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Continuous *in vitro* multiplication of shoot buds of Norway spruce (*Picea abies* L.) by intermittent application of growth regulators

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Abstract

A culture system was developed which allows continuous production of adventitious buds. Segments of seedlings germinated on media supplemented with different growth regulator combinations were used as explants. Cultivation was carried out in two phases, which alternate permanently: (I) without and (II) with growth regulators. Thus it was possible to establish meristematic tissue cultures from 5 - 10 % of the seedlings tested. They have produced buds now for over 3 years with multiplication rates of about 1.5 within 5 - 6 weeks.

Introduction

A major objective in *Picea abies* tissue culture is the development of a meristematic tissue culture system that allows indefinite subculture and continuous production of adventitious buds and finally plants. For *Picea abies*, regeneration systems *via* organogenesis have been published by Jansson and Bornman (1980), Bornman (1981), Bornman and Jansson (1982), Von Arnold (1982), Bornman and Vogelmann (1984) and Von Arnold and Hakman (1988).

The published regeneration systems describe principal solutions for the development of intact plants from juvenile explants but a method for permanent propagation of *Picea abies* was still missing. In order to develop efficient *in vitro* propagation methods several problems have to be solved. They concern (*1*) the kind of the somatic explant, (*2*) the regime of the *in vitro* culture allowing continuous multiplication and (*3*) the mode of *in vitro* plant formation.

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Abbreviations: BA - 6-benzylaminopurine; KIN - N^6-furfuryladenine; 2ip - 6-(γ, γ-dimethylallyl)-aminopurine; ZEA - zeatin; NAA - 1-naphthaleneacetic acid.

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In general, early embryonic stages were most suitable for morphogenic response. Besides the explant source, the morphogenic response is strongly influenced by the individual genotype which could be due to the respective content of endogenous hormones (Zel *et al.* 1988). Induction of shoot buds and their development into intact plants requires special conditions for each genotype (Bornman and Jansson 1982). Many experiments demanding a large amount of material have to be carried out in order to optimize the regeneration system for a defined genotype. Since the suitable explant material per genotype is strongly limited, a method had to be developed which provides sufficient meristematic tissue for extensive regeneration studies.

In this paper we describe a method for continuous multiplication of shoot buds induced on mature zygotic embryos of *Picea abies*.

Material and methods

Material: Seeds of Norway spruce (*Picea abies* L.) were kindly supplied by the Institute for Breeding of Forest Plants in Waldsieversdorf/Eberswalde (FRG). They had been collected from the forest in Elstra/Kamenz (FRG) and were stored at 4 °C until use. Seeds were surface sterilized in 0.2 % $HgCl_2$ for 6 min and washed carefully in sterilized double distilled water.

Culture media: The basic medium was developed by slight modification of the so-called MCM (medium for conifer morphogenesis) developed by Bornman and Jansson (1982). The concentrations of $(NH_4)_2SO_4$ and Fe-EDTA were reduced to 100 mg l^{-1} and 2.76 mg l^{-1}, respectively. The concentrations of the remaining MCM-components were reduced to one half. The medium contained 1 % saccharose and 0.7 % agar. Media with different concentraions of 1:1 combinations of BA and KIN or ZEA and media prepared without cytokinins or with 0.01 mg l^{-1} KIN were alternate during cultivation. Media referred to as differentiation phase were prepared without or with 0.01 mg l^{-1} KIN and were used for interphases.

***In vitro* culture:** Sterilized seeds were placed on agar-solidified growth regulator containing medium in Petri dishes. The growth regulator combinations used during germination and the following growth regulator phases were identical. The dishes were sealed with parafilm and germination was allowed to proceed at 25 °C in the dark for 4 weeks at which time the seedlings were dissected. In the case of seedlings larger than 2 cm, cotyledonary needles, hypocotyl segments and/or whorl with 2 - 3 mm hypocotyl and about 1 mm of the cotyledons were prepared. In the case of smaller seedlings only the roots were removed. These explants were placed separately on cytokinin-free medium or on medium supplemented by 0.01 mg l^{-1} KIN and kept in red light (650-700 nm, irradiance 40 µmol m^{-2} s^{-1}, 16 h photoperiod) for three to four weeks - the first growth regulator phase. Subsequently a growth regulator phase of 1 - 2 weeks and a 3 - 4 weeks differentiation phase alternated permanently. One growth regulator phase and one interphase will be referred to as one cycle. Clumps of buds or primordia larger than about 1 cm in diameter were dissected before transfer.

Results and discussion

Cytokinin application during germination of seeds proved a reliable and time-saving way for the initiation of *in vitro* meristem cultures. In combination with permanently repeating growth regulator phases and interphases a meristematic *in vitro* culture resulted from 5 - 10 % of the seeds (several thousands seeds were tested). Selected lines have produced shoot buds now for more than three years. The development of the shoot buds depends on the concentration of cytokinins contained in the medium. A concentration of 0.5 mg l^{-1} of both BA and KIN during germination inuced shoot buds during the first differentiation phase, whereas 0.1 mg l^{-1} of both cytokinins during germination and the following growth regulator phases stimulated development of buds after at least three cycles.

