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STUDY ON RESULTS OBTAINED BY DIFFERENT RESEARCHERS ON *IN VITRO* PROPAGATION OF HERBACEOUS PEONY

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Abstract

These researches highlights the advances made on various aspects of herbaceous peony (Paeonia lactiflora Pall.) tissue culture, including the in vitro culture of underground buds, leaves and petioles, the induction of cluster buds, and callus induction. Tissue culture has the ability of speeding up the rate of propagation, reducing breeding time and meeting the needs of mass production. Problems that are currently being faced in herbaceous peony tissue culture are highlighted and possible viable solutions are provided. More research is needed to make mass production of peony more commercially successful. Optimization of procedures are necessary, from selection of explants, decontamination, screening of medium, application of plant growth regulators (PGRs), induction of callus and shoots, subculture, rooting, and to final transplanting. The major goal of these studies is to evaluate shoot induction ability of peony explants and PGRs. The development of micropropagation methods for peonies is necessary to not only overcome this problem but also accelerate peony breeding progress. Tissue culture is one of the most effective approaches for rapid propagation of plants and is also providing a new approach for plant breeding in crops, ornamentals, fruits and vegetables. Effect of Gelling Agents on Tissue Culture of Herbaceous Peony. Comparison of Basal Medium in Tissue Culture of Herbaceous Peony. Effect of Pretreatment of Underground Buds on Tissue Culture of Herbaceous Peony. Investigation of Callus and Shoot Induction Ability of Flower Tissue. Effect of PPM on Decontamination and Rescue of Explants. Root Induction and Transplanting of Herbaceous Peony In Vitro.

Key words*: herbaceous peony, Paeonia lactiflora* Pall.*, tissue culture, in vitro propagation.*

INTRODUCTION

Peonies can be propagated by division, cutting, grafting, and layering to obtain true-to-type plants.

There are multiple traditional choices for propagation of peony. However, the limited number produced by these traditional methods can not meet the increasing demands in the market, especially for a quick release of a new cultivar and massive production of a specified variety. The development of micropropagation methods for peonies is necessary to not only overcome this problem but also accelerate peony breeding progress.

The field of plant tissue culture is based on the premise that plants can be separated into their component parts (organs, tissue, or cells), which can be manipulated in vitro and then grown back to complete plants. The first successful plant tissue and cell culture was accomplished by Gottlied Haberlandt near the turn of the 20th century when he reported the culture of leaf mesophyll tissue and hair cells (Steward, 1968; Krikorian and Berquam, 1969).

Micropropagation of peony began in the middle 1960s. During the last 40 years, much research on micropropagation of peony has been conducted with plants successfully produced from tissue culture labs. Planteck Biotechnologies Inc., a company based in Quebec, Canada, produced mass numbers of herbaceous Itoh peonies and made the tissue cultured plants available in the market for the first time in 2006 (Whysall, 2006).

However, there is still much work to be done in order to make mass production of peony more commercially successful. Optimization of procedure for each stage or step should be studied in depth, including selection of explants, decontamination, screening of medium, application of plant growth regulators (PGRs), induction of callus and shoots, subculture, rooting, and finally transplanting.

In peony, callus was induced successfully for the first time by Yamada and Sinotô (1966) from petal culture of *P. japonica*. Large variation in chromosome numbers and

characteristics within cells was observed during culture. Demoise (1967) and Demoise and Partanen (1969) induced callus from tree peony and investigated the effects of subculturing and physical conditions of culture on the mitotic cycle kinetics of a population of cells, particularly in relation to the degree of heteroploidy. Since then, many original studies on in vitro culture of peony have been reported. Several review papers have also been published. The first review on micropropagation of peony was published by Buchheim and Meyer (1992), where research on *in vitro* culture of peony before 1989 on has been mostly discussed. A summary of in vitro studies conducted on several species of *Paeonia* was included in a table consisting of plant species, inoculum, medium, growth response, and reference. This review is useful for a researcher working on peony tissue culture, although some medium formulations have not been cited correctly. The second review on in vitro culture peony was written by Gabryszewska (2004). The regeneration ability of different organs of both herbaceous and tree peony was reported in this paper. The role of exogenous and endogenous PGRs in differentiation and growth of shoots, roots, and somatic embryos was also discussed.

