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Oxygen Stress in Tulip Bulb Scale Micropropagation

By

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Summary

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In this report we describe the measurement of a number of physiological factors related to oxygen stress in the regeneration of tulip bulb scale explants with the purpose to determine the role of oxygen stress in this system. In order to get a good reference for the importance of the different physiological factors in regeneration we used a set-up that made it possible to distinguish between explants which showed a satisfying regeneration and explants showing no regeneration. Furthermore, we used tulip stalk explants, which show generally good regeneration, and incubated them at 2, 20 and 100 % oxygen. No indications were found for oxygen stress as an important cause for the unsatisfactory reaction of bulb scale tissue in micropropagation experiments. The degree of desaturation of lipid fatty acids remained high and the fraction of 18:3 fatty acids even increased considerably. The oxygen stress-related reaction of the scale explants which showed no regeneration was generally similar to that of scale and stalk explants showing cell division/ regeneration. However, the activities of some enzymes decreased (phenylalanineammonialyase, peroxidase, lipoxygenase) or showed a smaller increase (polyphenoloxidase) in regenerating scale explants, while they remained high in non-regenerating explants showing low viability. Apparently, the latter explants show an irreversible loss of the ability to regenerate, which is accompanied by a loss of vitality without a clear indication of oxidative stress.

Introduction

Propagation of tulip is strongly hampered by the long time (25-30 years) which traditional vegetative propagation methods take before a new variety can be introduced into the market; micropropagation *via* tissue culture might solve this

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problem. Although micropropagation using stalk explants as starting material has been quite successfully employed (TAEB & ALDERSON 1990), the use of bulb scale explants still yields a lot of problems. Although bulb scales are available throughout the year, successful regeneration appeared possible only during limited periods (NISHUICHI & KOSTER 1988). The bulb scale explants suffer from extreme browning, which eventually results in death of the explant. In this paper we studied the possible role of oxidative damage as a cause of this vitality loss of the explants. This was done by using an experimental set-up in which we were able to track the history of a particular bulb scale explant. It appeared that two types of bulbs existed; one showing a satisfying regeneration (adventitious bud/ shoot formation on at least two third of the explants) and one showing no regeneration at all (VAN ROSSUM, unpublished results). By comparing the physiological properties of the explants derived from these two populations of bulbs, we attempted to identify factors which determinine viability and regenerability.

To examine possible effects of oxygen stress on these processes we compared the reaction of explants under normal tissue culture conditions (20% O_2) with that of explants under low oxygen conditions (2% oxygen/ 98% nitrogen) and high oxygen conditions (100% oxygen) and measured the various physiological parameters. Another tool to investigate the physiological basis of the problems encountered in bulb scale explant micropropagation is comparison of this system with tulip stalk explants which provide us with a good regenerating system within the same species. Comparison of physiological changes in these systems in relation to regeneration and viability, was used to pinpoint factors that might play a role in the regeneration process.

Material and Methods

Tulip bulbs were fieldgrown and harvested in early July. For bulb scale explants, the bulbs were stored at 30°C in the dark prior to use. Bulb scale explants were cut from the basal part of the second and third outermost scales, just above the basal plate in the end of July and early August. Explants were ± 2.5 mm thick and weighed 70-100 mg. Explants were grown on full-strength MS medium (MURASHIGE & SKOOG 1962), containing 3% sucrose, 0.5 g/l casein hydrolysate (Gibco), 0.1 g/l myo-inositol, 0.1 mg/l thiamine-HCl, 0.5 mg/l pyridoxine HCl, 0.5 mg/l nicotinic acid, 1 mg/l 2,4-D, 1.5 mg/l BAP and 6 g/l agar (BBL); the pH was adjusted to 6.0 prior to autoclaving the medium. The explants were cultured for 10 weeks at 20°C in the dark, either under atmospheric (20%) oxygen conditions or in aeration chambers, in 2% oxygen/ 98% nitrogen or in 100% oxygen.

For stalk explants the bulbs were planted in October, grown for 2 weeks at 9°C and subsequently stored at -2°C for at least 6 weeks prior to use. After isolating the stalk from the bulb tissue, six explants were cut around the first node and cultured as described for bulb scale explants.

After 10 weeks all explants were evaluated: scored was for callus or shoot formation and for browning. The reaction of the explants was scored, according to the following classifications: normal callus formation (1 point), extensive callus formation (2 points), beginning of shoot formation (2 points) or action shoot formation (4 points). For browning a negative score was assigned to the explant (-1 point). Each explant was individually scored, and the mean vitality score for the explants derived from one bulb was calculated. If the explants were scored for shoot formation only a direct correlation with this method of scoring was found.

Enzyme extracts were prepared as described by BENSON & al. 1992. Catalase and peroxidase activities were determined according to BENSON & al. 1992, lipoxygenase (LOX) activity was determined according to SANZ & al. 1992, phenylalanineammonialyase (PAL) activity in a modified protocol according to ZUCKER 1965 and superoxide dismutase (SOD) in a modified protocol according to BEYER & FRIDOVICH 1987. Polyphenoloxidase (PPO) was determined according to SÖDERHÄLL 1995. Fatty acid composition determinations of phospolipid were performed according to HOEKSTRA & VAN ROEKEL 1988.

