**THE ROLE OF ANTIBIOTICS IN MANAGEMENT OF IN VITRO CONTAMINATION AND CYTOKININ EFFECTS IN SHOOT PRODUCTION IN TENDER AND HARDY ROSES.**

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**Running title: In vitro techniques in the micropropagation of roses**

**Abstract**

In vitro contamination of explants or cultures was a serious problem encountered during the course of study especially when the explants were collected from the field. Growth of both bacterial and fungi together posed difficulty in culture development. The most effective measure to control *in vitro* contamination was with treatment of HgCl2 solution and subsequent transfer of cultures on shoot induction medium containing antibiotics (Carbenicillin and Timentin). A detrimental effect on explants during culture was observed from NaOCl treatments. Explants supplied from *in vitro* derived plants grown under control environment produced zero percent contamination in the rose tissue culture. Amongst various cytokinins, namely BA, TDZ, Kinetin and 2ip, BA was most effective for production of number of shoots per explants based on their mean values. Cultures on medium supplemented with TDZ produced abnormal axillary shoots. Rooting easily occurred in all cultivars in a rooting medium based on half strength MS medium supplemented with 1.0mg/l IBA. Rooted plants were successfully transferred to soil. Well established plants from tender and hardy rose varieties can be obtained within 2-3 months. This micropropagation protocol with minimal contamination and fewer additives in tissue culture medium can be applied to establish a commercially viable rose nursery.

**Key words:**  antibiotics, contamination, cytokinins, micropropagation, roses

**INTRODUCTION**

Amongst ornamental crops, roses have gained popularity as garden, landscape and potted plants or cut flowers, as well as being used as a source of aromatic oils for the perfume industry (Gudin, 2000). Rose petals and hips (berries) are used to make rose tea, which has both nutritional and medicinal value (Krussmann 1981, Zlesak 2006). The aesthetic value of roses is well recognized and this plant family is one of the most economically important ornamental crops in the horticultural industry. Micropropagation in different species of roses has been described by several earlier workers (Bressan et al. 1982, Marcelis-van Acker and Scholten, 1995, Jabbarzadeh and Khosh-Khui 2005).

*In vitro* contamination by fungi, bacteria, or yeast is one of the most serious problems of commercial and research plant tissue culture laboratories (Leifert et al. 1994). The inability to adequately control contamination levels is the primary reason for failure of commercial laboratories (Leifert and Woodward 1997). Our goal is to multiply selected cultivars of tender and hardy roses through tissue culture with minimal contamination of *in vitro* cultures. To our knowledge, problem and control of *in vitro* contamination in roses is less addressed in rose literature. Contamination sources either from bacteria or fungi in explants obtained from rose plants grown in the field is still one of the early steps to be solved in rose micropropagation. We aimed to develop reliable and efficient *in vitro* shoot propagation protocols for a wide range of genotypes of this species with minimal contamination. The established protocols will provide the basis for commercial plant production to be used by local growers (Bressan et al. 1982, Pati et al. 2006). The micropropagation technique coupled with greenhouse facilities will boost the revenue of horticultural nurseries based in the prairie region of Canada instead of depending on the import of new nursery stock every year from neighbouring countries.

**MATERIALS AND METHODS**

***Collection of plant materials***

Plant materials including woody stem or juvenile stems along with actively growing shoot buds of tender and hardy roses were collected from rose growers in the city of Calgary during May-October, 2011.

***Storage of vegetative tissues***

Axillary shoot buds along with stems or branches were bagged into Ziploc® bags and stored in deli maintained at 4ºC temperature. Axillary shoot buds remain active for several weeks (8 weeks) and can be used for micropropagation without compromising their ability for further growth.

