German Society of Horticultural Sciences (DGG)
Lentzeallee 55/57
Humboldt-Universität zu Berlin
D-14195 Berlin

DGG-Proceedings, Vol. 5, 2015

Short Communications – Annual Conference DGG and BHGL
24.02. - 28.02. 2015, Weihenstephan, Germany

Peer Reviewed

DGG-Proceedings, Vol. 5, Sep. 2015, No. 12, p. 1-5. DOI: 10.5288/dgg-pr-05-12-cc-2015

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DGG-Proceedings, Vol. 5, Sep. 2015, No. 12, p. 1-5. DOI: 10.5288/dgg-pr-05-12-cc-2015

Effect of glutathione on the early differentiation of *Cyclamen persicum* somatic embryos

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1. Introduction, Knowledge, Objectives

Cyclamen persicum (Myrsinaceae) is an ornamental pot plant and is propagated by seeds. However, seed propagation requires manual pollination and in some cultivar groups results in the variability of the offspring. Somatic embryogenesis (SE) could become an alternative vegetative propagation pathway (Wicart et al., 1984; Schwenkel and Winkelmann, 1998). However, the use of SE for commercial scale propagation is still limited due to physiological disorders, such as low tolerance for desiccation (Seyring and Hohe, 2005), non-synchronized development of somatic embryos and the lack of a maturation step leading to low germination rates (Schmidt et al. 2006).
Among the factors that could be associated with the physiological obstacles above mentioned, reactive oxygen species (ROS) are the focus of recent studies. ROS are transient species that include free radicals, e.g. radical superoxide (O_2^-) and other reactive intermediates, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO). The important role of ROS as signaling and regulatory molecules during plant morphogenesis and development has been shown (Schmidt and Schippers, 2015). A complex network of low molecular weight molecules, among them glutathione, with an anti-oxidative capacity seems to control ROS concentration and oxidative repair. It has been demonstrated that alterations in the glutathione redox state, i.e. the ratio of reduced glutathione/oxidized glutathione (GSH/GSSG) by adding the respective form to culture media, resulted in positive effects on the quality and amount of somatic embryos (Belmonte et al., 2003, 2005; Stassola et al., 2008). In the initial development of somatic embryos, the supplementation of GSH to the culture medium resulted in a higher amount of pro-embryos, while in the later maturation step, the inversion of that ratio by the addition of GSSG led to an increased number of normal embryos (Stasolla, 2010). Therefore, the aim of this study was to investigate the effect of exogenous GSH on the early embryogenesis in *Cyclamen persicum* in order to increase the number and quality of somatic embryos.

2. Material and Methods

Embryogenic cell cultures were initiated from ovules of genotype 56/2 (one single plant of the F1 hybrid cultivar ‘Maxora Light Purple’ (Varinova BV), as described by Schwenkel and Winkelmann (1998). The suspension cultures were established following Winkelmann et al. (1998). In experiment 2, cell cultures of two other genotypes (2824 = plant of cv.

Miracle White, Syngenta, CNP 6 = plant of cv. Super Series Compact Neon Pink, Morel) were used that had been established in the same way.
The embryogenic suspensions were sieved through a 1000 µm mesh and the density was adjusted to 4 % packed cell volume using liquid plant growth regulator (PGR)-free differentiation medium (Schwenkel and Winkelmann, 1998) with different concentrations of GSH: 0, 4, 6, 8 and 10 mM GSH in the first experiment and 0 and 4 mM GSH in the second experiment. Each treatment consisted of three 100 ml Erlenmeyer flasks containing 20 ml of cell suspension. After 14 days of culture on a rotary shaker at 100 rpm and 24 °C in darkness, the cell cultures were collected on a sieve of 200 µm mesh and 200 mg were plated in 6 cm Petri dishes containing 15 ml of solidified PGR-free differentiation medium and cultured at 24 °C in darkness. The experiment was composed of three blocks and each block contained 3 Petri dishes. The number of somatic embryos in the torpedo stage was evaluated at day 28. The experiments were repeated twice.
After 28 days torpedo stage somatic embryos were collected and transferred to fresh differentiation medium for the conversion into seedlings (germination). After 30 days of culture at 24 °C in darkness, the percentage of converted embryos was evaluated by counting the somatic embryos with cotyledons of at least 1 cm length.
At day 14, i.e. at the end of the treatment in liquid culture, the cell viability was determined by staining with fluorescein diacetate (FDA) as described in Winkelmann et al. (1998). The localization of superoxide (O_2^-) was performed by staining with nitro blue tetrazolium (NBT) following the method described by Pietrowska et al. (2014). The data were subjected to F-max test to verify the homogeneity of variance. Percentage data were transformed to $\sqrt{(x + 0.5)}$. The data were subjected to ANOVA and mean separation was performed by the Student Newman Keul's (SNK) test, following Steel and Torrie (1980).