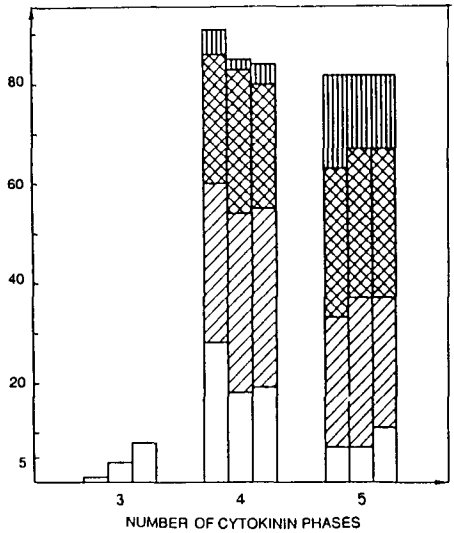

Fig. 1. Percentage of seedling producing adventitious buds after 3, 4 and 5 growth regulator phases on medium supplemented with BA and KIN each of 0.1 mg l^{-1} for the first (*a*), 0.25 mg l^{-1} for the second (*b*) and 0.5 mg l^{-1} for the third column (*c*). The seedlings were subdivided into classes of diferent bud forming capacity: *empty columns* - up to 5; *hatched columns* 5 to 10; *cross-hatched columns* 10 to 20, *vertically hatched columns* more than 20 buds per seedling. 80 to 120 seedlings were tested for each variant. Statistical analysis of the results was performed by the Fisher-test using the computer program developed by W. Jank. The statistical analysis showed that differences in the bud forming capacity between the third and the fourth (but not between the fourth and the fifth growth regultor phase) were significant but differences among the tested concentrations of BA and KIN were insignificant.

The most reactive centre for bud formation was located around the whorl. This includes the apex, the cotyledonary node with the basal parts of the cotyledon and a 2 - 3 mm hypocotyl part below the cotyledonary node. These segments are known to contain meristematic tissue (Bornman and Jansson, 1982 Bornman 1983).

The number of shoot buds which appeared during the first three cycles was low and was hardly influenced by an increase of the concentrations of both cytokinins from 0.1 to 0.5 mg l^{-1}. The efficiency of bud formation increased drastically during the fourth cycle. This concerns the percentage of seedlings producing buds as well as the number of buds formed per seedling. During the following cycles only the number of buds formed per seedling increased further (Fig. 1). The increase of the concentration of both cytokinins from 0.1 to 0.5 mg l^{-1} had no significant effect on the efficiency of bud formation, but it resulted in an increased callus formation during further culture (0.25 or 0.5 mg l^{-1} of both BA and KIN). Higher cytokinin concentrations (1 mg l^{-1} of both BA and KIN) induced callus formation during primary culture (about 50 % of the explants) and lowered the survival of the corresponding explants. The average multiplication rate for one cycle (5 to 6 weeks) is about 1.5.

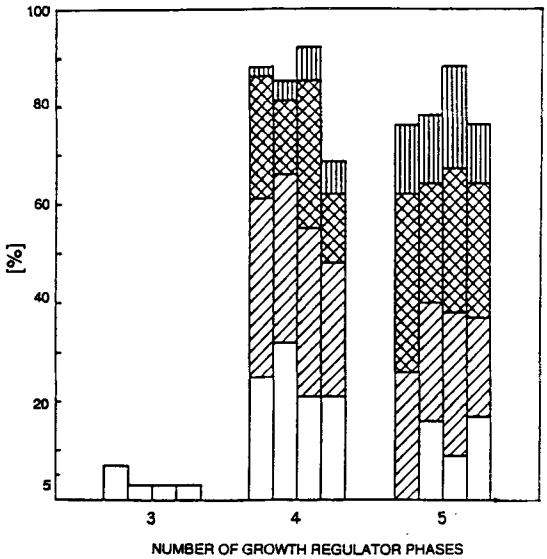

Fig. 2: Percentage of seedlings producing adventitious buds after 3, 4 and 5 growth regulator phases on medium supplemented with 0.25 mg l^{-1} of both BA and KIN + NAA as follows: 0 mg l^{-1} for the first (*a*); 0.001 mg l^{-1} for the second (*b*); 0.01 mg l^{-1} for the third (*c*) and 0.1 mg l^{-1} for the fourth column (*d*). Seedlings wee subdivided into classes of different bud forming capacity: *empty columns* - up to 5; *hatched columns* 5 to 10; *cross-hatched columns* 10 to 20, *vertically hatched columns* more than 20 buds per seedling. 80 to 120 seedlings were tested for each variant. Statistical analysis of the results was performed by the Fisher-test. This test showed analogous results to those described in Fig. 1 with one exception (0.1 mg l^{-1} NAA in the fourth growth regulator phase caused a significant lower portion of seedlings producing adventitious buds).

Substitution of BA/KIN by ZEA as sole cytokinin supply during the growth regulator phases did not increase the efficiency of multiplication. ZEA had to be applied at higher concentrations than BA/KIN to get the same results for induction and permanent multiplication of the shoot buds. 2 mg l^{-1} ZEA had similar effects as 0.1 mg l^{-1} of both BA and KIN.

Additional application of 0.1 mg l^{-1} NAA during the interphases had no permanent positive effects on the multiplication of buds (data not shown). Only after the fourth growth regulator phase this application caused significant more bud forming seedlings compared to the other NAA concentrations. But from the fifth growth regulator phase this growth regulator variant induced callus proliferation accompanied by rapid browning and finally death of the explants.

Application of 0.01 mg l^{-1} of the cytokinins KIN, ZEA, BA or 2iP during the interphases did not result in an increasing efficiency of multiplication.

In preliminary experiments we could initiate shoot elongation with an efficiency of up to 100 % from two clones characterized by high bud multiplication rates (manuscript in preparation).

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