***Explant preparation and Surface sterilization***

Freshly harvested or 1 to 8 weekold vegetative tissues of several rose cultivars were prepared for sterilization. Branches were cut into small pieces by maintaining 2-3 axillary buds per segment from healthy underdeveloped vegetative shoots. A single bud was left attached to a piece of stem 0.5-1 cm long. Shoot segments or cut branches were placed on stainless steel mesh and pre-washed with running tap water, in addition to deionised water for one and half to two hours to flush out dirt and fungal spores. Explants were put into a sterile 250-500 ml Erlenmeyer flask. Several cultivars of roses can be sterilized at one time in a single flask by separating them with different coloured cotton threads. Each individual cultivar will carry distinct colour ties. This allows reduced sterilization time for every individual cultivar. Several disinfectants alone or in combination were attempted during this study (Table 1).

In all these 4 approaches of sterilization, 2-3 drops of Tween 20 (Sigma) were added. Prior to sterilization with any disinfectants, explants were treated with 70% EtOH for 1 minute with vigorous shaking and a one-time sterile water rinse. Explants were rinsed with sterile water until detergent bubbles disappeared. About 1L of sterile H2O is used for rinsing explant material with 200 ml of disinfectant solution contained in a 500 ml flask. Disinfectant solutions were discarded into appropriate liquid disposal containers located inside the fume hood. After the surface sterilization procedure was completed, the flask containing explants was brought to the Laminar Flow hood. These clean explants were then ready for culture under aseptic conditions.

***Culture Medium***

Micropropagation was based on Murashige and Skoog (1962, MS) medium with Gamborg’s B5 Vitamins (Gamborg 1965). Medium was prepared from powder salts from Phytotechnology Laboratories (M404). Organics of B5 medium was added separately from stocks prepared in the lab. Sucrose 3% (Phytotechnology Laboratories S829) was added into micropropagation media except in the case of rooting medium. Rooting medium was based on MS salts with half strength and full strength of organics and iron supplemented with 2.0% sucrose. Different concentrations of 6-Benzylaminopurine (BA), Kinetin, 2ip and Thidiauron (TDZ) at 0, 1.0, 2.0. 3.0 and 4.0 mg/l were attempted for a few cultivars. All culture media was adjusted at pH 5.8 before gelling agent 0.65% agar (Duchefa) was added throughout the experiments. Two antibiotics, i.e. Timentin (Phytotechnology Laboratories T869) and Carbenicilin (Phytotechnology Laboratories, C346) were added to the culture medium in combinations or alone at low level of 100mg/l of each antibiotic. Activated charcoal (1.0%), AgNO3 (2.0 mg/l), and 1.0 mg/l Polyvinylpyrrolidon (PVP-40, Phytotechnology Laboratories, P728) were added to the culture medium.

***Shoot multiplication***

Sterilized explants were transferred to sterile petri dishes. Before transferring these explants to culture medium they were tapped on sterile filter paper in order to absorb excess water. Five explants were inoculated per culture plate (100 mm x 15 mm; Fisher Scientific) containing shoot multiplication medium of 15 ml of basal MS medium with B5 vitamins supplemented with one of the cytokinins, such as BA, Kinetin, 2ip and TDZ at different levels and 3.0% sucrose.

Sub-culturing of explants followed every week to fresh medium with the shoot multiplication medium in plates and plastic culture jars (SteriCon, Phytotechnology Laboratories) depending on the growth and size of the cultures. After 3-4 weeks on multiplication medium, cultures were transferred to shoot elongation medium contained in plastic culture jars. This medium has the same composition as shoot multiplication, but without growth regulators. Antibiotics and PVP 40 (1.0 mg/l) was still present in the medium. After 1-2 weeks, individual shoots were excised from multiple shoots originating from single axillary bud and were transferred to rooting medium. All cultures in this experiment were kept under a 16/8hr photoperiod cycle. The temperature of culture room was maintained at 24±1⁰C.

***Rooting***

Rooting medium was based on half strength MS medium supplemented with 1.0 mg/l IBA and 2.0% sucrose. IBA was added to the medium after autoclaving. Four shoots were transferred to a plastic culture jar. Rooting medium with or without Putresine was also included in the study.