3. Results

After 14 days of GSH treatment in experiment 1, the viability of cell cultures was examined by FDA staining (Figure 1 a-c). At the high concentrations of GSH (8 and 10 mM), hardly any viable cells were observed, whereas no differences were recorded between the control (0 mM GSH) and the cultures with 4 mM GSH (Fig. 1 a-c). The O_2^- formation was observed in the control and the lower GSH concentrations (0-6 mM) (Figure 1 d-e), whereas after treatment with 8 and 10 mM GSH NBT staining localized O_2^- only very rarely (Fig. 1f).
After 28 days, high numbers of globular embryos were formed in the control and especially in the 4 mM GSH treatment, but much lower numbers at the higher GSH concentrations (8-10 mM) (Fig. 1 g-i). A high variation in the number of torpedo stage somatic embryos was observed, not only between the experiments, but also between the blocks that were derived from different flasks (Table 1). Thus, the slightly increased number of embryos in the 4 mM GSH treatment was not significant when compared to the control ($p>0.05$). Significantly negative effects of GSH on differentiation were recorded in both replications at 8 mM GSH (Table 1). The conversion rates showed no differences between the concentrations of GSH (0-10 mM) in the first experiment.
Because the higher GSH concentrations had a strong negative effect on cell viability, in experiment 2 only the lowest concentration of 4 mM GSH was tested with two further genotypes (Table 2). While in genotype CNP 6 the number of torpedo stage somatic embryos was significantly increased by the treatment, GSH negatively affected the

development of somatic embryos in genotype 2824 which expressed a low embryogenic potential (Table 2).

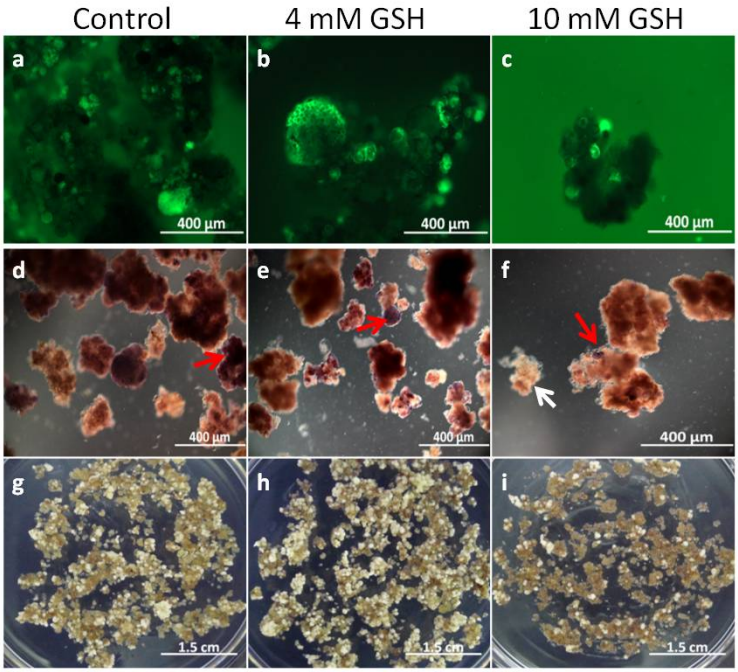

Fig. 1. Effects of GSH (0, 4 and 10 mM) during early somatic embryogenesis in *Cyclamen persicum*: a – c: Viability of cell cultures after 14 days of treatment. Viable cells express green fluorescence. d – f: Histochemical localization of O_2^- using NBT staining in cell cultures after 14 days of treatment; red arrows shows cell aggregates with NBT staining, white arrows show cell aggrerates negative in the NBT staining. g – i: Morphological aspects of the cultures after 28 days.

Table 1. Effect of GSH in the early differentiation phase of *C. persicum* on the number of torpedo stage somatic embryos after 28 days and their conversion rate after further 30 days of culture in genotype 56/2. Means plus-minus standard deviation followed by different letters indicate significant differences in the SNK test (5%). Rep = Replication.

