

INITIATION AND STABILIZATION OF A TRUMPET CREEPER (*Campsis radicans* (L.) Seem.) TISSUE CULTURES

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Abstract. A trumpet creeper (*Campsis radicans*) is a very decorative shrub propagated vegetatively through cuttings. So far, there is no available information on micropropagation of this beautiful species. Determination of the optimal sterilization methods as well as types and concentrations of plant growth regulators as medium constituents is one of the most important factors of successful micropropagation. With the aim of optimization of *in vitro* initiation and multiplication of *C. radicans*, the effect of different methods of disinfection and terms of explants isolation on contamination rate of cultures as well as the influence of cytokinins on growth and branching of shoots was studied. The cytokinins used in the experiments were: benzyladenine (BA), isopentenyl adenine (2-iP) and kinetin (KIN). The obtained results show that contamination rate is a very significant problem to overcome in order to initiate tissue cultures of *C. radicans*. The best results were observed when explants were excised in spring (May), shortly after the vegetation had started (88% contamination rate). Soaking initial the fragments in a mixture solution of Topsis M 500SC and streptomycine for 12 hours decreased the contamination rate of explants from 100 to 94%. The shoot tips are more suitable to establish the tissue culture of a trumpet creeper, in comparison to nodes with axillary buds. The multiplication rate after two subcultures was 2.6–3.7 for shoot tips (depending on the media) and 1.9–2.1 for nodes. The cytokinins used in the experiment had a significant influence on multiplication rate of *C. radicans*. The highest number of good quality shoots was obtained on the media supplemented with KIN in concentration of 2 mg·dm⁻³.

Key words: cytokinins, disinfection, explant type, micropropagation, tissue culture

INTRODUCTION

A trumpet creeper (*Campsis radicans* (L.) Seem.) belongs to the Bignoniaceae family. It is a woody, deciduous, perennial vine, commonly found in the midwestern and southeastern United States, where it is considered as a weed [Edwards and Oliver 2004, Beeler et al. 2012]. Leaves are opposite and composed of several similar leaflets. Stems get woody and may trail along the ground or climb. Showy orange-red scentless tubular flowers are clustered in inflorescences consisting of up to 14 flowers. In natural habitat it blooms from June till September [Bertin 1982, USDA 2004]. It produces nectar which is attractive to its pollinators. In natural habitat it is pollinated by hummingbirds, but in Poland they are substituted by honeybees [Kołodziejska-Degórska and Zych 2006]. *C. radicans* contains flavonoids that show free radical scavenging activity [Hashem 2007]. In Europe a trumpet creeper is regarded as an attractive ornamental vine often planted in private gardens. For many years the trumpet creeper was cultivated only in botanic gardens and arboreta, but nowadays it is available from many nurseries [Polish Nurserymen Association]. It is used as a cover for fences, arbors, walls or large trellises and as a groundcover. The cigar-like fruits may be decorative during winter [USDA 2004]. *C. radicans* is known to be generally self-incompatible, although in specific conditions pollination with self pollen may occur [Bertin et al. 1989]. Usually less than 10% of flowers produce mature seeds, [Bertin 1982], but individual plant may still produce up to 2800 viable seeds [Chachalis and Reddy 2000]. In climate conditions of Poland, however, it rarely sets seeds what is a limiting factor for the generative reproduction [Kołodziejska-Degórska and Zych 2006]. The trumpet creeper may be propagated vegetatively by stem cuttings with the use of rooting stimulators [Ban 2011, USDA 2004] or by rootstocks [Edwards and Oliver 2004].

