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Efficient *in vitro* plantlet regeneration in *Populus euphratica* Oliver

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Populus euphratica **Oliver is highly tolerant against salt stress and thus very important for afforestation efforts of saline soils in sandy desert regions. Even if protocols already exist for the regeneration of shoots from leaf explants for this species, all are based on the hormones 6-benzylaminopurin (BAP) and 1-naphthaleneacetic acid (NAA). An alternative and fast regeneration protocol was developed for** *P. euphratica* **starting from leaf explants by employing the cytokinin-active urea derivative thidiazuron (TDZ) in systematically performed callus induction and shoot regeneration studies. Furthermore, evidence is provided here for the first time that sensitivity of callus induction and shoot regeneration in** *P. euphratica* **against the antibiotic cefotaxime is different, a finding to be considered for the development of a protocol to genetically transform this species.**

Key words: Callus culture, organogenesis, plant growth regulators, transformation.

INTRODUCTION

Within the genus *Populus* (family *Salicaceae*), *Populus euphratica* Oliver belongs to the section Turanga (Eckenwalder, 1996). This tree species is naturally distri-buted in Eurasia and North Africa and is adapted to subtropical regions. This species accepts wet and dry conditions but requires a lot of light for normal develop-ment. Plants are medium to large in size, with very varia-ble leaves, usually broader than they are long. *P. euphra-tica* acts well as a colonizer on exposed soils, or as windbreaks and shelters, e.g., protecting crops from sun irradiation. The functional use of the wood is mainly as a source for fuel and fiber, but rarely as timber. A pronoun-ced characteristic of this species is the high tolerance against salt stress (Chen et al., 2002; Parida and Das, 2005; Sixto et al., 2006; Chen and Polle, 2010), thus this species is very appropriate for the afforestation of saline soils in sandy desert regions. *P. euphratica* is also of high interest for the identification of genes involved in abiotic stress responses under natural conditions (Brosché et al., 2005).

The first report on organogenesis and plantlet regene-ration in *P. euphratica* was published by Gu et al. (1999). The authors found that the ratio of the plant growth regulators 6-benzylaminopurin (BAP) and 1-naphthalene acetic acid (NAA) is important for successful plant rege-neration from embryogenic callus. Also Phan et al. (2004) used different combinations of these two hormones for plant regeneration from leaf explants and provided a pro-tocol for *in vitro* propagation of *P. euphratica* including transfer of regenerated plants into greenhouse. An opti-mized protocol for *in vitro* propagation of *P. euphratica* from leaf explants was developed by Ferreira et al. (2009), again using the two hormones NAA/BAP, but at a fixed 2:1 ratio. The regeneration systems described for *P. euphratica* so far lasted in total for about 17 weeks, start-ing from callus induction through the regeneration of new shoots.

Although efficient regeneration protocols are seemingly available for *P. euphratica* only two reports could be found in the literature, describing the transformation of

Table 1. Media including growth regulator used in the experiments.

Medium	Basic medium	TDZ (µg/L)	NAA (mg/L)	BAP (mg/L)
MS1	MS*	22	1	---
MS2	MS	2.2	1	---
MS3	MS	22	---	---
MS4	MS	2.2	---	---
Medium 4	WPM*	---	1	0.25
601½	WPM	1.1	---	---
601	WPM	2.2	---	---
608	WPM	10	---	---
609	WPM	20	---	---
610	WPM	50	---	---

*MS, Murashige and Skoog; WPM, Woody Plant Medium: both supplemented with 20 g/L sucrose and 6% agar (Murashige and Skoog 1962; Lloyd and McCown 1980).

this species after co-culture with different *Agrobacterium* strains (Ding et al., 2006; Li et al., 2007). These authors have provided optimum antibiotic concentrations for the selection of transgenic shoots as well for *Agrobacterium*-removal after co-culture. No inhibitory effect of cefotaxime was mentioned in these reports. Only one report has been made available describing inhibitory effects of high cefotaxime concentrations on callus induction (Fan et al., 2009). In this work, an alternative and fast regeneration protocol for *P. euphratica* starting from leaf explants by adopting thidiazuron (TDZ), a cytokinin-active urea derivative (Yip and Yang, 1986) are described as prerequisite for future transformation experiments. Evidence is provided here for the first time that callus induction in *P. euphratica* is sensitive to the antibiotic cefotaxime, a finding that has to be considered for the development of a transformation protocol.

