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# Direct organogenesis in hop - a prerequisite for an application of *A. tumefaciens*-mediated transformation

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## Abstract

The regeneration ability of primary explants derived from mericlones of two commercial Bohemian hops was investigated. It was found that these hops are able to regenerate shoots by direct organogenesis on media containing BAP or zeatin at concentrations 0.5 - 2 mg $dm^{-3}$. The highest regeneration of shoots was achieved from either petioles or internodes at frequencies 21 % and 52 %, respectively, on the medium containing zeatin (2 mg $dm^{-3}$), while relatively low amount of regenerated shoots (1.3 %) was observed for leaf blade explants. On the other hand, more efficient rooting occurred on the leaf blades then on other explants. A similar pattern of regeneration we observed for HLVd-infected mericlones of clone Osvald 31 even though viroid concentration in *in vitro* cultures was about 8-fold higher than in field-grown plants and was 31.1 pg $mg^{-1}$ of fresh mass in the average. These results suggest that HLVd infection did not impair organogenesis. We found that high 2,4-D concentration pretreatment (11 mg $dm^{-3}$) did not promote somatic embryogenesis. Although this treatment suppressed direct organogenesis, the inhibition was not complete and in low frequency the shoot regeneration was seen. Sensitivity of hop explants to antibiotics commonly used in *Agrobacterium*-mediated transformation was assayed. It was found that kanamycin (100 - 200 mg $dm^{-3}$) suppressed efficiently callogenesis, root formation and shoot proliferation. An estimation of effect of kanamycin (200 mg $dm^{-3}$) and ticarcillin (500 mg $dm^{-3}$) on morphogenesis was performed using regeneration medium. The inhibitory effects observed suggest that these conditions could be used in *Agrobacterium* transformation/selection system.

## Introduction

Although hop is an important crop having commercial value, only several reports have been published dealing with hop tissue cultures. Most of the data

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*Abbreviations:* 2,4-D - 2,4-dichlorophenoxyacetic acid; BAP - 6-benzylaminopurine; ZEA (trans-zeatin) - (6-[4-hydroxy-3-methyl- but-2-enylamino]purine); AMESS - 0.5 M sodium acetate buffer (pH 6.0) containing 10 mM $MgCl_2$, 20 % ethanol, 3 % SDS, and 1 M NaCl; HLVd - hop latent viroid; HSVd - hop stunt viroid

concern meristem culture and cloning, especially in relation to virus eradication (Vine and Jones 1969, Adams 1975, Samyn and Welvaert 1983, Popov *et al.* 1985, Svoboda 1988, 1991). Some authors inform about hop callus cultures (Paar *et al.* 1984, Connell and Heale 1986, Svoboda 1992), suspensions (Paar *et al.* 1984, Robins *et al.* 1985, Langezaal and Scheffer 1992), and protoplasts (Connell and Heale 1986, Furze *et al.* 1987). Successful plant regeneration was described for some foreign hop cultivars in few publications, only. For instance Motegi (1976) described a procedure for stem regeneration from leaf derived calli. Connell and Heale (1986) regenerated hop plants resistant to *Verticillium* wilt from calli grown in the presence of fungal culture filtrate in media. It seems that plant regeneration from dedifferentiated tissue is difficult to achieve. Probably due to this fact, up to now no information is available about hop transformation using *Agrobacterium tumefaciens* derived vectors. In the present study we described regeneration procedure for two Czech commercial clones. This regeneration occurred by direct organogenesis from primary explants of virus-free, viroid-free and HLVd-infected Bohemian hops. This fact together with the observations that hop tissue is sensitive to kanamycin are the prerequisites for the application of *A. tumefaciens*-mediated hop transformation using usual plant expression vectors.