Results and Discussion

Growth and regeneration

Clear differences in regeneration were found for bulb scale explants cut in July or August. Explants showing extensive callus or bud formation were only found during incubation at 20 % oxygen. Two populations of bulbs were found: one yielding explants showing satisfying regeneration (at least two third of all explants) and one giving rise to explants with a low regeneration potential when scored after 10 weeks of incubation. All explants incubated under 2% or 100% oxygen belonged to the group with no or low regeneration, indicating neither 100% nor 2% oxygen conditions had a positive effect. (Table 1)

Explants from tulip stalks reacted differently: the explants grown in a 100% oxygen environment showed the largest increase in fresh weight, while the explants that were incubated under 2% oxygen showed the smallest fresh weight increase. Two procent oxygen conditions resulted also in the formation of the lowest number of shoots, while the largest number of shoots was found on stalk explants incubated at 20% oxygen (data not shown).

Table 1. Vitality scores per explant determined as described in materials and methods section. The data are means and derived from more than 10 bulbs. The groups are significantly different from each other ($p<0.0005$).

Tissue type	oxygen condition	explant score
scale	2 %	-0.1
	20 % regenerating	2.7
	20 % non regenerating	1.0
	100 %	0.6
stalk	2 %	1.0
	20 %	3.7
	100 %	2.8

Fatty acid composition of phospholipids

The effect of the differences in tissue culture performance in various physiological parameters was only limited. For instance, the changes in fatty acid composition (Fig. 1), were identical in both regenerating and non-regenerating bulb scale explants. Linoleic acid, which was the most abundant fatty acid, decreased during the first ten days of incubation from 70% (in the starting material) to 50%, not changing appreciably afterwards. This was accompanied by an increase in the fraction of linolenic acid from less than 1 % to about 15 %.

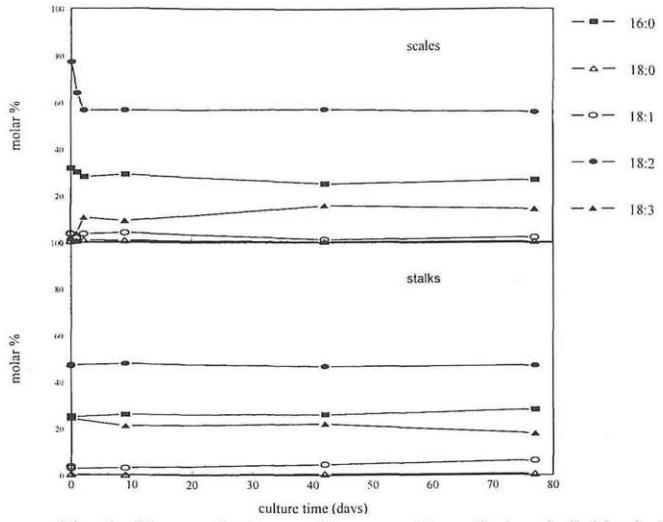

Fig. 1. Changes in fatty acid composition of phospholipids during incubation of tulip explants. For regenerating and non-regenerating bulb scale explants the changes were identical.

The fractions of the other fatty acids found (palmitic (16:0), stearic acid (18:0) and oleic acid (18:1)) did not alter upon incubation of the bulb scale explants. Very rapid changes (within the first hours) were not observed. In the well-regenerating stalk explants no changes in fatty acid composition were found upon incubation, the composition was more or less similar to that of bulb scale explants after several weeks of incubation. These results suggest that the changes in the fatty acid composition in bulb scale explants are not related to regeneration performance. Since the observed changes lead primarily to a exchange of linoleic acid for linolenic acid the unsaturation of the membranes increases (double bond index changed from 1.43 to 1.54). In the case of oxygen stress an opposite effect would be expected (HOEKSTRA & VAN ROEKEL 1988). The changes in the bulb scale explants lead to a composition similar to that of well regenerating stalk explants.

Changes in enzyme activities

In bulb scale explants not only a two-fold increase of the protein content per FW was observed, but also the activity of a number of enzymes upon cutting and incubation. The activity of LOX (Fig. 2) increased immediately after cutting bulb scale explants suggesting a wounding respons; it decreased again in regenerating explants but remained high in non-regenerating ones. In stalk explants no rapid increase upon cutting was found. SOD activity (Fig. 2) showed some changes (e.g. a rapid increase upon cutting) in both types of scale explants but no differences between non-regenerating and regenerating scale explants the activity remained high but in regenerating scale explants the increase was followed by a gradual decrease in activity of both enzymes. In stalk explants an increase in the activity of both enzymes was observed; highest activities were found for explants incubated under normal oxygen pressure. Polyphenoloxidase (Fig. 4) showed an increase but for this enzyme no clear decrease was found. However, the activity in non-regenerating explants and in stalk explants under 2 % oxygen was higher than the

