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EFFECT OF GLUTATHIONE ON MATURATION OF SOMATIC EMBRYOS DERIVED FROM VEGETATIVE SHOOT APICES OF MATURE TREES OF *PINUS ROXBURGHII*

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Redox reagents are powerful tools in the study of plant growth and development. The glutathione-glutathione disulfide redox pair has gained attention over the years as an important factor in plant growth and development. Overall embryo quality and quantity have yet to reach their optimum for coniferous species. This investigation highlights the use of glutathione redox pair in the maturation medium for improving the somatic embryo quality and quantity. The addition of glutathione redox pair induced the largest number of mature somatic embryos at 0.1 mM. Higher concentrations (0.5 mM and 1.0 mM) impeded embryo formation.

Keywords: Glutathione redox pair; Somatic embryogenesis.

Introduction

Forest productivity can be increased by planting tree farms with large numbers of elite, high value trees. Successful establishment of embryogenic cultures from mature vegetative tissues has been reported for various conifers[1-12]. Poor maturation of somatic embryos and difficulty in producing a large number of plants is still a limiting factor in large-scale application of conifer somatic embryogenesis in commercial forestry, particularly in pine species. Reports of maturation of somatic embryos for various conifer species emphasized the importance of factors such as carbohydrate source, gelling agent, organic nitrogen and amino acids[13,14]. Redox reagents are powerful tools in the study of plant growth and development. The glutathione-glutathione disulfide redox pair has gained attention over the years as an important factor in plant growth and development[15]. Although many studies have looked at the function of glutathione in mature plant development, very little is known regarding its effect on somatic embryogenesis. Overall embryo quality and quantity have yet to reach their optimum for coniferous species. An attempt was made to improve the protocol for somatic embryogenesis via the manipulation of maturation conditions through the glutathione redox pair. The present study reports optimized parameters for maturation of somatic embryos of *Pinus roxburghii* using the glutathione redox pair as a maturation promoter.

Materials and Methods

Plant material and source of embryogenic cultures- Apical shoots from mature trees (15-years-old) of *Pinus roxburghii* from 3 genotypes (PR11, PR105 and PR521) were collected from the Western Ghat Forest, India. Embryogenic tissue showing elongated cells with signs of cleavage polyembryony was used for the maturation experiments. The embryogenic tissue was raised from vegetative shoot apices of mature trees of *Pinus roxburghii* according to the previous established protocols[1-6, 16].

Maintenance of embryogenic cultures (Fig. 1. A, B.)- The callus producing embryonal suspensor masses or early embryos were subcultured onto maintenance medium. The DCR basal medium containing 120 mM maltose, 2 g l^{-1} Gellan gum supplemented with 2 µM 2,4-D, 2.5 µM NAA, and 1 µM BA (maintenance medium) was used for this purpose[1-6, 16]. All cultures were maintained in the dark at 25 ± 2° C. On this medium, embryonal suspensor masses or early embryos were cultured for 30 days with 2 subcultures. The presence of embryonal suspensor masses was determined by microscopic observation[1-6, 16]. For partial desiccation, the calli (after 15 days on the maintenance medium) were transferred to sterile empty Petri dishes containing two sterile Whatman No 1 filter paper disks. The Petri dishes were sealed with parafilm and kept at 25 ± 2° C in the dark for 24 h to obtain the desired desiccation.

Maturation of somatic embryos (Fig. 1. C)-The partially desiccated calli were transferred to maturation medium to induce cotyledonary embryo development. DCR basal medium with 175 mM maltose, 80 µM ABA and 9 g l^{-1} Gellan gum was used for this purpose [16]. All cultures were placed in the dark at 25 ± 2° C and maintained for 8 to 12 weeks. In another separate experiment the effect of reduced/oxidized

glutathione and a mixture of both (Redox pair) at concentrations of 0.1, 0.5 and 1.0 mM were also studied. Glutathione was filter-sterilized and added to the media at 50°C. The control was DCR basal medium without glutathione.

