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SOMATIC EMBRYOGENESIS AND ISOZYMES IN ROSE SPECIES *ROSA HYBRIDA* L. CV. KING'S RANSOM

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ABSTRACT

Somatic embryogenesis was obtained from long term callus cultures of leaf bud in half strength MS medium supplemented with both 0.5 mg/l 2,4-D +1.0 mg/l NAA and 0.5 mg/l 2,4-D. However, further development of the somatic embryos into plants was obtained only from the 0.5 mg/l 2,4-D supplemented cultures. Half strength MS medium supplemented with 0.5 mg/l 2,4-D + 1.0 mg/l NAA + 0.5 mg/l BAP yielded only non-embryogenic callus. Isozyme patterns were studied to distinguish between non-embryogenic and embryogenic calli and also between non-viable and viable somatic embryos in embryogenic calli. Specific isoperoxidases and isoesterases were found to be associated with the development of viable somatic embryos.

Key words : Somatic embryogenesis, isozyme analysis, rose, micropropagation

Rose micropropagation through shoot tip culture is a viable technique, but is a labour intensive and time consuming process. Recovery of plants from cells through somatic embryogenesis is regarded as an advance micropropagation technque. Somatic embryogenesis is useful in studies on somaclonal variation, genetic transformation, clonal propagation and production of synthetic seeds.

Multiple molecular forms of enzymes, termed isozymes [1], are known to be produced in a wide variety of higher plants [2]. They are readily demonstrable by gel electrophoresis, and are considered to be products of genes which segregate in a Mendelian manner [3, 4]. To study the gene controlled mechanisms that occur during development and differentiation of cells and tissues, enzymology is a very useful tool. Isozymes may be particularly useful as markers of embryogenesis because plants undergo changes in gene expression during embryo development and

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germination [5]. Esterases have been suggested as cytochemical markers of embryogenesis in carrot cell culture [6]. There is a need for biochemical markers to identify cells capable of undergoing somatic embryogenesis [7]. The above literature suggests that isozyme analysis can be utilized to delve into the differences at the callus level especially during somatic embryogenesis.

MATERIALS AND METHODS

The surface sterilization of leaf explants of *Rosa hybrida* L. cv King's Ransom was done using 0.01% mercuric chloride with 2 drops of Tween-20 for 90 sec. The explants were washed in sterile distilled water for 5 times and were placed in sterile filter paper to remove the excess water droplets. The explants were then cut into bits (leaf bits) and were inoculated into the culture media. Culture media consisted of half strength MS with growth regulators with different combinations. The growth regulators used for callus initiation were 0.5 mg/l 2,4-D individually and in combination with 1.0 mg/l NAA and also in combination with BAP at 0.5 mg/l. The long term cultures were maintained in the hope of obtaining viable somatic embryos with frequent subculturing. The different combinations of phytohormones yielded embryogenic calli with viable somatic embryos, embryogenic calli with non/viable somatic embryos and non-embryogenic calli. Analysis of activities of peroxidase and polyphenoloxidase and electrophoretic analysis of isoperoxidases and isoesterases were carried out in the three different calli.

Peroxidase activity was estimated according to the method of Kar and Misra [8] with minor modifications. The assay system contained 1 ml of 0.1 M phosphate buffer pH 6.8, 0.5 ml of 0.1% H_2O_2, 0.5 ml pyrogallol (0.1M) and 1 ml of diluted enzyme solution. The absorbance at 420 nm was recorded on UV, SHimadzu spectrophotometer. Protein in the sample was estimated by Lowry's method [9]. The reaction was terminated by adding 0.5 ml of 5% H_2SO_4 after incubating at 20°C for 5 min. The amount of purpurogallin formed was determined by using the extinction coefficient EC 420 = 3.162 cm/min and the enzyme activity expressed in terms of micromolecules of poduct/min/mg protein.

Polyphenol oxidase assay was carried out according to the method of Malik and Singh [10]. The absorbance was recorded at 495 nm. The amount of quinone formed was determined by using the extinction coefficient EC 495 = 24.9 cm/min.

The methodology of Davis [11] was adopted to analyse the isozymes by SDS-polyacrylamide gel electrophoresis. 700 mg each of non-embryogenic calli, embryogenic

calli with non-viable somatic embryos and embryogenic calli with viable somatic embryos were homogenised by grinding with 50 ml Tris buffer pH 7.4 and 250 mM sucrose. The homogenate was centrifuged for 15 min at 11,000 rpm. The clear supernatant served as the source of isozymes for SDS- PAGE. Aliquots of the extract were mixed with an appropriate volume of diluent consisting of 5% 2-mercaptoethanol and 0.05% bromophenol blue to obtain a final isozyme concentration of 1 μg/μl. Approximately 20 μl of the mixture was loaded in the wells. The electrophoresis was carried out at 4.5°C for about 5 hours till the bromophenol front reached almost the end of the slabgel at a constant current of 30 mA.