In 2007, Beruto and Curir summarized tissue culture of tree peony under a book chapter title, '*In vitro culture of tree peony through axillary budding*', which included most of the results published between 1969 and 2004. The review was well organized by introduction, experimental protocol consisting of stages of tissue culture, and conclusion. During this time, five other review papers on peony tissue culture were published by Chinese researchers (Buchheim and Meyer, 1992; Gabryszewska, 2004; Li and Luo, 2004; Li et al., 2006a; Jia et al., 2006b; Meng et al., 2007, Beruto and Curir, 2007 2007; Zhao et al., 2007). However, each of these papers is only 2 to 4 pages long and did not cover as much as information of previous work on micropropagation of peony. With the fast development of internet and database construction in the world and the benefit of language translation software, it is becoming much easier to access and understand the original research resources published by non-native languages.

More research is needed to make mass production of peony more commercially successful. Optimization of procedures are necessary, from selection of explants, decontamination, screening of medium, application of plant growth regulators (PGRs), induction of callus and shoots, subculture, rooting, and to final transplanting. The major goal of this study is to evaluate shoot induction ability of peony explants and PGRs.

EFFECT OF GELLING AGENTS ON TISSUE CULTURE OF HERBACEOUS PEONY

During this researches made in 2007 Torres et al., used as material and method the follouing:

Four types of agar products were used to compare responses of nodal stem explants of 'Yang Fei Chu Yu' ('YFCY') and 'Xi Shi Fen' ('XSF') in ½ MS + 1 mg l-1 TDZ medium with (1) A111 (Phytotechlab) 5 g l^{-1}; (2) A296 (Phytotechlab) 6 g l^{-1}; (3) A133 AgagellamTM (Phytotechlab) 4 g l^{-1}; and (4) A20020 (high gel strength) 5 g l^{-1} according to the recommended rates.

After this researches made in 2007 Torres et al., obtained the follouing result and discutions:

Explants responded to the types of agar gelling agents differently. Generally A111 (5 g l^{-1}) and A133 (4 g l^{-1}) were much better than A296 (6 g l^{-1}) and A20020 (5 g l^{-1}) for all indexes. Response was also genotype dependent. A133 and A111 significantly increased explant growth of 'YFCY' as compared to A296 and A20020 but this effect did not occur in 'XSF'. Similar results were noted on induction of callus and shoots. The highest rates of shoot initiation and callus production were seen on explants with treatments of A111 and A133, respectively, in 'YFCY', but no significant difference was observed in 'XSF'. For both cultivars, more phenolic compounds exuded into media with A296 and A20020, and explant color looked abnormal. Agar has long been used to solidify media for plant tissue culture. There are lots of brands of agar gelling agents.

The type of agar or other gelling agents significantly influences plant growth in tissue culture. Differences of results are even seen in the same product made at different times or locations (Torres et al., 2007).

COMPARISON OF BASAL MEDIUM IN TISSUE CULTURE OF HERBACEOUS PEONY

During this researches made in 2008 Daike Tian used as material and method the follouing

The choice of type of basal medium is important for tissue culture. MS, ½ MS and WPM have been mostly used in peony tissue culture for shoot induction. In this experiment, effects of MS (1/4, half, full strength) and WPM (half, full strength) medium with 1 mg l^{-1} TDZ + 0.1 mg l^{-1} BA + agar (A111, 4 g l^{-1}) was evaluated on callus and shoot induction of nodal stems from three herbaceous cultivars: 'Bin Shan' ('BS'), 'Fen Ling Hong Zhu' ('FLHZ') and 'Yang Fei Chu Yu' ('YFCY').

After this researches made in 2008 Daike Tian obtained the follouing result and discutions

The choice of type of basal medium is important for tissue culture. MS, ½ MS and WPM have been mostly used in peony tissue culture for shoot induction. Explants generally performed best in full strength medium MS followed by half strength MS, and worst in half strength WPM. Nodal stems of all three cultivars remained green in full strength MS medium, while explants turned pink and showed slight abnormalities in other MS media. Explants cultured in WPM turned pink, then red over time. MS was the most favorable medium for explant growth of 'BS'. Explants cultured in ½ WPM had the least increase in growth for all three varieties. Browning was more visual in WPM than MS. MS medium was more favorable than WPM for callus induction but results of shoot induction were complicated. Explants cultured on full strength WPM produced the highest shoot initiation rates for 'BS' and 'FLHZ' but the lowest rate for 'YFCY'. Explants of 'BS' generated the lowest shooting rate on half strength WPM while explants of 'FLHZ' produced the lowest shooting rate in ½ MS. Compared with the data collected on 24 and 40 days after inoculation, the difference in the initiation rate among basal medium types was consistent.