There is very little information on *in vitro* propagation of *Campsis radicans* (Wei et al. 2007, in Chinese with English abstract) and there are a few articles on micropropagation of plants belonging to the Bignoniaceae family. Initiation of the plant tissue culture may depend on the age of the donor plant, term of the explants isolation, conditions of growth of donor plants [Adamus 1996]. Most of woody plant species may be used to establish tissue cultures during juvenile growth [Evers et al. 1996]. Pawłowska [2005] studied regeneration of shoot tips of a climbing rose 'New Dawn' excised from one year old and older shoots. She noticed that the one-year-old shoots produced two times more adventitious shoots in comparison to the older ones. To initiate tissue culture of *Morus latifolia*, Lu [2002] used 6-month-old shoots. The term of an *in vitro* culture initiation is especially important in case of species that become dormant. It is known that regeneration ability increases in spring [Adamus 1996]. In case of *Syringa vulgaris*, the best term to collect explants was the beginning of April (40% of regeneration), in comparison to March and February (7% and 0% respectively) [Nesterowicz et al. 2006].

Different types of explants are used for micropropagation [Pati et al. 2006]. In the method of adventitious shoots, the shoot tips and nodal explants containing meristems, are usually used as the initial explants [Bach 2004]. The shoot tips were used to establish tissue cultures of such species as *Viburnum odoratissimum* [Schoene and Yeager 2005], *Lonicera japonica* and *Lonicera maackii* [Cheng and Osburn 2005]. In turn, the axillary buds were used to establish tissue culture of *Hydrangea macrophylla* [Pod-

wyszyńska et al. 2003], Rosa 'New Dawn' [Pawłowska 2005], Rosa sp. [Carelli and Echeverrigaray 2002, Ibrahim and Debergh 2000]. Nodal explants are also very often used to initiate tissue cultures of many other plant species such as: *Vitis thunbergi* [Lu 2005], *Olea europaea* [Santos et al. 2003] or *Jatropha curcas* [Sujatha et al. 2005]. Wei et al. [2007] also used shoot tips and stems as explants to optimize the tissue culture initiation of *Campsis radicans*.

In order to obtain sterile cultures of plants, the method of sterilization must be developed experimentally for each species. The reagents most often used contain active chlorine, fungicides or ethanol. The concentration of each reagent and duration of each step is very important and may decide about further success of *in vitro* propagation.

Micropropagation needs specific medium, growth regulators and culture conditions [Giri et al. 2004]. BA is the most frequently used cytokinin to stimulate growth of axillary shoots *in vitro* [Sahoo and Chand 1998, Nobre et al. 2000]. Wei et al. [2007] observed proliferation of *Campsis radicans* cultures when BA in concentration of $1.5 \text{ mg} \cdot \text{dm}^{-3}$ was added to the media together with NAA in concentration of $0.05 \text{ mg} \cdot \text{dm}^{-3}$. However, high concentration of BA may cause shoots hyperhydricity [Huang et al. 1998]. Kim et al. [1998] used BA and TDZ on shoots induction of three clones of *Fraxinus pennsylvanica*. The good results were obtained with the use of $2.2 \text{ mg TDZ} \cdot \text{dm}^{-3}$ or $9 \text{ mg BA} \cdot \text{dm}^{-3}$. Both cytokinins had also positive effect on propagation of *Acacia sinuata* shoots [Vengadesan et al. 2002]. It happens that cytokinins added to media have a disadvantageous influence on explants growth in tissue cultures. Palacios et al. [2002] described that kinetin added to the media used for initiation of *Lonicera tatarica* shoots lowered effectiveness of induction from 55 to 30%.

The aim of the study was to estimate the best possible method to initiate and establish a tissue culture of *C. radicans*.

MATERIAL AND METHODS

Culture initiation. The tissue culture was initiated from *Campsis radicans* shoots obtained from plants growing in a private garden. The single node pieces were excised in summer and disinfected with three methods (tab. 1).