Staining of peroxides isozymes was according to the method of Smith [12]. The staining solution ontained a mixture of saturated benzidine, 30% ammonium chloride and 0.4% H_2O_2 in the proportion of 50:10:2. The gel was completely immersed in the staining solution for 15 min at room temperature and then fixed in 2% acetic acid solution. Esterases isozymes were stained according to the method of Payne and Koszykowski [13]. Staining solution contained 75 mg fast blue RR slat. 2.25 ml substrate solution, 10 mg alpha-naptyl acetate in 10 ml of 50% chilled acetone in 150 ml of 0.6 M phosphate buffer (pH 6.2). The gels were completely immersed in the staining solution for 45 min at room temperature. Then they were removed and fixed in 2% acetic acid solution. The distance travelled by the isozymes was recorded on graph paper as Rm values.

$$Rm = \frac{\text{Distance travelled by a band}}{\text{Distance travelled by tracking dye}}$$

RESULTS AND DISCUSSION

The three types of calli used for biochemical analysis were non- embryogenic callus (produced in half strength MS medium supplemented with 0.5 mg/l 2,4-D + 0.1 mg/1 NAA and 0.5 mg/1 BAP), embryogenic callus with non-viable somatic embryos (produced in half strength MS medium supplemented with 0.5 mg/1 2,4-D + 1.0 mg/1 NAA) and embryogenic calli with good viable somatic embryos (produced in half strength MS medium supplemented with 0.5 mg/1 2,4D) (Table 1).

In the present study, embryogenic callus contained relatively higher values of both peroxidase activity and polyphenol oxidase activity, than the non-embryogenic callus. The embryogenic callus with viable embryos further had higher values than in the embryogenic callus with non-viable somatic embryos (Table 2). It is generally reported that there is higher enzyme activity in embryogenic calli compared to non-embryogenic or proembryogenic stages [14, 15]. Such studies provide more

information for distinguishing between embryogenic and non-embryogenic calli and can also be used as markers of embryogenesis because plants undergo dramatic changes in isozyme expression during embryo development [16].

Table 1. Response of long term cultures to different hormonal combinations (somatic embryogenesis)

Media* + Growth regulators	Mean no. of days for growth of callus	Appearance of callus	Fresh weight (mg)	Dry weight (mg)
2,4-D 0.5 Mg/1	9.00	Embryogenic callus with green viable globular embryos	1407	86
2,4-D 0.5 mg/1	9.50	Embryogenic callus with non-viable globular embryos	2817	165
2,4-D 0.5 mg/1 + NAA 1.0 mg/1	9.50	Embryogenic callus with non- viable globular embryos	2817	165
2,4-D 0.5 mg/1 + NAA 1.0 mg/1 + BAP 0.5 mg/1	12.62	Non- embryogenic compact callus	2674	103

*half strength MS medium

Table 2. Activity of peroxidases and polyphenol oxidases in non-embryogenic calli ** embryogenic calli ** with non-viable somatic embryos and embryogenic calli ** with viable somatic embryos

Calli	Peroxidase*	Polyphenoloxidase*
Non-embryogenic calli	0.2560	1.232
Embryogenic calli with non-viable somatic embryos	0.3261	2.631
Embryogenic calli with viable somatic embryos	0.4923	3.213

*per mg protein; ** 180 day old calli

In the present isozyme study, embryogenic calli, from which healthy somatic embryos were isolated, subsequently exhibited more number of bands (peroxidases and esterases) compared to the embryogenic callus with non-viable somatic embryos and non- embryogenic callus. Embryogenic callus with viable somatic embryos exhibited a total of 7 bands of peroxidases compared to 5 bands in the embryogenic callus with non-viable somatic embroys and non-embryogenic callus. Embryogenic callus with viable somatic embryos exhibited certain specific peroxidases with Rm values of 0.42 and 0.55 which were absent in both other calli indicating a possible association of specific isozymes with embryogenesis. However two specific peroxidases with Rm values of 0.34 and 0.59 were further absent in the non-embryogenic callus which might be an indication that the peroxidases with Rm values of 0.34 and 0.59 are essential for the formation of embryogenic callus, whereas those with Rm values of 0.42 and 0.55 are absolutely essential for successful differentiation of viable somatic embryos. Embryogenic callus with viable somatic embryos exhibited a specific isoesterase with Rm values of 0.59, whereas, the band was absent in the other two calli. However, the enzymes with Rm values of 0.51 and 0.54 were further absent in the non-embryogenic callus and an additional enzyme with Rm value of 0.80 was present in the embryogenic callus with non-viable somatic embryos. This also shows that the enzymes with Rm values of 0.51, 0.54 and 0.59 may be essential for embryogenesis, whereas the specific enzymes with Rm value 0.59 seems to be absolutely essential for production of viable somatic embryos.

Everett *et al.* [17] reported different isozymic pattern in embryogenic and non-embryogenic calli lines and Chibbar *et al.* [18] located two major groups of esterase isozymes based on their mobility in *in vitro* cultured carrot tissue. Rao *et al* [19] reported specific isoperoxidases and isoesterases in embryogenic calli which were absent in non-embryogenic calli indicating a possible association of these isozymes with somatic embryo differentiation.

The result of the present study supports all the above statements and provides more evidence to the possibility of some isozymes being associated with somatic embryogenesis and differentiation of somtic embryos. Further, differentiation of embryogenic callus into viable somatic embryos also was shown to be associated with the presence of certain isozymes and the absence of these crucial isozymes may be the reason for non-viability of somatic embryos.

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