EFFECT OF PRETREATMENT OF UNDERGROUND BUDS ON TISSUE CULTURE OF HERBACEOUS PEONY.

During this researches made in 2008 Daike Tian used as material and method the follouing:

Responses of underground buds to three pretreatments were evaluated in tissue culture of two varieties of herbaceous peony. Underground buds (Figure 1) from containerized stock plants of **'**Da Fu Gui' ('DFG') were treated in three ways: (1) plants remained untreated; (2) plants were treated in cooler for 20 d beginning Nov. 20, 2006; and (3) plants from outside were washed by tap water and repotted in container with perlite, then directly moved to greenhouse to break dormancy and force growth.

Figure 1. Underground buds of herbaceous peony, (after Daike Tian, 2008)

In this treatment, the stock plants were also rinsed with 1% Zerotol for two times, once per week, to decrease contamination. After surface sterilization of 20 sec in ethanol (75%) following by 25 min in 10% Chlorox bleach, the buds (tips) were inoculated in test tubes with medium ½ MS + 1 mg l^{-1} BA + 0.1 mg l^{-1} GA3 or ½ MS + 0.1 mg l^{-1} BA + 0.1mg l^{-1} GA3 on Dec 10, 2007. Shoot multiplication medium was ½ MS + 0.1 mg l^{-1} BA + 0.1 mg l^{-1} TDZ + 0.1 mg l^{-1} GA3.

After this researches made in 2008 Daike Tian obtained the followuing result and discutions

A previous experiment produced 100% contamination of 'DFG' and 'Xi Shi Fen' ('XSF') dormant underground buds with a sterilization treatment of 10 sec in ethanol (75%) following by 10-20 min in 10% Chlorox bleach. After 15 d of culture, no callus formed and buds did not grow. In this experiment, contamination and phenolic exudation 281 were visual within one day of culture and increased over the time of the 15-d experiment. All explants (100%) treated in greenhouse were contaminated within 3 d of culture. The cooler treated explants performed better. Only 2 of 16 explants were contaminated within three days although contamination rate went up to 75% after 15 d culture. More explants were contaminated by bacteria than fungi. Contamination with bacteria also occurred earlier. There was significant difference in fungi caused infection among treatments. At the end of experiment, only one bud from the outside treatment and 4 buds from the cooler treatment were sterile for use of shoot induction after transfer. Before culture, buds treated in the cooler for 20 d were already slightly elongated (dormant release) and looked better in quality than both outside and greenhouse treated buds. After inoculation these buds grew very fast and main shoots as well as some lateral shoots elongated (Figure 2). These elongated shoots could be cut into nodal sections and transferred to fresh medium for shoot multiplication. Buds from the outside also elongated but did not grow as fast as the cooler treated buds. The shoots were much shorter than those generated from the cooler treated buds (Figure 2). The greenhouse perlite medium treated buds performed worst and all nearly stopped growing. These buds finally died and no axillary shoots generated. For all treated buds, no callus formed in the early stages. Only very little green callus was induced at the cutting side of buds after more than 15 d of culture. A more effective sterilization approach must be developed. Dormant buds were not effective for shoot induction.

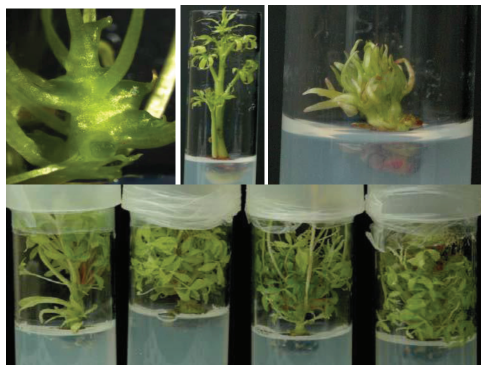

Figure. 2. After culture in ½ MS + 1 mg l^{-} BA + 0.1 mg l^{-} GA3, the cooler treated buds (left) grew fast and produced elongated shoots (main and lateral). After cutting of shoots and subculture in ½ MS + 0.1 mg l^{-} BA + 0.1 mg l^{-} TDZ + 0.1 mg l^{-} GA3, the number of shoots was multiplied, each bud eye region formed one to several new shoots (middle). While bud explants from outside stock plants (right) grew slow and shoot stem did not elongated much (after Daike Tian, 2008)

If the buds were treated in cooler, contamination decreased significantly. Shoot or bud explant could readily grow and axillary shoots were easily induced after bud dormancy was broken.