***Plant to soil:***

Rooted plantlets were removed from culture jars and washed carefully to remove agar from the roots. They were then transferred to pots (2x2x4 inch) containing soil mixture of 2 soil:1 vermiculite:1 perlite. Pots were placed in a plastic tray covered with a transparent plastic dome. Pots were also covered with transparent disposable plastic cups. Each plastic tray was partially filled with tap water. Fertiliser (Rose Mix) application was followed throughout the growth phase of the roses at 2 week intervals. These tissue culture derived plants were maintained in a plant growth chamber. The maximum and minimum temperature of the growth chamber was maintained at 25ºC and 21ºC respectively.

***Experimental Design:***

Forty explants from a tender rose cultivar, Playboy, were equally distributed among 8 different petri dishes containing 5 explants per petri dish. At the same time control was also running in parallel with the experiment. The culture regime of the control is the same as the experiment except for exclusion of plant growth regulators. This experiment was repeated twice. Selection of explants was based on the uniformity of the explants size and growth stage. After 1 week, cultures were transferred to culture jars. After 6 weeks of initial culture, the number and length of shoots were recorded. Collection of data for these two parameters was recorded from all 75 explants pooled from two experiments. The average mean value and their SE were calculated from total pooled value. However, 25 samples were randomly pooled from the population for estimation of mean and SE estimation for shoot length.

**RESULTS AND DISCUSSIONS**

***Decontamination procedures***

Bacterial and fungal contamination of *in vitro* cultures was a major problem when explants were collected from the field. Timing of explant collection played an important role in minimizing contamination. One hundred percent contamination was experienced when the explants were collected during July to September. Collection May to June and in October experienced reduced contamination from 100 to 80%. However, this frequency of explant contamination was also unpredictable, depending on variety and location where the explants were collected. The most effective measure was treatment of explants with 0.2% HgCl2 solution for 15 min and addition of 100 mg/L each of Timentin and Carbenicillin in combinations to culture medium for a week. Prior to this step, explants treated with 70% Ethanol (v/v) for 1 minute and addition of 2 drops of Tween 20 helped to reduce contamination. Any disinfectant solutions containing commercial bleach (NaOCl) led to the death of tissues. Therefore, this disinfectant was removed from the list of disinfectants used in the study. Besides its detrimental effects on tissues, it could not control the growth of bacteria or fungus. However, surface sterilization in 70% ethanol for few seconds, followed by 20 min. in very low concentration of 1% NaOCl was reported in rose *in vitro* culture (Marcelis-van Acker and Scholten 1995). High concentration of NaOCl (10%) in rose tissue culture without damaging the tissue of explants was also reported (Jabbarzadeh and Khosh-Khui 2005). Vigorous shaking of the solution along with the explants continued for the prescribed sterilization time with magnetic stirrer bar is a very important step.

The cultures were moved to 50 mg/L each of Timentin and Carbenicillin after one week of culture in shoot initiation and multiplication medium. In the subsequent cultures, both antibiotics at 50 mg/L were maintained in the culture medium until the shoots reached to rooting stage. From initial experiments using different hardy and tender roses (Table 2) about 10-20% of the cultures/explants from all tested varieties can be recovered and established complete rose plants by adopting these contamination control measures. This recovery percentage can be further improved by good timing of collection of plant material from the field (during May-June or October month). Later this contamination problem was completely solved by using explant materials derived from tissue culture plants raised in a plant growth chamber environment. Disinfection of explants from field-grown roses was less effective than explants from the greenhouse (Randall and Michael 2004). Control of fungal and bacterial contamination was very effective using 0.2% HgCl2 for 15 minutes in most of the varieties used. Increasing the level of both antibiotics showed detrimental affects to the growth of axillary buds.

***Plant growth regulators for shoot multiplication***

Initially only a cytokinin BA with different levels 0, 1.0, 2.0, 3.0 and 4.0 mg/l was added into the micropropagation medium. Conducting an experiment to maximize the number of shoot multiplications with different levels and types of cytokinin was impossible due to massive loss of explants due to contamination. Most of the explants were lost due to contamination and failed to complete the experiment. Shoots were counted before the cultures were discarded. Amongst the levels of BA tested, 2.0 mg/l was found to be the best concentration to produce maximum shoots per explant with single axillary shoot (data not shown). After minimizing the contamination, rose plants derived from tissue culture were raised in a plant growth chamber. Explants obtained from these plants were used for further experiments to optimize the method of shoot multiplication.