MATERIALS AND METHODS

In vitro culture and sub-culture of *P. euphratica* sterile plants

Sterile *P. euphratica* plants were kindly provided by A. Polle (University of Goettingen, Germany) and cultured in Magenta® vessels (Sigma-Adrich, Germany). Shoot tip explants were cut every four to eight weeks for *in vitro* propagation and sub-cultured on WPM medium (Lloyd and McCown, 1980) as described by Ahuja (1986) for aspen. Leaf explants were cut from 4 to 6-week old sterile plants. To eliminate bacterial release from cultured *P. euphratica* explants, the antibiotic cefotaxime (Duchefa, Haarlem, The Netherlands) was added to the media.

Media used and culture conditions

The media used for the callus induction and regeneration experiments are summarized in Table 1. Either solidified WPM (Woody Plant Medium) (Lloyd and McCown, 1980) or MS (Murashige and

Skoog, 1962) were supplemented with NAA and BAP, or with the cytokinin-active urea derivative TDZ (Yip and Yang, 1986). Stock powder of MS and WPM were purchased from Duchefa (Haarlem, The Netherlands). All media were supplemented with 20 g/L sucrose, had their pH adjusted to 5.8 prior addition of 0.6% w/v agar, and were autoclaved at 15 lbs and 120°C for 20 min. Influence of cefotaxime (Duchefa, Haarlem, The Netherlands) on callus formation and plant regeneration in *P. euphratica* leaf explants was studied by using the two media MS1 and 601 (Table 1). Both media contain either 250 mg/L (C½) or 500 mg/L (C) cefotaxime.

Callus induction and regeneration

In total, 7 experiments were conducted to optimize callus induction and regeneration in *P. euphratica* in terms of frequency and incubation/culture time (Table 2). For callus induction and shoot regeneration, periods of 2, 4, 10 and 6, 17, 30 weeks, respectively, were chosen. For callus induction, different TDZ concentrations alone or in combination with 1 mg/L NAA (Table 1) were tested in different experiments for callus induction and subsequent shoot regeneration (Table 2).

In addition, Medium 4, with 1 mg/L NAA and 0.25 mg/L BAP, was included for callus induction. In further experiments, the combination of callus induction and plant regeneration was studied. Here, first callus was induced on the medium given in Table 2, and plant regeneration was subsequently initiated on the mentioned second medium (Table 2). Callus induction and regeneration experiments were carried out in Petri dishes containing the different media. Cut leaves were divided into 0.5 to 1 cm long segments and transferred to the different media.

The experimental conditions tested for callus induction and shoot regeneration are summarized in Table 2. The cultures were kept at 24 ± 2°C under white fluorescent light (10 to 12 µE/ m²/s) with a 24 h photoperiod.

Evaluation of results

At the end of the experiments, the results were estimated according to the following scheme: Callus: 1=no callus; 2=few, small amount; 3=many, small amount; 4=many, large amount. Regeneration: 1=no regeneration; 2=few, single shoots; 3=many, single shoots;

Table 2. Experiments for callus induction and plantlet regeneration conducted in this study.

Callus induction and plantlet regeneration	Media tested	Duration of culture (weeks)	Results shown in Figure
Callus induction	MS1, MS2, MS3, MS4, medium 4	All media: 6	1
Shoot regeneration 1	MS1, 601	Both media: 14	3
Callus induction and shoot regeneration 1	MS1C*, 601C, 608C, 609C, 610C	MS1C: 2 (callus induction) 601C, 608C, 609C, 610C: 17 (regeneration)	4
Callus induction and shoot regeneration 2	4/601½, 4/601, MS1/601½, MS1/601	Medium 4: 6 (callus induction) MS1: 10 (callus induction) 601½: 30 (regeneration) 601: 30 (regeneration)	5
Callus induction and shoot regeneration 3: effect of cefotaxime 1	MS1, MS1C½**, MS1C, 601, 601C½, 601C	All media: 14	6
Callus induction and shoot regeneration 4: effect of cefotaxime 2	MS1, MS1C½, MS1C, 601, 601C½, 601C	MS1, MS1C½, MS1C: 4 (callus induction) 601, 601C½, 601C: 6 (regeneration)	7

*C,500 mg/L cefotaxime; ** C½,250 mg/L cefotaxime.