The best term of explants excision from mother plants was also studied. Single node explants were excised in three seasons of the year: May, June (shoots of the current year) and October (shoots of the second year) and they were surface sterilized with the most effective method of disinfection from the first experiment (Method III) (tab.1). Isolated explants were placed in $25 \times 100 \text{ mm}$ test tubes containing 10 ml of basal medium consisting of Murashige and Skoog medium (MS) [1962] mineral salts, thiamine – $0.4 \text{ mg} \cdot \text{dm}^{-3}$, pyridoxine – $0.5 \text{ mg} \cdot \text{dm}^{-3}$, nicotinic acid – $0.5 \text{ mg} \cdot \text{dm}^{-3}$, glycine – $2 \text{ mg} \cdot \text{dm}^{-3}$, myo-inositol – $100 \text{ mg} \cdot \text{dm}^{-3}$, sucrose – $30 \text{ g} \cdot \text{dm}^{-3}$ and Agar-Agar (Lab-Agar™ Biocorp) – $6.5 \text{ g} \cdot \text{dm}^{-3}$ and supplemented with isopentenyl adenine (2-iP) in concentration of $2 \text{ mg} \cdot \text{dm}^{-3}$ and indole-3-acetic acid (IAA) in concentration of $0.5 \text{ mg} \cdot \text{dm}^{-3}$. The medium pH was adjusted to 5.7 before autoclaving. The cultures were maintained in a culture room at a temperature of 22°C during the day and 20°C at night in 16-h photoperiod with irradiance of $35 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

Table 1. Disinfection methods of initial explants of *Campsis radicans*

Successive treatments	Treatment description	Method I	Method II	Method III
1.	Soaking in solution of Topsin ($1 \text{ g} \cdot \text{dm}^{-3}$) and Streptomycine ($1 \text{ g} \cdot \text{dm}^{-3}$) for 12 hours			X
2.	Rinsing in water with detergent 3 times \times 20 minutes	X	X	X
3.	Dipping in 70% ethanol alcohol for 5 seconds	X	X	X
4.	Shaking in the solution of Topsin ($1 \text{ g} \cdot \text{dm}^{-3}$) for 60 minutes	X		
5.	Shaking in the solution of Topsin ($1 \text{ g} \cdot \text{dm}^{-3}$) and Streptomycine ($1 \text{ g} \cdot \text{dm}^{-3}$) for 60 minutes		X	
6.	Shaking in the solution of NaOCl (0.5% of chlorine) for 30 minutes	X	X	X
7.	Rinsing in sterile distilled water for 3 times	X	X	X

The following features were evaluated during the experiment: a survival rate, a contamination rate, number of regenerating shoots.

All shoots with symptoms of contamination were removed and sprouting shoots were further multiplied on the same medium to produce plant stock.

Multiplication. Shoot tips and one-node shoot pieces with one pair of leaves were excised from aseptically grown tissue cultures of *Campsis radicans* and placed in test tubes containing basal medium containing 2-iP in concentration of $2 \text{ mg} \cdot \text{dm}^{-3}$ and IAA in concentration of $0.2 \text{ mg} \cdot \text{dm}^{-3}$ or without growth regulators (control medium). The culture conditions were the same as during the initiation stage. After 4 weeks of culture, shoots were divided and subcultured into the fresh media of the same composition for another 4 weeks.

The following features were evaluated during the experiment: multiplication rate after two subcultures (counted as a total number of shoots obtained from nodal fragments and axillary shoots), number of axillary shoots per plant, length (mm), weight (mg) and number of leaves of the main shoot, length (mm) and weight (mg) of axillary shoots, weight of callus (mg). Some specific issues, such as colour, leaves and callus size, leaf roll, incidence of chlorosis, necrosis or malfomation were also monitored.

The influence of cytokinin type and concentration. The plant material was excised from aseptically grown tissue cultures of *Campsis radicans*. The explants used in the experiments were 2-node shoots pieces with one pair of leaves. Explants were placed into 300 ml Erlenmeyer flasks filled with control medium supplemented with: kinetin (KIN), isopentenyl adenine (2-iP) and benzyladenine (BA) in concentrations of 2 or $5 \text{ mg} \cdot \text{dm}^{-3}$. Medium without growth regulators was used as a control. Each combination included 21 shoots. One flask with 7 shoots was treated as a replication.