Effect of different cytokinins on shoot induction and height of shoots was included in the present study (Tables 3 and 4). Counting of shoots per explants and measurement of height of shoots was performed on 6 passages of cultures. Subculturing of cultures on a weekly basis was followed throughout the experiment. Frequent subculturing prevented yellowing and browning of lower leaves of shoots during the culture period. Data were collected from a single experiment with replication of two from a single tender rose variety, Playboy.

Amongst the cytokinins tested for shoot induction and multiplication, BA containing medium produced 204 shoots from 75 explants, the highest number with mean value of 2.6 and lowest number was observed in medium containing Kinetin and 2ip with 1.2 mean values in both treatments (Table 3). Number of shoots produced from TDZ containing medium was very close to BA with mean value of 2.4. However, most of the shoots obtained from TDZ containing medium could not be maintained further due to abnormal growth behaviour and vitrification. Pulse treatment for short period of time for TDZ treatment could avoid this kind of response from TDZ. All rose cultivars (tender and hardy) responded in similar growth behaviour in *in vitro* environment in culture media supplemented with different cytokinins, except TDZ. The number of shoots produced per axillary shoot ranged from 3 - 5. In the best scenario, some cultivars can produce up to 6 shoots. MS medium with B5 vitamins supplemented with BA (2.0 mg/l) induced the highest number of shoots amongst the cytokinins tested and was found to be the best cytokinin for multiplication of healthy shoots.

Shoots which originated from explants cultured on cytokinin-containing medium were more elongated than the shoots which originated from culture medium without cytokinin (Table 4). Comparison of the mean values of the control (2.0 cm in length) and treatments indicated that effect of 2ip (2.4 cm in length) was more pronounced as compared to other cytokinins tested. BA and Kinetin produced similar response (2.2 cm in length). Explants cultured on TDZ alone proliferated but shoots did not elongate. However, of the several cytokinins tested, BA appeared most suitable for shoot proliferation and elongation, although elongation was slightly lesser than shoots derived from 2ip.

It is important to consider the orientation and preparation of explants to achieve the maximum number of shoots per explant. In this study, the best method of explant preparation was - axillary adventitious buds attached to the main stem and leaf petiole. The cut stem will allow supplying nutrients to the adventitious bud without touching the shoot bud onto the medium. If the bud was separated from the stem and plated onto the medium directly, it suffered due to browning and led to necrosis and yellowing of leaves. This could be avoided if the buds are supported by the stem tissue on the medium. The growth was tremendous when these shoot buds are attached to the stem. This applied to all of the genotypes tested. This unique position of the shoots above the medium has another advantage of avoiding browning problem in the culture. These shoots can grow continuously even when these cut stems produced phenols in the medium. The effect of shoot size on growth of the axillary buds might be nutritional and/or hormonal. The effect of stem tissue attached to the axillary bud suggests a nutritional effect of the maternal tissue (Marcelis-van Acker and Scholten 1995).

Anti-polyphenolic compounds like PVP 40 (Polyvinyl- pyrrolidon) at 1.0 mg/l added to the medium was essential to reduce tissue browning due to secretion of polyphenols into the medium. In the present study, this compound was more effective than activated charcoal. Cultures from activated charcoal medium did not grow as vigorously as in PVP. Activated charcoal and AgNO3 was supplemented to the micro-propagation medium but these compounds did not minimize browning nor improve the growth of the cultures.

Rooting was easily induced at 1.0 mg/l IBA alone with 100% response in all cultivars after 2 weeks of culture. No preconditioning for rooting is required. When shoots were allowed to remain in the rooting medium for more than 2 weeks, root tips turned brown, and the leaves of plantlets started yellowing and falling, which led to the death of the plantlets.