INVESTIGATION OF CALLUS AND SHOOT INDUCTION ABILITY OF TISSUE

During this researches made in 2008 Daike Tian used as material and method the followuing

Callus and shoot induction ability of 'Da Fu Gui' ('DFG') and 'Paula Fay' ('PF') were studied in this experiment using petals, anthers, filaments, and pistils as explants from the tight flower buds (TFB), one week before blooming, newly opening flowers (OF) with a small mouth or the fully open flowers (FOF). The petals of TFB, OF and FOF were from 'DFG', and the petals, anthers and filaments of TFB were from 'PF'. Three media were tested: A: ½ MS (PGR-free); B: ½ MS + 1 mg l^{-1} TDZ; and C: ½ MS + 1 mg l^{-1} TDZ + 0.1 mg l^{-1} GA3.

After this researches made in 2008 Daike Tian obtained the followuing result and discutions

After 14 d of culture, contamination occurred on the petals but not on either anthers or

filaments. Petals from FOF had higher contamination rate than those from TFD and OF in 'DFG'. Browning was obvious in all treatments and it was more severe on the petals of FOF followed by OF in 'DFG'. Petals from TFB had the least browning problem. However, in 'PF' nearly all of the petals from TFB turned brown. The least browning rate was seen on anther culture of this cultivar. No difference in browning was found among PGR treatments. There were large differences in growth of explants among types of petals. Petal sections from both TFD and OF grew fast, whereas those from FOF grew slow and lost their original color within two days of culture. Callus generated very slowly and no callus formed within 15 d. Following 30 d of culture, very little callus was induced from the base cutting side of the petals from TFB and OF in both B and C media.

EFFECT OF PPM ON DECONTAMINATION AND RESCUE OF EXPLANTS

During this researches made in 2008 Daike Tian used as material and method the follouing

Effect of PPM on decontamination of nodal stem segments from 'Da Fu Gui' (treated 3 months in the cooler) and rescue of contaminated explants was investigated in media with ½ MS + 1 mg l^{-1} BA + 0.2 mg l^{-1} GA3 with supplement of 0, 0.05, 0.1, and 0.2% (v/v) of PPM, respectively. The explants were surface sterilized with a 12–15 sec quick soak in 70% Ethanol followed by 20 min in 10% Bleach. For rescue of contaminated explants, 12 of bacteria contaminated explants were cleaned by a soft brush under running tap water following by a 12 min soak in 50% PPM and then were inoculated on fresh medium.

After this researches made in 2008 Daike Tian obtained the follouing result and discutions

PPM had effect on minimizing contamination of explants and the contamination rate decreased in 0.2% PPM-treated medium. However, lower concentrations (<0.2%, v/v) of PPM only delayed occurrence of contamination. No difference was observed in browning, the callus and shoot induction rate, and shoot growth of posttransferred explants between PPM treatment and the control explants. This indicated application of PPM at low level in medium had no side effect on tissue culture of peony.

ROOT INDUCTION AND TRANSPLANTING OF HERBACEOUS PEONY *IN VITRO*

During this researches made in 2008 Daike Tian used as material and method the follouing

Several trials on root induction were conducted on vitroshoots from the varieties: 'Xi Shi Fen', 'Da Fu Gui', and 'Cytherea'. The treatments included following variables: light and darkness; liquid (paper bridge method, Hosoki et al., 1989), half solid and solid ½ MS 285 medium; activated charcoal (AC); temperature; and IBA with different concentrations or a quick soak in high concentration of IBA (10 mg l^{-1}).

After this researches made in 2008 Daike Tian obtained the follouing result and discutions

Following 7–10 d of culture in rooting medium, all vitroshoots generated callus. Callus even formed sometimes on the petioles.

Plantlets rooted following about 20 d of culture and up to 20 roots developed on a shoot. Roots grew up to 3 cm after 45 d of shoot inoculation. The vitroshoots grew very fast in semi-solid rooting medium possibly benefiting from an easy absorption of nutrients (Figure 3). AC treatment increased the shoot length but not significantly. IBA shock treatment (10 mg l^{-1}) did not influence growth of *in vitro* shoots on rooting medium. IBA at 1 mg l^{-1} in a continuous treatment was too strong for small younger vitroshoots and caused their petioles to calluse. It remains not clear how long vitroshoots should be treated in medium with high concentration IBA before transfer to root growth medium with either lower concentration of IBA or no IBA. High-quality shoots are the basic requirement to obtain high rate of rooting. Several studies have reported treatment of darkness and chilling was beneficial for rooting of peony in vitro (Kunneman and Albers, 1989;

Habib and Donnelly, 2001; Beruto et al., 2004; Chen 2005).