4=many, high number of shoots.

RESULTS AND DISCUSSION

Few days after the leaf cut and transfer to callus induction or the regeneration media, unfortunately, white mucus was released from the cut sites of the *P. euphratica* leaf explants. This mucus turned out to be endogenous bacteria (bacterial endophytes), and as described by Ulrich et al. (2008), bacterial endophytes (mainly belonging to the bacterial genus *Paenibacillus*) are ubiquitously distributed in diverse tissue cultures. After adding the antibiotic cefotaxime (500 mg/L) to the plant culture media, no further bacterial release from the leaves was observed. Bacteria-free *P. euphratica* plants were obtained after several sub-culture rounds. This was the prerequisite to study more systematically callus induction and shoot regeneration in *P. euphratica* leaves.

Callus induction

Dissected *P. euphratica* leaves were transferred to MS1, MS2, MS3, MS4, and medium 4, and screened for callus formed after 6 weeks of incubation (Figure 1). As expected, Medium 4 was very efficient in terms of callus induction on *P. euphratica* leaf explants after 6 weeks of incubation, however, calli started to get brownish at the outer, older parts of the callus (Figure 2A). In contrast, leaf explants on MS1 and MS2, containing 2.2 and 22 µg/L TDZ, respectively, and 1 mg/L NAA formed lower amounts but solid, dark green callus (Figure 2B). Media MS3 and MS4 without NAA did not reveal any callus induction on leaf explants, thus it can be concluded that NAA is the responsible hormone for callus induction irrespective of added TDZ concentrations. The culture of leaf explants with formed calli on Medium 4 for more than 10 weeks led to a browning of the whole calli. Calli formed on leaf explants cultured on MS1 and MS2 kept their solid appearance and white-to-green colour for more than 10 weeks. Few callus pieces cultured only on MS1 (and mot MS2) started to regenerate after 8 to 10 weeks in culture. Thus, MS1 was taken as a callus induction medium in all further experiments.
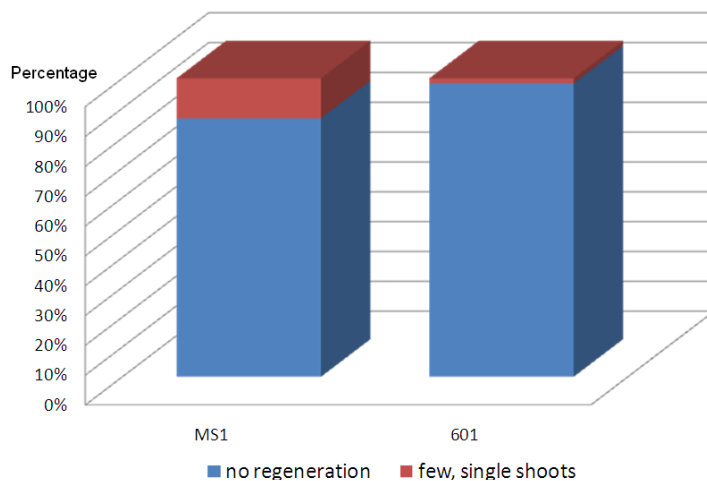
Shoot regeneration from callus