Germination and plantlet recovery (Fig. 1. D.) - After 10 to 14 weeks of maturation in the presence of 80 µM ABA and a higher concentration of maltose (175 mM), advanced cotyledonary somatic embryos were isolated from the cultures for germination. The germination medium used was half DCR basal medium with 2 gl^{-1} gellan gum[16]. Somatic embryos were considered germinated as soon as radical elongation occurred. Conversion to plantlets was based on the presence of epicotyls. After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite. Plantlets were placed in a growth room with a 16 h photoperiod (50 µmol m^{-2} s^{-1}) for hardening.

Data analysis- In all experiments each culture tube contained a single explant. Each replicate contained 50 cultures and each treatment included at least 2 replicates (100 cultures). All the experiments were repeated 3 times (total 300 cultures). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA and the differences contrasted using Fishers pair wise comparisons using GENSTAT® release 4.21(Rothamsted Experimental Station, United Kingdom). All statistical analysis was performed at the 5% level.

Results and Discussion

In this study, both reduced and oxidized glutathione did not have a significant effect on the maturation of somatic embryos when added alone in the maturation medium at all the tested concentrations (0.1, 0.5 and 1.0 mM) (data not shown). This paper presents only optimum results. On the other hand incorporation of both forms (glutathione reduced/oxidized) had a marked effect on maturation of somatic embryos when compared to the control (Table 1). The addition of the mixture not only increased the number of somatic embryos but also promoted the development of better quality embryos. The higher concentrations (0.5 and 1.0 mM) of glutathione (reduced/oxidized) inhibited embryogenesis, resulting in poor maturation of somatic embryos in all three genotypes of *P. patula* (data not shown). The role of glutathione (reduced/oxidized) in plant development has been investigated by many workers[17] and species, including wild carrot suspension cultures[18]. In carrot suspension cultures, addition of 0.3 mM buthionine sulfoximine (a glutathione synthesis inhibitor) to developing cultures decreased the cellular reduced glutathione levels and enhanced somatic embryogenesis while addition of 0.6 mM reduced glutathione increased the cellular reduced glutathione levels and inhibited embryogenesis. These results provide evidence that the levels of reduced glutathione are important in determining whether carrot cells develop into somatic embryos or grow prolifically[19].

Similarly the addition of reduced/oxidized glutathione at a concentration of 0.1 mM during the maturation of white spruce (*Picea glauca*) somatic embryos not only increased the total number of somatic embryos but also promoted the development of superior quality

Table 1. Effect of redox pair (glutathione reduced/oxidized) at a concentration of 0.1 mM on maturation and germination of somatic embryos of three genotypes of *Pinus patula.*

Genotypes	Total number of somatic embryos recovered per gram fresh wt of embryogenic callus	Total number of somatic embryos germinated per gram fresh wt of embryogenic callus	Total number of somatic seedlings recovered per gram fresh wt of embryogenic callus
Control	20.0 ± 1.8 a	17.0 ± 2.8 a	10.0 ± 1.7 b
PR11	30.0 ± 1.8 d	27.0 ± 2.8 b	20.0 ± 1.0 c
Control	36.0 ± 2.0 b	32.0 ± 1.8 b	29.0 ± 1.5 g
PR105	42.0 ± 3.0 c	38.0 ± 1.5 d	35.0 ± 1.3 d
Control	29.0 ± 1.6 d	26.0 ± 1.5 b	20.0 ± 1.4 c
PR521	35.0 ± 2.0 b	32.0 ± 4.0 d	29.0 ± 1.5 g

Data recorded after 8 weeks represents the mean±SE of at least 3 different experiments. In each column, values with different letters are significantly different ($P < 0.05$).