Figure 3. Shoots grew fast in half-solid medium and there was no difference in shoot length between treatments (with or without IBA shock, AC), (after Daike Tian, 2008)

Roots were successfully induced from plantlets in solid medium (Figure 4) or using paper bridge method (Figure 5) in some cases.

Figure. 4. Roots were only induced on the shoots with a 10-min pretreatment in high concentration of IBA (10 mg l^{-}) before inoculation on medium with ½ MS + 1 mg l^{-} IBA ± 5% AC (w/v) (after Daike Tian, 2008)

The present experiment showed that the shoots with a 40-d darkness treatment at 25ºC nearly stopped growth and completely died later. The shoots with a 40-d darkness treatment at 10ºC survived with chlorotic tissue. However, no roots were obtained in both treatments. For both of the rooted and non-rooted shoots, the earlier developed leaves showed obvious necrosis after more than two months culture but the later formed young leaves remained healthy. It was not clear if necrosis problem could be avoided or minimized by an increase of transfer frequency. Limited number of shoots with roots or root primordia was transplanted to jars with non-sterile peat moss. These vitroplants grew in the first week but quickly died because of infection with fungi (Figure 6).

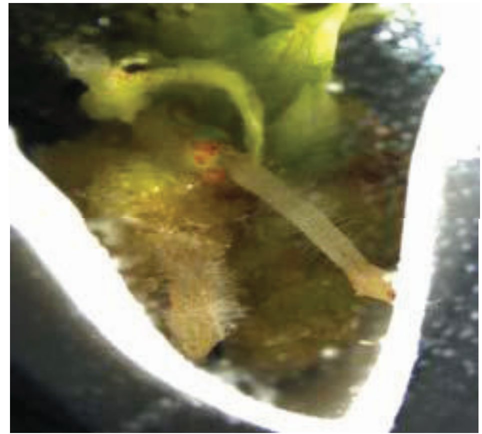

Figure 5. Roots were induced from vitroshoots of ‘Da Fu Gui’ by paper bridge (white material) method with liquid medium: ½ MS + 1 mg l^{-1} IBA (after Daike Tian, 2008)

Figure. 6. After transplanting, rooted shoots grew in the first week but then died with contamination of fungi or rot of roots and stem (after Daike Tian, 2008)

CONCLUSIONS

As a result of their researches made in 2007 Torres et al., reached follouing conclusion

The type of agar or other gelling agents significantly influences plant growth in tissue culture. Differences of results are even seen in the same product made at different times or locations.

As a result of their researches made in 2008 Daike Tian, reached follouing conclusions

1. The highest shoot length was observed in explants of 'BS' cultured on 1/2 MS and 'FLHZ' on MS. For these two cultivars, shoot growth of explants performed worst on ½ WPM. Shoot growth generally increased from ¼, ½, to 1 MS. Quality of generated shoots was better in MS than WPM medium.

2. It has been widely reported that underground buds of herbaceous peony are difficult to disinfect compared with aerial tissues. Response of underground buds was different in tissue culture between stages of growth or when different pretreatments are used. . Contamination was a big problem with the use of underground buds as explants in tissue culture of herbaceous peony.

3. No callus was seen in A (PGR-free) medium for petals. Anthers and filaments also did not produce callus. Results indicated 1 mg l^{-1} TDZ could induce callus from petal culture. However the induction rate as well as the amount of callus was very low. There was no difference in callus induction between B and C media. It showed that GA3 at 0.1 mg l^{-1} concentration had no effect on induction and production of callus compared with the control. No adventitious shoots developed.

4. Contamination of the rescued contaminated explants significantly decreased following PPM treatment but black phenolics exuded quickly to medium from explants. Browning was a big problem which quickly caused death of plant material. Therefore, it was not a viable option to rescue contaminated peony explants using PPM even though successful in decontamination.

5. Treatment with a spray of 1% Hydrogen peroxide (H2O2) did kill fungi but could not save the plantlets. Four plantlets transplanted to sterile peat-moss mix died quickly with rot of roots and stem base.

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