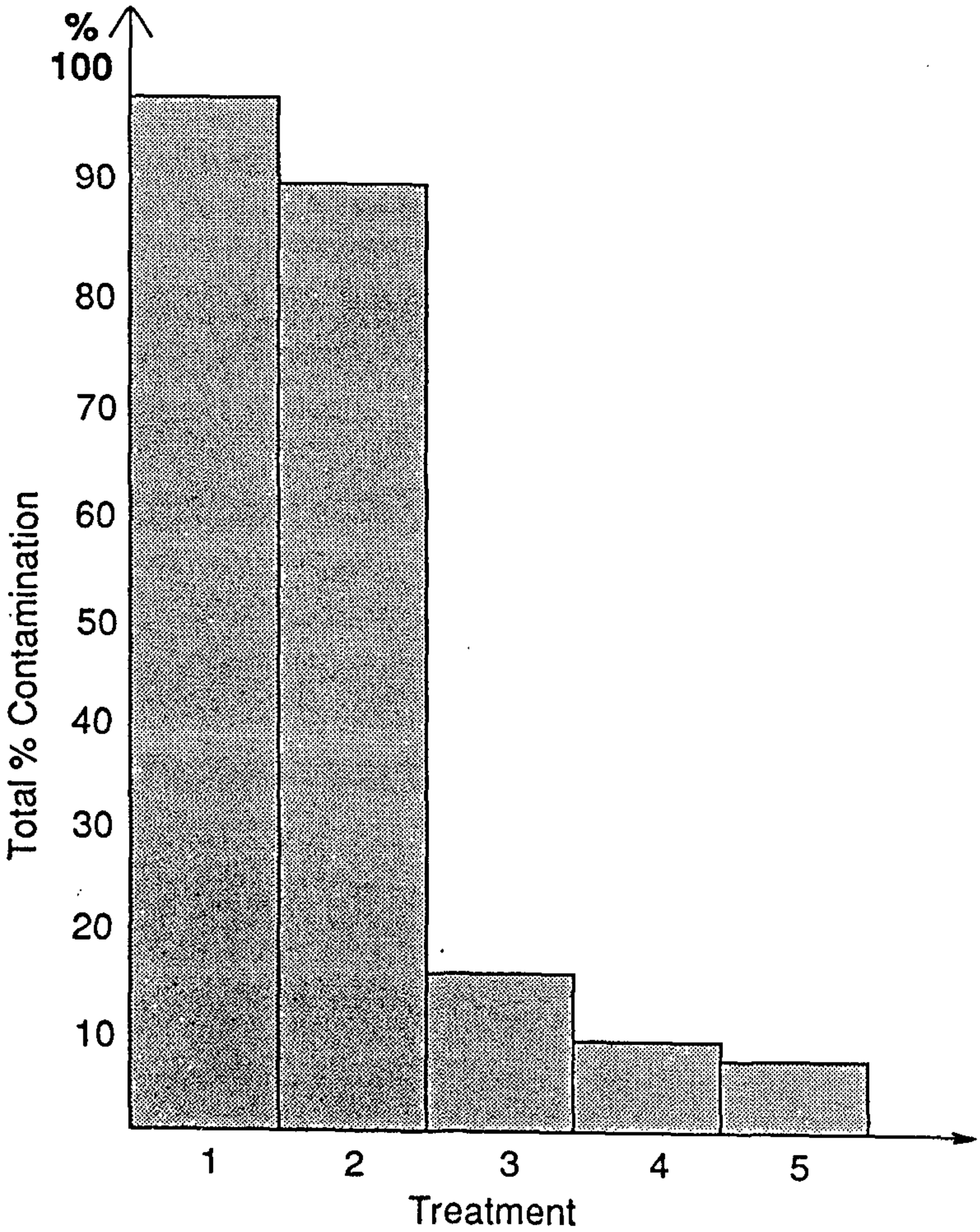


Figure 3. Percentages of contamination for Experiments 1 to 5.

DISCUSSION/CONCLUSIONS

Because bulb tissue is directly in contact with the medium or soil, the risk of contamination from soil pathogenic fungi and bacteria is greatly increased. Bulb tissue is difficult to free from contamination for tissue culture purposes. Up to 60% of double scale segments and 40% of basal plate tissue from hyacinth can be contaminated (1). This rate is high compared with the adventitious plantlet regeneration from floral stem explants of *Nerine bowdenii* W. Watts. The contamination rate for this method was much lower at 5 to 10% (5).

The first method for twin-scaling of *Nerine bowdenii in vitro* (4), used a sterilizing technique by immersion of quarters in 70% ethanol for a few seconds. The quarters were then rinsed in 20% bleach for 25 min., then three times in sterilized water for 25 min. When this procedure was followed carefully an infection rate of 10% was found (4). In the experiment reported here ethanol treatment was not used because of the risk of damaging the delicate twin-scales. Also, in the preliminary experiments 1 and 2, fungal contamination was the main pathogen, thus the reason for introduction of a fungicide treatment.

From the experiments conducted here it is evident that surface sterilization of the excised twin-scales is necessary to reduce the contamination rate of *Nerine sarniensis* hybrid bulb tissue. Even with the treatment of bulbs with 20% bleach *and* 0.2% Benlate, a 90% contamination rate was still recorded. Compare this with the next treatment in experiments 3 to 5, with treatment of bulbs *and* resterilized excised twin-scales where only 6 to 15% contamination was found.

This project, however, has only researched the sterilization of twin-scales of *Nerine sarniensis* hybrids, and further research is needed to induce bulblet information on the twin scales before this method can be safely used on other members of the Amaryllidaceae.

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