The basal medium and culture conditions were the same as during the initiation stage. The similar parameters as during multiplication stage were evaluated.

Statistical analysis. The results obtained in the experiments were analyzed statistically with the use of analyses of variance and Tukey t-test at $p = 0.05$ level of significance.

RESULTS AND DISCUSSION

Culture initiation. The method of sterilization did not have a significant influence on the contamination rate of *Campsis radicans* explants used to initiate the tissue cultures (tab. 2).

Table 2. A contamination rate of *Campsis radicans* explants depending on the method of sterilization

Method of sterilization	Number of explants	Contamination rate (%)
I	42	100
II	38	97
III	35	94

No matter the method of sterilization most of the explants had symptoms of fungal or bacterial contamination. It was observed that about 86% of explants were contaminated with fungi. The most contamination free explants (6%) were obtained when the shoot pieces were soaked in a mixture solution of Topsin and streptomycine for 12 hours prior to proper disinfection. Topsin was also used for disinfection of *Myrica esculenta* [Bhatt and Dhar 2004]. In the research of Podwyszyńska et al. [2003], spraying the donor plants with fungicide and streptomycine increased the number of contamination free explants from 17.9% to 30.8%. The mixture solution of streptomycine and Bavistin was used to disinfect *Carissa carandas* initial explants before proper disinfection. After 3 weeks of culture the survival rate was 74.3%. [Rai and Misra 2005].

The term of shoots excision from mother plants had a slight effect on contamination rate of *Campsis radicans* shoots used to initiate the tissue culture (tab. 3).

Table 3. Contamination rate of *Campsis radicans* explants depending on the term of shoots excision

Term of shoots excision	Number of explants	Contamination rate (%)
October	43	94
May	100	88
June	71	93

Nevertheless the highest number of contamination free shoots was obtained when explants were excised from donor plants in May (88%). Spring was also the best time to excise pieces of *Rosa* 'New Dawn' [Pawłowska 2005]. Rai and Misra [2005] initiated tissue cultures of *Carissa carandas* from shoot tips taken during winter, spring, summer and autumn. They observed that the most explants formed adventitious shoots in spring, when vegetation started. On the contrary, Bhatt and Dhar [2004] observed that explants of *Myrica* were the best when the tissue cultures were initiated in winter. Dormant buds were also used to initiate tissue cultures of *Hydrangea quercifolia* [Preece and Ledbetter 2003].

Multiplication. The growth and development of the shoot tip explants of *Campsis radicans* was not affected by the growth regulators added to the media (tab. 4).

Table 4. Branching of *Campsis radicans* shoots depending on the explant type and medium composition after two subcultures

Medium	Explant type	Multiplication rate	Main shoot length (mm)	Number of leaves on the main shoot	Main shoot weight (mg)	Number of axillary shoots per explant	Length of axillary shoots (mm)	Callus weight (mg)
Control	shoot tips	3.3 _a *	27.0 _a	2.5 _a	19.03 _a	1.5 _a	8.3 _b	23.58 _{ab}
	nodes	2.1 _{ab}	–	–	–	1.0 _b	1.0 _c	15.26 _b
2-iP 2 + IAA 0.2	shoot tips	2.6 _{ab}	27.0 _a	2.5 _a	16.23 _a	1.4 _{ab}	10.0 _b	33.41 _a
	nodes	1.9 _b	–	–	–	1.2 _{ab}	17.0 _a	14.65 _b

*means followed by the same letter do not differ significantly at $\alpha = 0.05$

On the basis of the obtained results it was observed that multiplication rate of *Campsis radicans* shoots after two subcultures depended on the type of explant used and the media composition. The highest multiplication rate (3.3) was obtained when the shoot tips were put on the control medium without growth regulators. Good results were also obtained in case of nodal explants cultured on the control medium (2.1) or shoot tips cultured in presence of 2-iP and IAA (2.6). On the control medium the shoot tips explants formed significantly more and longer axillary shoots (1.5 and 8.3 mm) than nodal explants (1.0 and 1.0 mm). The longest axillary shoots were obtained from nodal fragments cultured on the basal medium (17.0 mm).