## Material and methods

**Plant material:** Clones Osvald 31 and Osvald 72 of Žatec semi-early red-bine hop (*Humulus lupulus* L.), grown in commercial and experimental gardens in the Žatec area were used as source materials. *In vitro* cultures (mericlones) were established from these clones maintained in the experimental garden of the Hop Research Institute, Žatec (Svoboda 1991). These mericlones grown *in vitro* were examined for Apple mosaic virus (ApMV), Prunus necrotic virus (PNRV), Hop mosaic virus (HMV) and Hop latent virus (HLV) in the Hop Research Institute and were shown to be virus-free. This material was kindly provided as *in vitro* cultures by Ing. P. Svoboda (Hop Research Institute, Žatec). Additional analyses for viroid infection performed in our laboratory revealed that the *in vitro* clones are not infected with hop stunt viroid (HSVd). Hop latent viroid (HLVd) was detected in mericlones of clone Osvald 31 (see the Results), while supplied material of mericlones of clone Osvald 72 was HLVd-free. The level of HLVd was compared in *in vitro*- and field-grown plants of clone Osvald 31.

**Media composition and culture conditions:** *In vitro* cultures (8 mericlones, 4 for each clone number) derived from clones Osvald 31 and 72 were maintained by regular transfers of stem cuttings (nodal segments) in 8 week intervals on fresh agar medium H. The medium based on half strength MS salts (Murashige and Skoog 1962), vitamins MS, 100 mg $dm^{-3}$ myo-inositol, 2 % glucose, and 0.7 % agar (pH 5.6 - 5.7) was one of those used by Svoboda (1991) for clonal propagation of hop. Plants were cultured in 120 $cm^3$ glass jars covered with aluminium foil. All cultures were kept in cultivation chamber at temperature of 25 ± 1 °C and 16-h irradiation

(100 μmol(PAR) $m^{-2}$ $s^{-1}$) supplied by fluorescent tubes *Tesla,* type *Day.* For regeneration experiments and sensitivity tests transversal sections of leaf blades (2 - 3 mm wide), petioles, internodes (2 - 4 mm long) and nodes of 4 - 8 week-old plants were used. The amount of each type of explants evaluated ranged in different experiments from 200 to 500 per variant. Individual experiments were repeated at least four times. The viability, character of explants, and number of explants forming calli, roots and shoots were visually evaluated. In order to induce organogenesis, the H medium was supplemented with 0.5 mg $dm^{-3}$ BAP (regeneration medium HR1), 2.0 mg $dm^{-3}$ BAP (HR2) or 2.0 mg $dm^{-3}$ zeatin (HR3). In some experiments pretreatment of explants with a high concentration of 2,4-D (H medium with 11 mg $dm^{-3}$ 2,4-D and 1.125 mg $dm^{-3}$ BAP) was done for the selected period (5, 14, 35 d) and then the explants were transferred to HR1 or HR2 media. The effect of antibiotics on explant viability and different morphogenic events were evaluated on media H supplemented with either 100 or 200 mg $dm^{-3}$ of kanamycin. HR1 medium supplemeted with ticarcillin (500 mg $dm^{-3}$) and 200 mg $dm^{-3}$ of kanamycin was used for the evaluation of the morphogenic response to this medium which is supposed to be a possible medium for hop transformation/selection system. For more details see the Results.

**Viroid detection and quantification of HLVd using molecular hybridization:** For detection of hop viroids, we used cDNA clones kindly provided by Prof. Sänger (Max-Planck-Institut für Biochemie, Martinsried bei München, Germany). HLVd clone contained a dimeric cDNA insert of HLVd; clone HSVd contained a dimeric sequence of cucumber pale fruit viroid cDNA, which resembles to cucumber isolate of HSVd. Both cDNAs were inserted in the Blue scribe M13+ plasmid. Extraction and hybridization procedures described originally for PSTVd diagnosis were adopted for HLVd and HSVd detection. Viroids were analyzed in the leaves using a modified dot-blot hybridization procedure as described by MacQuaire *et al.* (1984). HLVd was extracted from leaves and denatured as described by Palukaitis *et al.* (1985) for PSTVd using AMESS. The hybridization procedure was similar as described by Matoušek and Rakouský (1993). The [α-32-P]UTP (110 TBq $mmol^{-1}$, *Amersham*) labelled transcripts from plasmids containing dimeric inserts either HSVd or HLVd cDNAs were used as the hybridization probes. The autoradiograms were scanned and quantified using 2D-spots program as described above. The amount of viroids was estimated according to a calibration curve obtained for the HLVd standard. Three independent measurements were performed per sample. The mean value of the amount of HLVd was always re-calculated per unit fresh mass.