As mentioned before, few *P. euphratica* callus pieces started to regenerate after 8 to 10 weeks culture on MS1. To test the regeneration capacity of MS1, this medium was compared to the standard regeneration medium 601, which has already been established in the laboratory for many years and is known to efficiently induce shoot regeneration in various poplar species and clones (Hoenicka et al., 2006). After 14 weeks of culture on 601, small
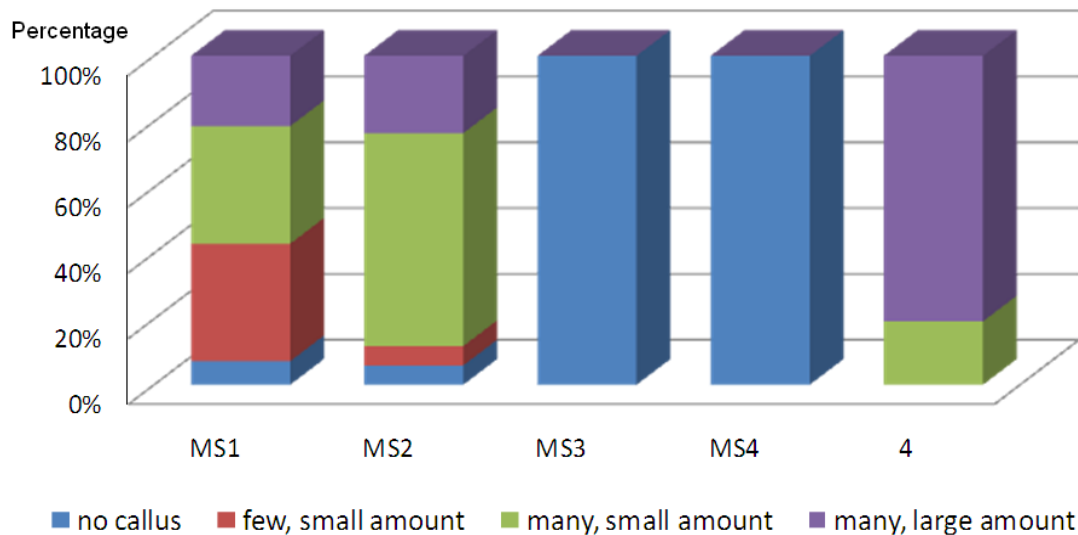
Figure 1. Callus formation in *P. euphratica* leaf explants cultured on MS1, MS2, MS3, MS4 and Medium 4 for 6 weeks. Amount of callus formation was scored at the end of this experiment after 6 weeks.
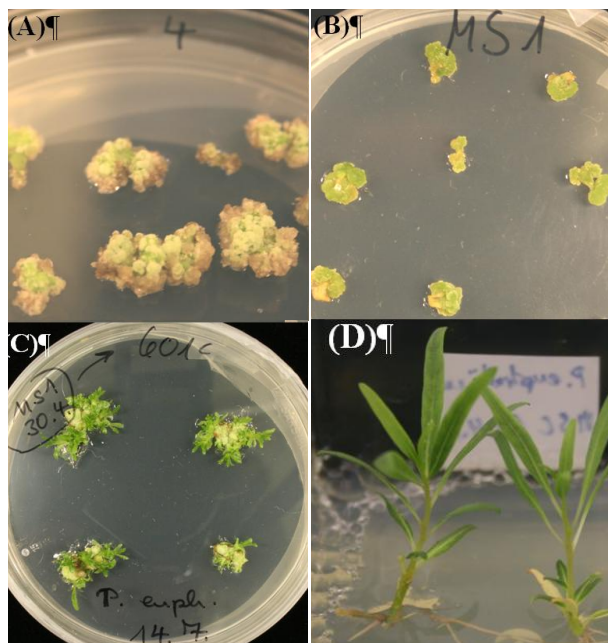
Figure 2. Callus formation (a, b) and shoot regeneration (c) on *P. euphratica* leaf explants. (A) Callus formed after 6 weeks of culture on medium 4. (B) Callus formed after 10 weeks of culture on MS1. (C) Regeneration of shoots after 10 weeks of culture on MS1 and subsequent 10 weeks of culture on 601C. (D) Plantlet *in vitro* culture.
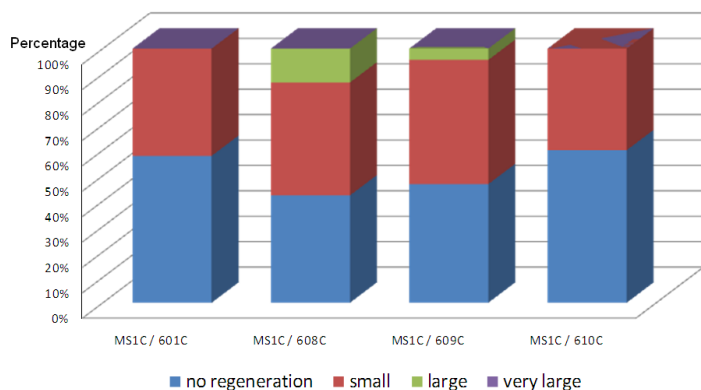