The obtained results are opposite to those given by many other authors. According to Horn [1992] regeneration abilities of nodal explants are better than shoot tips. It is confirmed in many experiments. Sansberro et al. [1999] used nodes, shoot tips and tip meristems to establish tissue cultures of *Ilex paraguariensis*. The most regenerating explants were obtained from nodes. Better regeneration abilities of this kind of explants also observed Bach et al. [1996] in research on micropropagation of *Chaenomeles japonica*. However many authors obtained regeneration from different types of explants.

Many woody plant species were propagated both from shoot tips and fragments with nodes, for example *Hedera helix* [Marcinek et al. 2004] and *Cerbera odollam* (Bignoniaceae) [Salwa et al. 2011].

The influence of cytokinin type and concentration. The cytokinins used in the experiment had an effect on growth and development of *Campsis radicans* shoots in tissue culture (tab. 5).

The longest shoots were observed in the presence of 5 mg KIN·dm⁻³ (10.0 mm) and on the control medium (8.6 mm). Long shoots were also formed on the media containing KIN in concentration of 2 mg·dm⁻³ (7.7 mm) and 2-iP in concentration of 5 mg·dm⁻³ (6.5 mm). The shortest ones appeared on the media supplemented with BA used in higher concentration (3.1 mm). Shoots cultured on the media with BA in both concentrations formed significantly less leaves in comparison to other treatments.

The branching of *Campsis radicans* shoots also depended on the type and concentration of cytokinin added to the media. Shoots cultured on the media supplemented with BA in concentration of 5 mg·dm⁻³ or 2-iP in concentration of 5 mg·dm⁻³ formed definitely more axillary shoots per explant (2.0) in comparison to those cultured on the media with KIN in concentration of 5 mg·dm⁻³ (1.0) or control medium (1.0). The axillary shoots were the longest in the presence of 2 mg KIN·dm⁻³ (37.7 mm). The long axillary shoots were also obtained on the media supplemented with BA in concentration of 2 mg·dm⁻³ (22.1 mm). The shortest ones were obtained on the control media (1.0 mm). Taking into consideration the number of nodes that could be obtained and the number and length of axillary shoots formed it seems that medium supplemented with KIN in concentration of 2 mg·dm⁻³ would allow to obtain 3–4 nodes, with 1.8 good quality axillary shoots each.

Table 5. Growth and development of *Campsis radicans* shoots depending on the cytokinin type and concentration after 4 weeks of culture

Cytokinin	Concentration (mg·dm ⁻³)	Main shoot length (mm)	Number of leaves on the main shoot	Main shoot weight (mg)	Number of axillary shoots per explant	Length of axillary shoots (mm)	Callus weight (mg)
Control	0	8.6 _a *	6.0 _{ab}	75.31 _{b-d}	1.0 _b	1.0 _d	6.83 _b
BA	2	4.1 _{cd}	2.0 _c	92.46 _{bc}	1.9 _{ab}	22.1 _{ab}	9.2 _{ab}
	5	3.1 _d	2.0 _c	73.91 _{cd}	2.0 _a	8.9 _{b-d}	13.05 _{ab}
2-iP	2	4.9 _{b-d}	5.6 _{ab}	69.22 _d	1.0 _b	5.0 _{cd}	22.41 _a
	5	6.5 _{a-c}	5.0 _b	99.12 _b	2.0 _a	7.5 _{b-d}	23.74 _a
KIN	2	7.7 _{ab}	6.0 _{ab}	97.75 _{bc}	1.8 _{ab}	37.7 _a	13.64 _{ab}
	5	10.0 _a	7.6 _a	133.91 _a	1.0 _b	20.0 _{bc}	6.83 _b

