Protocol for callus induction of *Camellia japonica* L. (Tea rose)

H. L. T. Dilrukshika, L. M. H. R. Alwis
Faculty of Animal Science and Export Agriculture, Uva Wellassa University of Sri Lanka

Introduction

Camellia japonica (the Japanese Camellia) is one of the best known species of the genus *Camellia*. Among the *Camellia* species, the economic value of the *C. japonica* ranks the highest due to its beautiful ornamental flowers, edible uses (dried flowers, oil), medicinal uses (astringent, antihaemorrhagic, haemostatic, salve and tonic) and material uses (dye, oil) (Salinero *et al.*, 2012).

Although *C. japonica* has a high ornamental and medicinal value, it is not popular in tea cultivating tropical agricultural country like Sri Lanka yet. Further, it was revealed that the difficulties of propagating Tea Roses are significant and therefore growers discourage to propagate them. Also *C. japonica* multiplication and improvement through seeds is rare due to poor seed set in the white and pink varieties present in Sri Lanka. *C. japonica* is usually propagated only using stem cuttings in Sri Lanka at present. But rooting was very poor in both pink and white varieties (Fernando and Alwis, 2013). But a good economic potential can be achieved in Sri Lanka due to its beautiful ornamental flower which is having long life span if it is scientifically developed to get different colors and shapes. Therefore, it is very important to *in vitro* propagation of *C. japonica* in large scale to commercially enhance its real value especially in the up country and mid country regions of Sri Lanka.

Therefore this study was aimed to develop a protocol to induce the callus culture of *Camellia japonica* L (Tea Rose).

Material and Methods

This research study was conducted at Tissue Culture laboratory at Uva Wellassa University during the period of 22.04.2014 to 15.08.2014. The explants were collected from the Ury estates in Balangoda Plantations and Hakgala Botanical Garden, Hakgala, Nuwara Eliya.

This study was conduct to develop an efficient protocol for rapid and prolific callus induction of *Camellia japonica* (Tea Rose). In the first experiment, leaves and nodal segments used as explants. Nine different combinations of 20% sodium hypochlorite for three different time durations (20 minutes, 30 minutes, 40 minutes) and 70% ethanol for three different time durations (30 seconds, 1 minute, 1 and half minutes) were used to select the best sterilization method. Number of contaminated vessels were counted after one week. Above nine treatment combinations were succeeded only for *C. japonica* leaves. Because of again used another nine different treatment combinations for surface sterilization of nodes by adjusting soaking time duration in the 20% sodium hypochlorite (35 minutes, 40 minutes, 45 minutes).

In the second experiment, leaves, nodal segments and un opened flower bud flower petals used as explants. The sterilized explants were cultured on MS medium with three different hormone combinations of 3-indolebutric acid (IBA) and 6-benzylamino purine (BAP) to investigate the effect on callus induction.

Table 1: Nine different treatment combination used for callus induction of white and pink varieties of *Camellia japonica*

Medium / Explant	Leaves	Nodal Segments	Petals
MS + 1mg/L IBA+2mg/L BAP	T_1	T_4	T_7
MS + 1mg/L IBA+3mg/L BAP	T_2	T_5	T_8
MS + 1mg/L IBA+4mg/L BAP	T_3	T_6	T_9

Shortest time duration was recorded as minimum number of days for callus initiation for each treatment separately. After three weeks from establishment of explants morphology of callus was observed.

Results and Discussion

Selection and Preparation of Explants

Developmental stage of an explant is an important factor for initiation of cultures for *in vitro* propagation. Younger the tissues better the *in vitro* response. Age of stock plant, physiological age of the explant and its developmental stage, as well as its size can determine the success of a procedure. Mature plant derived explants reported to be highly recalcitrant *in vitro*. Moreover, high degree of contamination in mature tissues poses problem in the establishment of culture. Juvenile explants are more responsive in culture than the mature explants from mature trees (Ahuja, 1993).

Accordingly, young, disease free, healthy fully expanded light green color leaves and light brown color nodal segments were selected as explant.

Experiment 1

Table 2: Contamination percentages of C. japonica leaves

Treatment	Contamination %
20% NaOCl for 20 min + Ethanol for 30 seconds (T_1)	90%
20% NaOCl for 20 min + Ethanol for 60 seconds (T_2)	50%
20% NaOCl for 20 min + Ethanol for 90 seconds (T_3)	50%
20% NaOCl for 30 min + Ethanol for 30 seconds (T_4)	60%
20% NaOCl for 30 min + Ethanol for 60 seconds (T_5)	40%
20% NaOCl for 30 min + Ethanol for 90 seconds (T_6)	40%
20% NaOCl for 40 min + Ethanol for 30 seconds (T_7)	40%
20% NaOCl for 40 min + Ethanol for 60 seconds (T_8)	20%
20% NaOCl for 40 min + Ethanol for 90 seconds (T_9)	40%

Contamination percentages of *Camellia japonica* leaves in each treatment were showed in table 2. The results showed that 20% NaOCl for 20 minutes with 70% ethanol for 30 seconds showed 90% contamination. All other treatments showed the contamination, below 60%. Among the nine treatments 20% NaOCl for 40 minutes with 70% ethanol for 60 seconds showed the lowest contamination percentage (20%) for leaves. That was acceptable for sterilization of leaves (T_8).