## Results

Regeneration ability of different primary explants derived from mericlones of Bohemian hop clone Osvald 72 was investigated (Fig.1). It was found that these hops are able to regenerate shoots by direct organogenesis on media containing BAP or zeatin at concentrations 0.5 - 2 mg $dm^{-3}$. Zeatin application lead to a slightly higher

increase of shoot regeneration than application of BAP to the regeneration media. On the other hand, the shoots regenerated on zeatin supplemented medium were soft and watery, while on BAP media regenerated plants were of normal appearance (Fig. 2). For this reason, BAP enriched medium was prefered in other regeneration experiments.

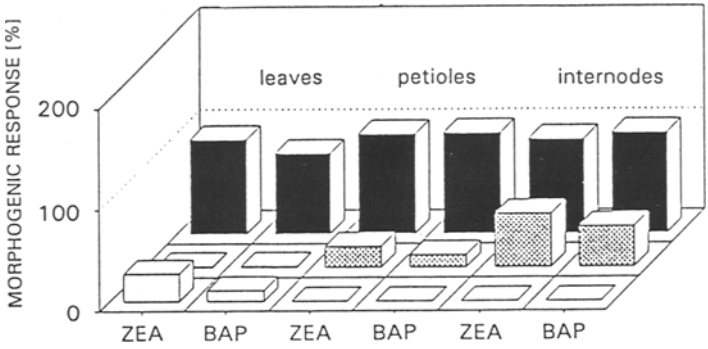


Fig. 1. Comparison of zeatin (ZEA) and BAP effects on regeneration of clone Osvald 72. Explants were cultivated on the media HR3 and HR2 containing zeatin and BAP, respectively. One subcultivation was made after 8 weeks. Regeneration response was evaluated four months after the culture establishment. Roots - *empty columns*; shoots - *chequered columns*; calli - *full columns*

Fig. 2. An example of hop stems regenerated by direct organogenesis from internodes on medium HR2.

It was shown that transfer of plants from HR3 medium onto H medium led to their normal morphology and growth. First regenerated shoots appeared 5 - 6 week after

the culture initiation. Shoot formation continued up to 10 - 12 weeks, and than substantially decreased. Shoots were predominantly formed on distal parts of nodal or petiolar explants, apparently without intervening callus stage. Sometimes shoots emerged on both ends of an explant. Occasionally multiple shoot formation was observed. The highest regeneration of shoots was achieved from either petioles or internodes at frequencies 21 % and 52 %, respectively, on the medium containing zeatin (2 mg $dm^{-3}$), while relatively low amount of regenerated shoots (1.3 %) was observed for leaf blade explants on the same medium (Fig. 1). On the other hand, more efficient rooting occurred on the leaf blades then on other explants. Compact and rather slowly growing calli were formed frequently on both, BAP and zeatin containing media (Fig. 1).

In further experiments a possible induction of somatic embryogenesis was investigated after 2,4-D shock pretreatment. 2,4-D pretreatment is known to be one of the inducers of somatic embryogenesis (Ammirato 1983, Tisserat 1985). However, in our experiments 2,4-D did not promote induction of somatic embryogenesis (Table 1). Our data demonstrate, that 2,4-D treatment inhibits direct organogenesis, but the inhibition was not complete, maybe due to the presence of BAP. Regeneration was seen even on induced calli but at low frequency. This regeneration according to the morphological characteristics of plant shoots formed, occurred also by organogenesis (*i.e.* formation of stem buds and stems appeared first and then roots appeared in some of regenerated shoots only). A similar morphogenic response was found when explants were pretreated with 2,4-D for shorter 14 or 5 d period (data not shown).