*means followed by the same letter do not differ significantly at $\alpha = 0.05$

The superiority of kinetin over other cytokinins was proven in many experiments. It was used for micropropagation of many ornamental plants [Ibrahim and Debergh 2000, Sansberro et al. 2003]. Kinetin had a positive effect on branching of other woody climber species, *Clematis integrifolia* and *Clematis viticella* [Dąbski and Parzymies 2006, Parzymies and Dąbski 2012]. The most often used cytokin in tissue cultures is BA. Its effectiveness is well proven. It was used for *in vitro* propagation of *Arbutus unedo* [Mereti et al. 2002], *Vitis thunbergii* [Lu 2005], *Forsythia koreana* [Shim and Ha 1997]. In presented research the presence of BA also caused good branching of shoots but many axillary shoots were hyperhydric with morphological abnormalities. They were thick, brittle and translucent. Similar effects of BA, especially in higher concentrations, was observed by other authors [Debergh et al. 1992, Hosoki et al. 2003, Parzymies and Dąbski 2012].

CONCLUSIONS

1. Soaking the shoot fragments of donor plants in a mixture solution of Topsin and Streptomycine ($1 \text{ g} \cdot \text{dm}^{-3}$) for 12 hours prior to a proper sterilization decreases the contamination rate of cultures.

2. The best term to excise *Campsis radicans* explants is Spring, when vegetation starts.

3. Shoot tips are more suitable to establish tissue culture of *Campsis radicans* than nodal explants.

4. The media supplemented with kinetin in concentration of $2 \text{ mg} \cdot \text{dm}^{-3}$ allows to obtain the highest number of good quality shoots.

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INICJACJA I STABILIZACJA KULTUR TKANKOWYCH MILINU (*Campsis radicans* (L.) SEEM.)

Streszczenie. Milin (*Campsis radicans*) to bardzo dekoracyjny krzew, który rozmnaża się wegetatywnie za pomocą sadzonek. Obecnie nie ma informacji dotyczących mikrorozmnażania tej pięknej rośliny. Określenie najlepszej metody dezynfekcji oraz optymalnego rodzaju i stężenia regulatorów wzrostu w pożywce to jedne z najważniejszych czynników udanego mikrorozmnażania. W celu optymalizacji inicjacji oraz rozmnażania w warunkach *in vitro* *C. radicans* badano wpływ różnych metod dezynfekcji i terminów pobierania eksplantatów na poziom zakażeń oraz wpływ cytokinin na wzrost i rozkrzewianie pędów milinu. W badaniach wykorzystano następujące cytokininy: benzyloadeninę (BA), izopentyloadeninę (2-iP) i kinetynę (KIN). Uzyskane wyniki wskazują, że zakażenia to bardzo poważny problem przy inicjacji kultur tkankowych milinu. Najlepsze rezultaty uzyskano, gdy eksplantaty inicjalne były pobierane w maju (wiosną), krótko po rozpoczęciu wegetacji (88% zakażonych kultur). Moczenie fragmentów inicjalnych w mieszaninie Topsin M 500SC oraz streptomycyny przez 12 godzin zmniejszyło procent zakażeń z 100 do 94. Stwierdzono też, że do inicjacji i stabilizacji kultur milinu bardziej nadają się wierzchołki pędów niż węzły. Współczynnik rozmnażania po dwóch pasażach wyniósł 2,6–3,7 dla wierzchołków pędów (w zależności od pożywki) oraz 1,9–2,1 dla węzłów. Cytokininy użyte w doświadczeniu miały znaczący wpływ na rozmnażanie *C. radicans*. Najwięcej dobrej jakości pędów uzyskano na pożywce zawierającej KIN w stężeniu $2 \text{ mg} \cdot \text{dm}^{-3}$.

Słowa kluczowe: cytokininy, dezynfekcja, kultury tkankowe, mikrorozmnażanie, rodzaj eksplantatu

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