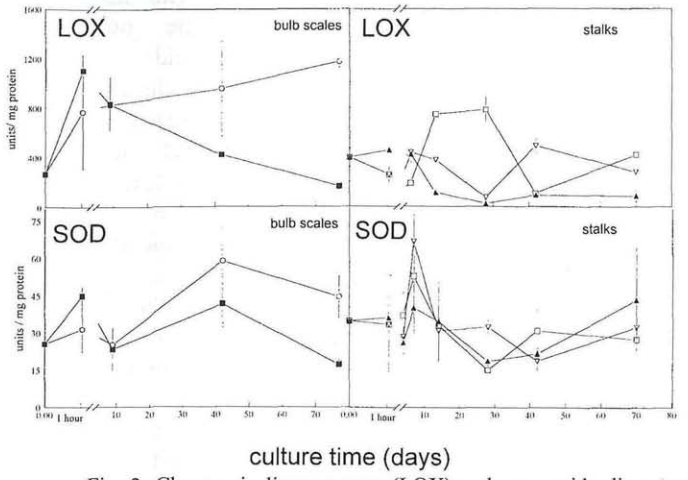

Fig. 2. Changes in lipoxygenase (LOX) and superoxide dismutase (SOD) activities during incubation of tulip explants: regenerating (■) and non-regenerating(❍) bulb scale explants (left), and stalk explants (right) incubated under 20% (▲), 100% (▽) or 2% (□) oxygen conditions.

activity in regenerating bulb scale explants or stalk explants incubated under either 100% or 2% oxygen. The activity of PAL (Fig. 4) showed a strong increase in the first week of incubation (not immediately upon cutting) and the activityof this enzyme remained high in non-regenerating scale explants.

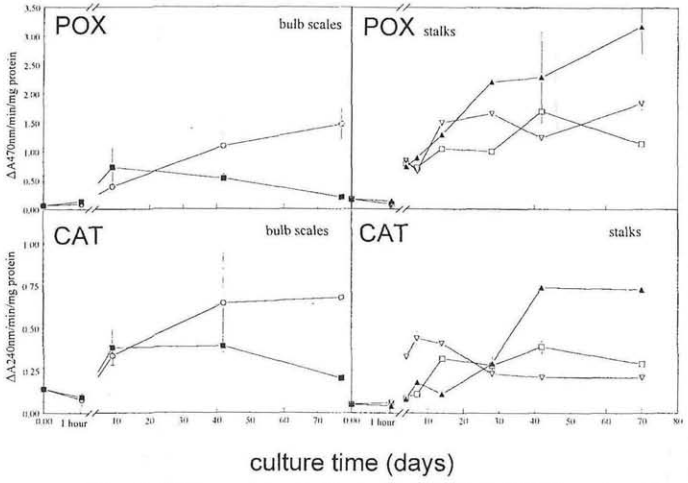

Fig. 3. Changes in activities of peroxidase (POX) and catalase (CAT) during incubation of tulip explants. For legends, see Fig. 2. Values are means ± SD and derived from four replicate determinations.

A gradual decrease occurred in regenerating scale explants as well in all stalk explants, incubated either under 100%, 2%, or 20% oxygen con-ditions.

Since several parameters of the oxidative stress response did not differ consistently bet-ween regenerating tissue (bulb scale and stalk explants) and non-regenerating bulb scale explants, no clear indications for oxidative stress as the cause of the differences in vitality were found. Also, when the bulb or stalk explants were incubated at 2% or 100% oxygen

conditions, to increase or decrease observed were especially found in later stages of incubation when the activities of oxygen stress, no clear differences in stress parameters were seen. Differences several oxygen stress related enzymes decreased in the regenerating sca-le tissue and remained high in non-regenerating explants. These differences might therefore be secondary effects and not directly related to the morphogenic reaction of the tissue. We suggest that the problems encountered during micropropagation of tulip bulb scale explants are not directly caused by a general insufficient protection against oxygen stress-related damage.

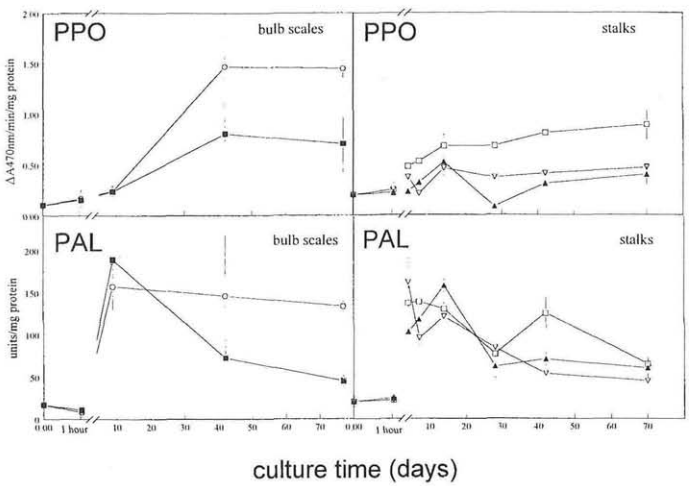

Fig. 4. Changes in activities of polyphenoloxidase (PPO) and phenylalanine ammonia lyase (PAL) during incubation of tulip explants. For legends see Fig. 2. Values are means ± SD and derived from four replicate determinations.

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