Figure 3. Regeneration of shoots in *P. euphratica* leaf explants cultured on MS1 and 601 for 14 weeks. Frequency of shoot regeneration was scored at the end of this experiment after 14 weeks.

lus were formed on leaves after 14 weeks of culture, but regeneration of shoots took place on only about 15% of the *P. euphratica* leaf discs (Figure 3), even of 10- times higher TDZ concentration compared to 601. Thus, both MS1 alone and 601 are not appropriate as regene-ration media for *P. euphratica*. Regeneration was never detec-ted on calli formed at the leaf discs cultured on Medium 4 (not shown).

Experiments combining callus induction and plant rege-neration revealed promising results. First, a callus induc-tion period of 2 weeks was tested for 17 weeks on MS1C

amounts of calli were detected on about 90% of the leaf explants but only low regeneration capacity could be observed (Figure 3). On MS1, again high amounts of cal-

Figure 4. Culture of *P. euphratica* leaf explants on MS1C for 2 weeks for callus formation and subsequently on 601C, 608C 609C 610C for 17 weeks for regeneration of shoots. Frequency of shoot regeneration was scored at the end of this experiment after 19 weeks.
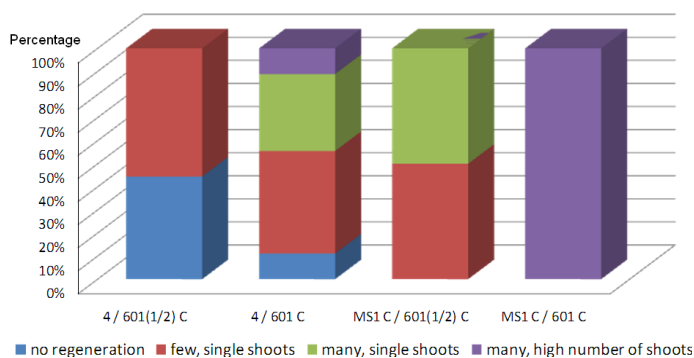
Figure 5. Regeneration of shoots in *P. euphratica* leaf explants cultured on Medium 4 and MS1C for callus induction for 6 and 10 weeks, respectively, and subsequently on 601C for 30 weeks. Frequency of shoot regeneration was scored at the end of this experiment after 36 or 40 weeks.

(C=containing 500 mg/L cefotaxime) and different TDZ concentrations in the regeneration medium ranging from 2.2 to 50 µg/L (Figure 4). The highest regeneration capacity of about 60% was found for the combination MS1C / 608C. Seemingly, 2 weeks are to short for callus induction. To further improve plantlet regeneration, *P. euphratica* leaf explants were cultured on both Medium 4 and MS1C for 6 and 10 weeks, respectively to form "many, large amount" of callus (highest score, Figure 1). Leaf discs and formed calli were then transferred to either subculture media 601C or 601½C (Figure 5). It could clearly be shown that the combination MS1C/601C is appropriate for high frequency of shoot regeneration in *P. euphratica* (Figures 2C and 5) even if the time period for callus induction lasted longer for MS1C than for Medium 4. The latter finding is very important, as when *P. euphra-*

tica leaf explants were transferred to 601 after 4 weeks culture on MS1 (thus harbouring lower amounts of callus (score "few, small amount, Figure 7), a significantly reduced regeneration capacity was found. Regenerated plantlets were easily cut and rooted on WPM medium without hormones (Figure 2D).