Table 1. Effect of 2,4-D pretreatment on *in vitro* morphogenesis of different hop tissues. All experimental variants were evaluated 4 months following culture initiation.

| 2,4-D application | Type of explants | Frequency of callogenesis [%] | shoot formation+ [%] | root formation [%] |
|---|---|---|---|---|
| without 2,4-D* | leaves | 79.9 | 1.5 (0.049) | 10.6 |
| | petioles | 100.0 | 12.2 (0.233) | 0.0 |
| | internodes | 100.0 | 39.9 (0.744) | 0.0 |
| pretreatment 35 d** | leaves | 93.0 | 6.7 (0.015) | 66.6 |
| | petioles | 99.1 | 7.3 (0.241) | 15.9 |
| | internodes | 100.0 | 35.7 (0.696) | 72.5 |
| continuous*** | leaves | 91.6 | 0.4 (0.006) | 67.3 |
| | petioles | 100.0 | 0.5 (0.009) | 25.1 |
| | internodes | 100.0 | 4.2 (0.053) | 61.4 |

* - the explants were grown on HR2 medium.

** - the explants were grown for 35 d on H medium, supplemented with 11 mg $dm^{-3}$ 2.4-D and 1.125 mg $dm^{-3}$ BAP, and then the tissues were transferred to HR2 medium.

*** - the explants were grown on H medium supplied with 2,4-D and BAP, as described above.

+ - number of shoots per explant in parentheses. In some cases, shoot regeneration occurred on calli (see text for the details).

The clone Osvald 31 and corresponding *in vitro* cultures derived from this clone were found to be completely infected with HLVd (Fig. 3). Surprisingly, level of HLVd as counted per mg of fresh mass was very high in *in vitro* plants, 31.1 pg $mg^{-1}$ in the average. Viroid content was about eight times higher in *in vitro* cultures, than

Table 2. Morphogenic response of HLVd-infected clone Osvald 31 on medium containing 0.5 mg $dm^{-3}$ BAP. Data were evaluated following 4-months cultivation.

| Type of explants | Frequency of callogenesis [%] | shoot formation+ [%] | root formation [%] |
|---|---|---|---|
| leaves | 93.7 | 4.2 (0.053) | 0.3 |
| petioles | 99.3 | 16.1 (0.245) | 1.6 |
| internodes | 80.1 | 44.6 (0.672) | 3.1 |

+ - number of shoots per explant in parentheses

Table 3. Effect of antibiotics and *in vitro* selective conditions on the response of different hop explants. The effect of antibiotics was evaluated either on H medium, six weeks after initiation of the culture, or on HR1 medium, four month after culture establishment. In the last case the explants were subcultured once using the same medium.

| Medium/ Antibiotics | Type of explant | Explants exhibiting green colour [%] | necrosis partial [%] | total [%] | callogenesis [%] | formation of roots [%] | shoots [%] |
|---|---|---|---|---|---|---|---|
| H/O | leaves | 44 | 43 | 13 | 9 | 19 | <1 |
| control | petioles | 68 | 28 | 4 | 24 | 2 | 0 |
| | internodes | 83 | 17 | 0 | 48 | 5 | 0 |
| | nodes | 96 | 4 | 0 | 18 | 42 | 83 |
| H/100 mg $dm^{-3}$ | leaves | 66 | 27 | 7 | 0 | 0 | 0 |
| kanamycin | petioles | 42 | 58 | 0 | 0 | 0 | 0 |
| | internodes | 76 | 23 | 0 | 0 | 0 | 0 |
| | nodes | 83 | 17 | 0 | 0 | 0 | 22** |
| H/200 mg $dm^{-3}$ | leaves | 35 | 57 | 8 | 0 | 0 | 0 |
| kanamycin | petioles | 38 | 58 | 4 | 0 | 0 | 0 |
| | internodes | 75 | 21 | 5 | 0 | 0 | 0 |
| | nodes | 72 | 27 | <1 | 0 | 0 | 23** |
| HR1/500 mg $dm^{-3}$ | leaves | 18 | 82 | <1 | <1* | 0 | 0 |
| ticarcillin and | petioles | 99 | <1 | <1 | 4* | 2** | 0 |
| 200 mg $dm^{-3}$ | internodes | 93 | 5 | 1 | 11* | 3** | 0 |
| kanamycin | nodes | 98 | 2 | <1 | 13* | 0 | 33** |

* - only small and slowly proliferating calli were observed.