Table 3: Contamination percentages of *C. japonica* nodal segments

Treatment	Contamination %
20% NaOCl for 20 min + Ethanol for 30 seconds (T_1)	100%
20% NaOCl for 20 min + Ethanol for 60 seconds (T_2)	80%
20% NaOCl for 20 min + Ethanol for 90 seconds (T_3)	100%
20% NaOCl for 30 min + Ethanol for 30 seconds (T_4)	100%
20% NaOCl for 30 min + Ethanol for 60 seconds (T_5)	100%
20% NaOCl for 30 min + Ethanol for 90 seconds (T_6)	100%
20% NaOCl for 40 min + Ethanol for 30 seconds (T_7)	90%
20% NaOCl for 40 min + Ethanol for 60 seconds (T_8)	80%
20% NaOCl for 40 min + Ethanol for 90 seconds (T_9)	70%

Contamination percentages of *Camellia japonica* nodes in each treatment were showed in table 3. The results revealed that all treatments showed more than 70% contamination for nodal segments. T1, T3, T4, T5, T6 shows 100% contamination for nodal segments. That was doubtful for surface sterilization of nodal segments. None of surface sterilization method could be recommended. Therefore, again another nine different treatment combinations were used for surface sterilization of nodes by adjusting soaking time duration in the 20% sodium hypochlorite. Table 4 shows the results of adjusted treatments.

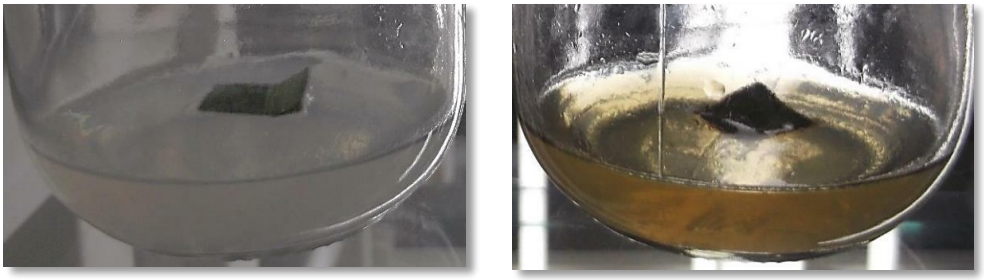
Table 4: Contamination percentages of *C. japonica* nodal segments

Treatment	Contamination %
20% NaOCl for 35 min + Ethanol for 30 seconds (T_1)	100%
20% NaOCl for 35 min + Ethanol for 60 seconds (T_2)	90%
20% NaOCl for 35 min + Ethanol for 90 seconds (T_3)	90%
20% NaOCl for 40 min + Ethanol for 30 seconds (T_4)	80%
20% NaOCl for 40 min + Ethanol for 60 seconds (T_5)	90%
20% NaOCl for 40 min + Ethanol for 90 seconds (T_6)	80%
20% NaOCl for 45 min + Ethanol for 30 seconds (T_7)	90%
20% NaOCl for 45 min + Ethanol for 60 seconds (T_8)	70%
20% NaOCl for 45 min + Ethanol for 90 seconds (T_9)	30%

Contamination percentages of *Camellia japonica* nodes in each adjusted treatments were showed in table 4. The results revealed that among the nine treatments only one treatment (T_9) showed 30% contamination where as all the other eight treatments showed more than 70% contaminations. 20 % NaOCl for 45 minutes with 70% ethanol for 90 seconds showed the lowest contamination (30%) for nodal segments of *C. japonica.* Thus T9 was accepted for surface sterilization of nodal segments of *C. japonica.*

Seran *et al.* (2007), Bidarigh and Azarpour (2013) reported that the surface sterilization of *Camellia sinensis* leaf and nodal explants were treated with 70% ethyl alcohol for two to three minutes time duration and 20% sodium hypochlorite for 30 minutes. The surface sterilization of the present study was strongly success with the using above chemicals with changing soaking time duration. 20% NaOCl for 40 minutes and ethanol for 60 seconds for leaves and 20% NaOCl for 45 minutes and ethanol for 90 seconds for nodal segments were succeeded for surface sterilization of *Camellia japonica*.

Browning Effect of Explant in Culture Establishment



(a) **(b)**

Plate 1: Browning of explants (a) Just after establishment of the explant b) Browning after establishment of the explant

One of the most common problems associated with the *in vitro* establishment of *Camellia japonica* is the deleterious effects of oxidized phenols (Forrest, 1969).The oxidation of exuded phenolic cause darkening or browning of explants of *Camellia japonica*. Leaf explants margins were light brown in the beginning, after it has become dark brown. Some leaf explant totally became brown color after the establishment and the oxidation of exuded phenolic cause browning of culture media after established the explants of *Camellia japonica* as shown in plate 1.