The results confirm the finding by Gu et al. (1999) and others who studied the effects of various concentrations as well as combination of the plant growth regulators NAA and BAP on callus induction, adventitious bud regeneration and proliferation. These authors show that the hormone concentration was not strictly required for induction of organogenesis, but the ratio of BAP and NAA was important. It was found that callus induction is crucial for subsequent regeneration efficiency when replacing the cytokinin BAP with 2.2 or 22 µg/L TDZ. With the given protocol (6 to 10 weeks for callus induction on MS1 + 10 to 30 weeks for regeneration on 601) regeneration capacity in *P. euphratica* has been significantly improved. When regeneration is aimed at a shorter time period (e.g. 2 weeks for callus induction on MS1 + 19 weeks for regeneration), the concentration of TDZ in the regeneration medium can be increased to about 10 µg/L, however, higher TDZ concentrations are detrimental. An efficient *in vitro* regeneration system starting, e.g. from leaves is a prerequisite for *Agrobacterium*-mediated transformation.

Influence of cefotaxime on callus induction and shoot regeneration

In some cultures, white mucus (endogenous bacteria) was released from the cut sites of the *P. euphratica* explants. No further bacterial release was observed from the leaves after adding 500 mg/L of the antibiotic cefotaxime (Duchefa, Haarlem, The Netherlands) to the media. However, at the point that *P. euphratica* plants were free of endophytic bacteria, it was surprisingly observed that media MS1 and 601 containing 500 mg/L cefotaxime turned out to have an opposite effect on callus induction and plant regeneration in *P. euphratica* leaf explants. The inhibitory influence of cefotaxime on callus induction is shown in Figure 6 scored after 14 weeks of culture. Callus formation decreases with increasing cefotaxime concentration (250 and 500 mg/L), found on both MS1 and 601, confirming the recent results obtained by Fan et al. (2009). However, in the same experiment it was observed that regeneration capacity is increased on Medium 601 with an increasing cefotaxime concentration (250 and 500 mg/L).

To systematically investigate this phenomenon, *P. euphratica* leaves were cultured for 4 weeks on MS1 with different cefotaxime concentrations (250 and 500 mg/L), and then sub-cultured for 6 weeks on 601 with the same
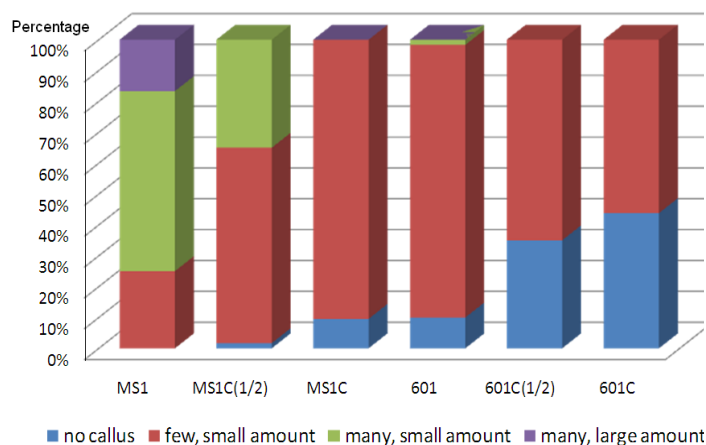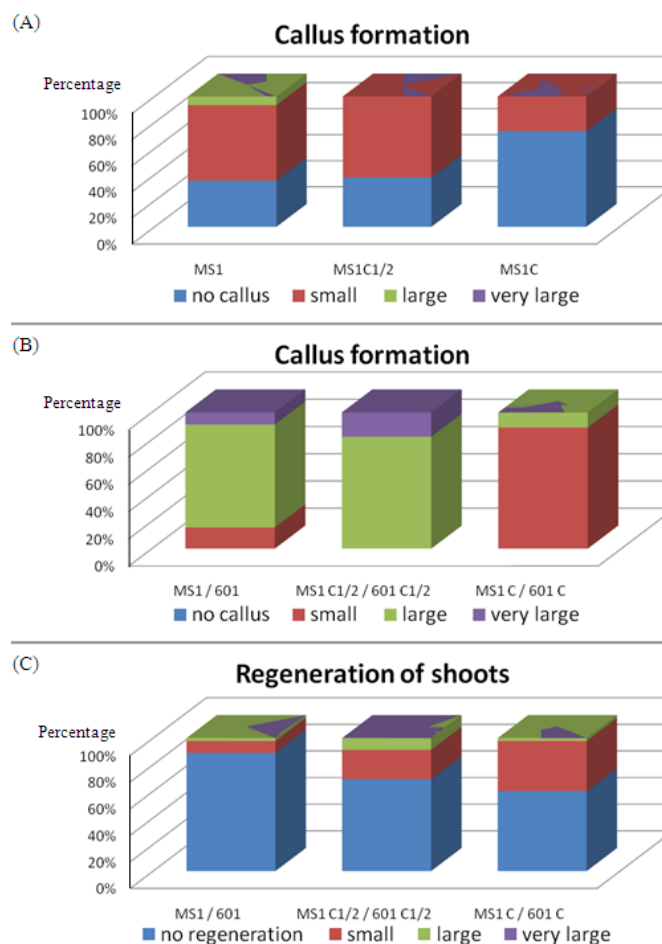
Figure 6. Influence of cefotaxime on callus formation in *P. euphratica* leaf explants cultured on MS1 and 601. Both media contain either 250 mg/L (C½) or 500 mg/L (C) cefotaxime. Amount of callus formation was scored at the end of this experiment after 14 weeks.