** - some of proliferating shoots were bleached and all of shoots formed reached height maximally 10 mm, then the growth was arrested. Only weak rooting was observed, the growth of single roots was subsequently arrested.

in young shoots collected from field-grown plants (Fig. 3). As viroid infection could impair the regeneration process, it was of interest to investigate regeneration ability of clone Osvald 31 (Table 2). According to our results, no significant difference in morphogenic response was found between Osvald 31 and Osvald 72 grown on similar media (compare the results in Fig. 1, Table 1 and 2). A similar pattern of regeneration for both HLVd-free and HLVd-infected mericlones suggests that HLVd infection had no influence on regeneration process under experimental conditions used.

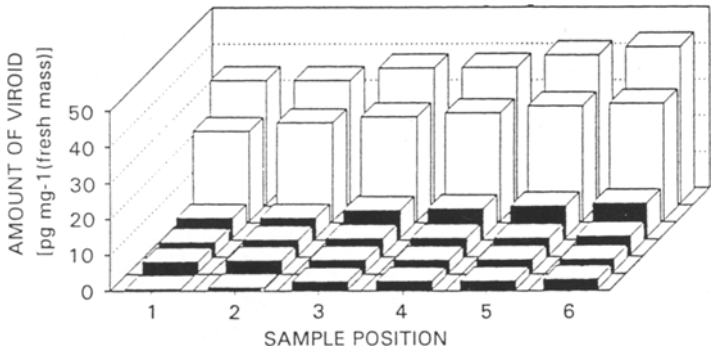


Fig. 3. Levels of HLVd in the leaves of clone Osvald 31 grown in the field conditions (*full columns*) and *in vitro* (*empty columns*). After hybridization of the samples the autoradiograms were scanned, and HLVd was quantified as described in the Materials and methods. Three dimensional histogram represents data obtained for individual samples sorted in ascending order.

The sensitivity of hop explants to antibiotics commonly used in *Agrobacterium*-mediated transformation such as kanamycin and ticarcillin was assayed. It was found that hop explants are sensitive enough to kanamycin. 100 - 200 mg $dm^{-3}$ of kanamycin (Table 3) efficiently suppressed callogenesis, root formation and shoot proliferation from buds on H medium. Explants grown on media with kanamycin but lacking phytohormones tended towards necrosis and culture deteriorated, especially if cultured more than six weeks. The important fact for development of a plant transformation/selection system is efficient suppression of nodal or dormant bud proliferation, which could occasionally contaminate the culture with non-transformed shoots. The data in Table 3 demonstrate that bud germination is highly suppressed and decreased from 83 to approximately 20 %. Moreover, some of proliferating shoots soon turned yellow and then became bleached. The growth of all of the proliferating shoots was soon arrested, reaching a maximum length of about 10 mm. The effect of kanamycin (200 mg $dm^{-3}$) and ticarcillin (500 mg $dm^{-3}$) on morphogenesis was analyzed on regeneration medium HR1, which is supposed to be used for the regeneration of transformed plants. The inhibitory effects were assayed four months after the culture establishment (Table 3). Our data suggest that all processes like callogenesis, root and shoot formation were significantly suppressed (compare data in Fig.1 with the data presented in Table 3). For instance, the frequency of callogenesis was decreased by about 80 - 90 % and in low frequency small and slowly proliferating calluses were observed, only. Most importantly, shoot

proliferation from either leaves, petioles or nodes was completely blocked, suggesting that direct organogenesis was efficiently inhibited in this non transformed material.