Experiment 2

Callus were initiated from *Camellia japonica* leaves and nodal segments, but among the three explants, flower petals did not respond to any of the treatment tried for callus initiation.

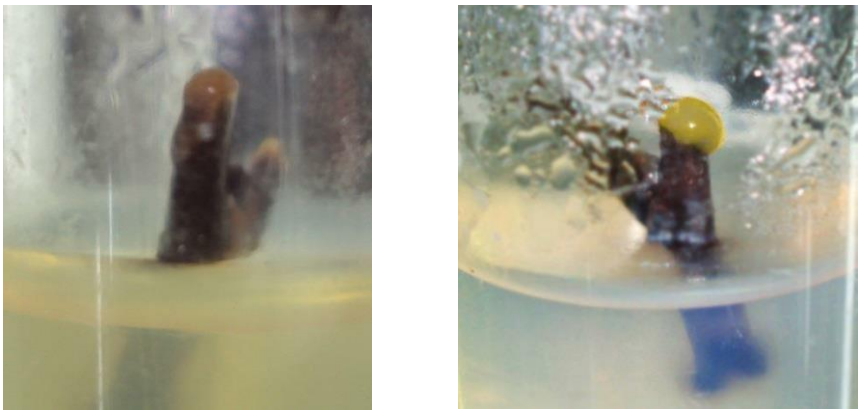
The minimum number days to callus initiation was 25 days from the leaves on Murashige and Skoog medium (MS) supplemented with the 1mg/L IBA + 4mg/L BAP (T3). Color of leaf pieces turned into light yellow in the beginning and gradually became dark brown and then initiated callus from the uncut surfaces.

Plate 2: Calli of leaves after three weeks

Leaves callus tissue was yellow in the beginning, it has become friable and greenish patches appeared on the top of the callus. Friable in texture and irregular shaped calli formed from *C. japonica* leaves explants.

The minimum number days to callus initiation was 19 days from the nodal segments on MS medium supplemented with the 1mg/L IBA and 2mg/L BAP (T4). MS medium supplemented with 1 mg/L

IBA and 3 mg/L of BAP (T5) took 23 days to initiate the calli. The minimum number days to callus initiation was 25 days from the leaves on MS medium supplemented with the 1mg/L IBA + 4mg/L BAP (T3).



(a) (b)

Plate 3: Nodal callus (a) Initiated calli of nodal segments after three weeks (b) Calli of nodal segments after four weeks

The color of nodal segments turned in to brownish orange and gradually became light yellow and then initiated callus from cut surfaces. When the callus aged, nodal callus was appeared as light yellow in color, watery and soft in texture and globular shaped.

Arulpragasam *et al.* (1988) reported on the successful production of callus from the tissues of cotyledons, nodal segments with axillary buds and leaves of *Camellia sinensis*. In the present study nodal segments with axillary buds and leaves of *C. japonica* were found effective explants for callus initiation.

Camellia sinensis friable calli were first initiated on cultured leaf segments in the presence of BAP (2.0 mg/L) and NAA (3.0 mg/L) after 21 days of incubation. A combination of BAP (2.0 mg/L) and NAA (1.0 mg/L) also induced greenish yellow friable calli but at a low frequency (Seran *et al.,* 2007). In the present study, MS supplemented with 4mg/L BAP and 1mg/L IBA was found effective for leaf callus formation of *Camellia japonica*. Callus color also greenish yellow and minimum number of days for callus initiation was 25 days. This is in accordance with the findings of Seran *et al.* (2007).

Conclusion

According to the results obtained, the protocol developed for initiation of the callus culture of *Camellia japonica;*

Semi mature light brown nodal segments (1cm) and light green leaves (1cm) as explant are favorable to induce callus on MS medium.

Camellia japonica leaves can be treated with 20% NaOCl for 40 minutes and 70% ethanol for 60 seconds (T8) and nodal segments can be treated with 20% NaOCl for 45 minutes and 70% ethanol for 90 seconds (T9) for proper surface sterilization.

MS supplemented with 0.9% agar, 3% sucrose, 0.001% myo-inositol, with 1mg/L IBA and 2mg/L BAP or 1mg/L IBA + 3mg/L BAP is better hormone combination for earlier callus formation from *Camellia japonica* nodal segments and MS supplemented with 0.9% agar, 3% sucrose, 0.001% myo-inositol, with 1mg/L IBA and 4mg/L BAP is better hormone combination for earlier callus formation

from *Camellia japonica* leaves. Among the different explants, nodal segments with axillary bud is the best explant for earlier callus formation for *Camellia japonica*.

Vertically placed nodal segments and leaves with a cut surface in contact with the medium is better for callus induction. Cultures should be maintained at 25±2 C temperature under completely dark conditions in an incubator. The callus formed should be subcultured on the MS medium of same combination after two weeks of induction.

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