Number of torpedo stage somatic embryos /200 mg (±SD)

	Control	4 mM	6 mM	8 mM	10 mM	Average
Rep 1	9.8 ± 1.7 ab	12.4 ± 2.1 a	7.8 ± 2.5 bc	3.6 ± 1.3 de	2.6 ± 0.5 de	7.2
Rep 2	7.7 ± 1.6 bc	10.0 ± 1.7 ab	4.3 ± 2.8 cd	2.7 ± 1.0 de	0.0 ± 0.0 e	4.9
Average	8.7 ± 1.9	11.2 ± 2.2	6.1 ± 3.0	3.1 ± 1.1	1.3 ± 1.4	

Conversion rate (%)* (±SD)

	Control	4 mM	6 mM	8 mM	10 mM	Average
Rep 1	61.6 ± 11.2 a	62.4 ± 7.3 a	37.6 ± 15.3 a	57.4 ± 8.5 a	63.9 ± 37.6 a	56.6
Rep 2	58.3 ± 3.8 a	63.9 ± 12.3 a	34.2 ± 29.6 a	58.6 ± 36.7 a	0.0 ± 0.0 b	43.0
Average	59.9 ± 7.7	63.2 ± 9.1	35.9 ± 21.2	58.0 ± 23.8	31.9 ± 42.3	

Means followed by different letters indicate values which differed to the SNK test (5%). * Data transformed in √ (x + 0.5).

Table 2. Effect of GSH in the early differentiation phase of *C. persicum* on the number of torpedo stage somatic embryos after 28 days and their conversion rate after further 30 days of culture in genotypes CNP 6 and 2824. Means plus-minus standard deviation followed by different letters indicate significant differences in the SNK test (5%). Rep = Replication. Rep 2 of genotype 2824 was contaminated.

Genotype CNP 6	Number of torpedo stage somatic embryos /200 mg (±SD)		Conversion rate (%) (±SD)	
	Control	4 mM	Control	4 mM
Rep 1	3.2 ± 0.5 b	31.2 ± 12.8 a	42.6 ± 23.1 AB	69.2 ± 6.7 A
Rep 2	10.2 ± 2.3 b	25.0 ± 4.4 a	34.8 ± 0.4 B	52.3 ± 5.0 AB
Average	6.7 ± 4.1	28.1 ± 9.2	38.7 ± 11.2	60.7 ± 8.5

Genotype 2824	Number of torpedo stage somatic embryos /200 mg (±SD)		Conversion rate (%) (±SD)	
	Control	4 mM	Control	4 mM
	3.7 ± 0.6 a	1.2 ± 0.4 b	35.8 ± 13.2 A	27.8 ± 25.5 A

4. Discussion

The cellular glutathione pool consists of two interchangeable forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). The reduced glutathione has a high reduction potential, which eliminates the reactive oxygen species, thereby being oxidized (Stassola, 2010). When the GSH was supplemented to cyclamen differentiation medium in higher concentrations in the present study, lower contents of superoxide anions were observed in our qualitative localization assay at day 14 (Figure 1 d-f). This reactive oxygen species was reduced drastically at 10 mM GSH resulting in the death of the cell culture.

Several authors have reported the crucial role of a high redox state of glutathione in the early stages of the development of somatic embryos (Belmonte et al., 2003, 2005; Stassola et al., 2008; Viera et al., 2012). In *Cyclamen persicum* the supplementation of GSH during the early stages of somatic embryogenesis seemed to have an effect on the number of somatic embryos formed, but in a genotype-dependent manner (Table 1, Table 2). However, the high variation between the two replications of the experiments as well as in the reaction of the different genotypes might indicate that the endogenous content of glutathione is also variable. In general, the regulation of generation and detoxification of ROS in plants is considered to be highly complex and interdependent involving many different pathways (Schmidt and Schippers, 2015).

In the present study, the supplementation of GSH during the early stages of SE did not have pronounced influence on the conversion rate. Other studies showed that the maturation and progression of embryos to subsequent germination was improved when the pool is shifted towards glutathione in the oxidized form (Stasolla, 2010).

5. Conclusions

In two of three cyclamen genotypes under investigation, a promoting effect of 4 mM GSH during the first 14 d of differentiation on the formation of somatic embryos was shown, but the reaction of the embryogenic cell cultures was found to be highly variable. Thus, further investigations are needed to study the effects of GSH in concentrations lower than 4 mM and in different time windows. Measuring endogenous GSH and GSSG concentrations during SE will improve our understanding of their role and offer new opportunities to adjust their ratio in different developmental phases accordingly.

6. Literature

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