## Discussion

A method for direct *in vitro* organogenesis of commercially important Bohemian hops, clones Osvald 31 and 72 was established. To our knowledge, this is the first report on successful plant regeneration induced on hop primary explants. Limited literature data related to hop regeneration are available. Mostly stem explants with pre-existing buds or primordia were used to micropropagate cultivars or clones of breeding interest or to eradicate plants of hop viruses using meristem culture (Vine and Jones 1969, Adams 1975, Samyn and Welvaert 1983, Popov *et al.* 1985, Svoboda 1988, 1991, 1992). It seems, that plant regeneration from undifferentiated tissues is difficult to achieve, and the efficiency of procedures used is relatively low. Only a few reports described plant regeneration from calli (Motegi 1976, Connell and Heale 1986, Heale *et al.* 1989). These approaches are used often for the selection of toxin- (pathogen)-resistant plants (for review see Heale *et al.* 1989). Up to now, no attempts have been made to work out methods enabling an application of *Agrobacterium*-mediated transformation technology to hop. Direct organogenesis favours the breeding of such crops where the retention of specific characteristics (*e.g.* bitter acids, volatile compounds) is strongly desired. This fact is very important because tissue cultures are known to be source of enhanced genetic variability (for review see *e.g.* D'Amato 1978, Evans and Sharp 1986). In our work we attempted to induce somatic embryogenesis by treatment of the explants with an elevated concentration of 2,4-D in media. We did not observe possible initiation of somatic embryogenesis. However, in low frequency, organogenesis was observed on induced calli. Although we did not performe additional histological analysis, the observations mentioned above suggest that dedifferentiated tissues formed on Osvald 72 explants are able to produce regenerated shoots, too.

*In vitro* cultures derived from clone Osvald 31 were completely HLVd-infected. Viroid concentration was much higher in *in vitro* cultures than in symptomless field-grown plants. It was demonstrated that this hop viroid occurs worldwide in hops (Puchta *et al.* 1988). This viroid has been detected in high (95 - 100 %) incidence in field-grown plants of both commercial clones Osvald 31 and 72 (unpublished results) and, therefore, it was of interest to include also HLVd-infected material in our tissue culture experiments. Despite of the absence of obvious morphological symptoms of disease in HLVd infected hop plants, this viroid was recently described to be deleterious to production in certain hop cultivars in the UK (Barbara *et al.* 1990, Adams *et al.* 1991). Therefore, HLVd infection could cause some degree of pathogenesis in certain hop cultivars. However, in our experiments we did not find significant differences in regeneration abilities between HLVd-free mericlones 72 and viroid infected mericlones Osvald 31. Our results suggest that there is probably no significant influence of HLVd infection on organogenesis under our experimental

conditions. It was described for hop stunt viroid (HSVd)-infected hop tissues that there were critical differences between tissue cultures derived from HSVd infected and uninfected leaf segments with respect to IAA requirement. A reduced rooting was also observed for HSVd-infected tissue explants probably due to aberrant IAA utilization in infected tissues (Takahashi *et al.* 1992). These differences were observed during cultivation of tissues under the dark conditions, but not under continuous light. We did not attempt to grow our cultures in complete darkness. It should be noted, in this context that HSVd (for characterization see *e.g.* Ohno *et al.* 1983, Sano *et al.* 1986) differs from HLVd, which forms a new viroid group (Puchta *et al.* 1988, 1989) in many respects.

High sensitivity of hop explants and direct organogenesis to kanamycin suggest using this antibiotic for hop transformation mediated by some of *A. tumefaciens* expression vectors. A combination of kanamycin and ticarcillin (or other antibiotics inhibiting *A. tumefaciens* growth on plant regeneration media) was used by many authors for different plants (*e.g.* Horsch *et al.* 1985, McDonnell *et al.* 1987). Our observations suggest that these conditions could be used also for *Humulus lupulus* transformation/selection system.

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