Control = Maturation medium without glutathione

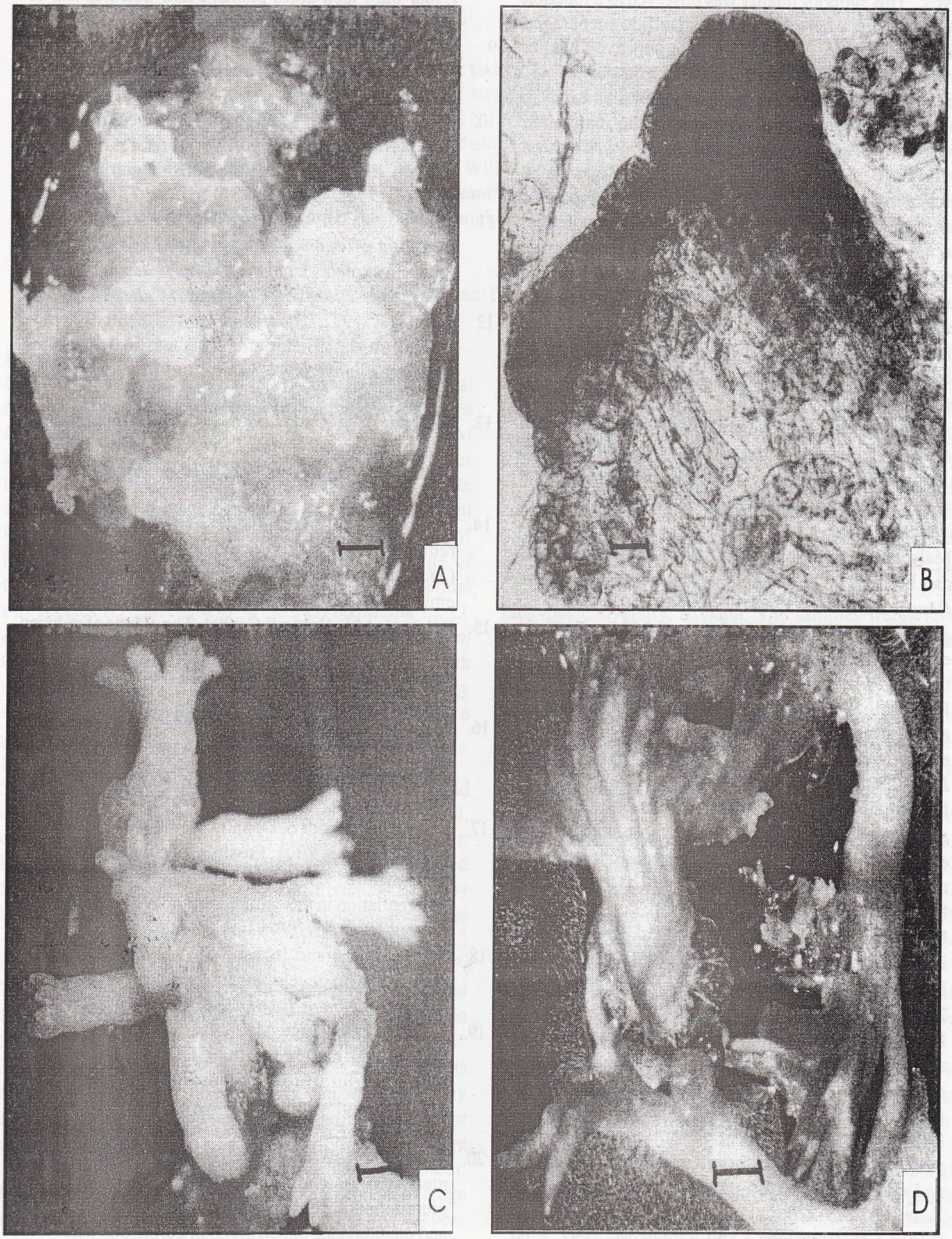

Fig.1. Effect of glutathione on maturation of somatic embryos in *Pinus roxburghii* (A) Embryogenic callus on maintenance medium (Bar=0.11 cm). (B), Early stage embryo showing head formation (Bar=0.023 cm). (C) Microscopic view of various developmental stages of somatic embryos on maturation medium (Bar=0.093 cm). (D) Somatic embryos successfully germinated on DCR basal medium (germination medium) (Bar = 1 cm).

embryos. This superior quality was observed as a function of the shoot pole, where glutathione disulfide encouraged cotyledon formation. In addition, this improved quality was reflected by an increased conversion frequency[17, 19]. The beneficial effect was the result of major alterations in morphology and gene expression during the maturation period. It was also reported that glutathione-treated embryos showed a differential accumulation of storage products and a preferential deposition of starch, reduced formation of protein bodies and increased cell vacuolation. These morphological changes correlated with extensive alternations of gene expression occurring throughout the maturation of white spruce somatic embryos. These results indicated that several genes involved in a variety of signal-regulating pathways were differentially expressed in developing glutathione-treated embryos[19, 20].

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