Figure 7. Influence of cefotaxime on callus formation and plant regeneration in *P. euphratica*. For callus induction, leaf explants were cultured on MS1, MS1C½ and MS1C for 4 weeks, and transferred to 601, 601C½ and 601C for plant regeneration for 6 weeks. The media contain either no, 250 mg/L (C½) or 500 mg/L (C) cefotaxime. Amount of callus formation was scored after 4 weeks (A) and at the end of this experiment after 10 weeks (B). Frequency of shoot regeneration was scored after 10 weeks (C).

cefotaxime concentration (Figure 7). MS1 with no cefotaxime and moderate concentration of 250 mg/L was appropriate, while the highest cefotaxime concentration (500 mg/L) was inhibitory for callus formation, (Figure 7a) at the same time promoted regeneration capacity (Figure 7b). Thus, for transformation of *P. euphratica*, the intermediate cefotaxime concentration of 250 mg/L seems sufficient for efficient removal of bacteria and at the same time without significant inhibitory effects on the frequency of callus induction. Only two reports could be found describing the transformation of *P. euphratica* explants after co-culture with different *Agrobacterium* strains (Ding et al., 2006; Li et al., 2007). The recommended concentration of 250 mg/L cefotaxime is in the range of the concentrations described as optimal to inhibit bacterial growth, that is 200 to 400 mg/L (Li et al., 2007), but a bit higher than the 100 to 150 mg/L suggested by Ding et al. (2006). No inhibitory influence of any antibiotic on callus induction and/or the regeneration process in *P. euphratica* was reported in either study. Possibly, the presence/absence of endophytes has an influence on the response of *P. euphratica* leaf explants to cefotaxime. No information is given on presence of endophytes in any other study published on *P. euphratica* regeneration.

Rooting of *P. euphratica* shoots

Regenerated shoots of *P. euphratica* were cut and transferred to solidified WPM medium (with 20 g/L sucrose and 0.6% agar) without hormones. After 2 to 4 weeks, roots are formed spontaneously into the medium.

Conclusions

In this study, the regeneration capacity in *P. euphratica* has been significantly improved. The protocol with TDZ as a growth regulator offers two options: either high efficiency of plantlet regeneration for 40 weeks (combination of MS1 and 601) or lower plant regeneration capacity, but in a shorter time of about 20 weeks (combination of MS1 and 608). An inhibitory effect of 500 mg/L cefotaxime on callus induction on MS1 was observed. However, inhibition of *Agrobacterium* strains normally used for plant transformation without influencing the regeneration process of putatively transformed plantlets by antibiotics like cefotaxime and others is essential for successful plant

transformation. The strategy of using half concentration of cefotaxime to remove *Agrobacterium* provides a solution to obtain transgenic *P. euphratica* plantlets.

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