Similar observation was reported by earlier workers (Jabbarzadeh and Khosh-Khui 2005). They attempted to induce roots from in vitro shoots in Damask roses using different auxins except 2, 4-D. This could be due to genotypic differences. Plantlets can be removed and transferred to soil. About half a cm length of roots can easily support further growth of these plants in the soil. Putrescin in the medium along with IBA helps callus induction at the base of the shoots. Similar results were also reported by Campos and Pais (1990). All rooted plants from this study survived (100%) under acclimatized condition. This survival percentage is higher than those reported by many rose workers (Campos and Pais 1990, Dubois et al. 1988, Khosh-Khui and Sink 1982, Davies 1980). Acclimatization of *in vitro* micropropagated roses was reported to be difficult due to rapid desiccation of plantlets (Messeguer and Mele 1986). This problem is easily overcome in the present study. Well established rooted plants derived from tender and hardy roses could be obtained after 2-3 months of the initial culture.

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Table 1. Type of disinfectants used in the study:

|  |  |  |  |
| --- | --- | --- | --- |
|  | Name of the disinfectant | Concentrations used | Time of treatment |
| 1 | NaOCl (Sodium hypochloride;  Commercial bleach, Javex) | 7%, 10%, and 15% (v/v) +  Tween 20 (2drops) | 10-15 minutes |
| 2 | HgCl2 (Mercuric Chloride) | 0.1- 0.2% (wt/v) +  Tween 20 (2drops) | 5 - 15 minutes |
| 3 | NaOCl and HgCl2 | 15% and  0.2% +  Tween 20 (2drops) | for 15 minutes |

Prior to sterilization with any disinfectants, explants were treated with 70% EtOH for 1 minute with vigorous shaking and rinsed with one time sterile H2O

Table 2. List of rose cultivars used in the study:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Cultivar/common name | Rating /10 |  | Cultivar/common name | Rating /10 |
| Tender roses | |  | Hardy roses | |  |
| 1 | Olympiad | 8.5 | 1 | Prairie Peace | NR |
| 2 | Strike It Rich | 7.7 | 2 | Prairie Princess | 8.4 |
| 3 | Playboy | 8.5 | 3 | John Davis | 8.7 |
| 4 | Cinco de Mayo | 7.8 | 4 | John Cabot | 8.8 |
| 5 | Last Tango | NR | 5 | *Rosa hugonis* | 8.7 |
| 6 | Climbing Piñata | 7.5 | 6 | *R. spinosissima* Scotch /Burnet | 8.4 |
|  |  |  | 7 | *R. acicularis* | % |

Ratings based on the 2013 American Rose Society Handbook for Selecting Roses

NR – not yet rated by the American Rose Society

% - An insufficient number of reports have been received to establish a proper rating

Table 3. Effect of different types of cytokinins on shoot induction using a cv., Playboy, a tender floribunda rose.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Treatment | Total No. of explants  cultured | Total No. of shoots produced | Range  (No.) | Mean  (No.) | ± SE |
| Control | 50 | 42 | 1-2 | 0.8 | 0.16 |
| BA | 75 | 204 | 2-3 | 2.6 | 0.62 |
| Kinetin | 75 | 90 | 1-2 | 1.2 | 0.35 |
| 2ip | 75 | 93 | 1-2 | 1.2 | 0.35 |
| TDZ | 75 | 180 | 1-3 | 2.4 | 0.53 |

± SE : Standard Error

Table 4. Effect of different types of cytokinins on shoot elongation of micropropagated shoots of cv. Playboy.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Treatment | No. of shoots measured | Range  (cm) | Mean  (cm) | ± SE |
| Control | 25 | 0.8-2.1 | 2.0 | 0.09 |
| BA | 25 | 1.7-3.4 | 2.2 | 0.07 |
| Kinetin | 25 | 1.5-3.3 | 2.2 | 0.04 |
| 2ip | 25 | 1.5-3.5 | 2.4 | 0.24 |
| TDZ | 25 | 0.7-2.1 | 1.3 | 0.28 |

± SE : Standard Error