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Illumination-dependent effects of gibberellin on *in vitro* developing European larch shoots

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Some morphological differences were observed between European larch shoots developing from isolated axillary buds (buds were collected in the first decade of April) on the nutrient medium enriched with 0.2 μM gibberellin GA_3 and that developing on the medium without GA_3. These differences were closely related with cultivation conditions, especially with the photoperiod and light intensity. Exogenous gibberellin had a slight negative effect on the development of needles under a long photoperiod but a more significant positive effect under a shorter photoperiod and slighter light intensity. On the medium without GA_3 larch needles sprouted more intensively under a more intensive illumination, but light intensity had no effect on this developmental feature when explants were cultivated on the medium with gibberellin. Light intensity significantly increased the development of long axial needles if gibberellin was not applied to the nutrient medium. Exogenous gibberellin, by contrast, caused a more intensive development of axial needles under slighter illumination as compared to intensive illumination, but this development was generally reduced. A large part of larch explants on the medium without GA_3 formed long-shoot primordia (instead of elongating axial needles) in the apical zone of sprouting buds under short-day conditions, but no increase in the formation of long-shoot primordia caused by a lower light intensity occurred in explants treated with gibberellin. A season can have a major influence on the response of larch buds to a certain gibberellin: the total mass of larch explants collected in the second decade of October and cultivated on the medium with gibberellin did not develop and kept browning.

Key words: gibberellin, larch, bud, explant, photoperiod

INTRODUCTION

Bioactive gibberellins, or gibberellic acids (GA), are known as important plant growth regulators (PGR) that promote leaf expansion, internode elongation, flowering, seed development and are involved in the regulation of some other developmental events during plant life cycle [1–3]. In many of these systems, gibberellin modulates the transcription of specific genes [3, 4]. Gibberellins form a large group of compounds with a similar chemical structure, but only several gibberellins are able to act as plant growth regulators [1, 2]. The effects of gibberellins are well investigated in both herbaceous and woody plants. Gibberellin-mediated regulation of flowering was examined in various species of woody plants, both angiosperms [5] and gymnosperms [6, 7]. But the role of gibberellins in tree development is not restricted to the promotion of flowering. These plant growth regulators are important in vegetative growth of trees too. Gibberellins, together with auxin, control longtinual and cambial growth in conifers [8]. As GA_4 is the main form of gibberellins found in conifers, most reports about the action of gibberellins in gymnosperm species are based on the use of the gibberellin mixture $GA_{4/7}$ [6, 7]. $GA_{4/7}$ was found to be much more effective in promoting flowering in conifers than GA_3 [9], though the latter form of gibberellins is most widely used for plant cultivation. In the case of conifer species, $GA_{4/7}$ is generally used for cone induction [6, 7, 10]. But all three main forms of gibberellic acid that are known as bioactive compounds (GA_1, GA_3, GA_4) promoted shoot elongation or the terminal bud development in seedlings of conifer species [11], suggesting that GA_3 can be successfully used to study vegetative growth response in conifers to exogenous gibberellin.

The total majority of reports about the effect of exogenous gibberellins on the development of conifer buds and shoots are based on *in vivo* experiments. Hormones are applied by drenching the shoot tip, injecting the stem or spraying the foliage [11]. Gibberellins are not

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generally used in conifer tissue cultures for plant regeneration. This causes the lack of knowledge about gibberellin action *in vitro*. The nutrient medium used for conifer cultivation *in vitro* is usually enriched with cytokinins and auxins. The effects of these plant growth regulators on *in vitro* developing conifer tissues were intensively investigated for practical purposes. Different combinations of cytokinins and auxins are used for larch micropropagation too [12–14]. The data revealing the positive effect of gibberellins on the development of conifer shoots *in vivo* argue in favour of a thorough investigation of respective effects of exogenously applied gibberellin *in vitro*. The aim of this research was to study the primal response of European larch axillary buds to exogenous GA_3 applied to the nutrient medium.

MATERIALS AND METHODS

Abbreviations

ABA – abscisic acid
BAP – 6-benzylaminopurine
GA_3 – gibberellic acid-3 (gibberellin-3)
PGR – plant growth regulator

Plant material and nutrient medium

Current year twigs were collected from the lower one-third of the crown of a 30-year-old European larch (*Larix decidua* Mill.) tree. Two gatherings took place in different seasons: the first in the first decade of April and the second in the second decade of October. After removal of needles the twigs were cut into short pieces (1–2 cm, each segment with an unburst vegetative bud). Segments of twigs were soaked for 3 min in 75% ethyl alcohol and then for 4 min in 0.1% silver nitrate solution. After sterilization larch explants were prepared as follows: all tissues except green bud meristems were removed from woody cores using sterile pincers. Bare buds were cultivated in plastic Petri dishes (90 mm or 55 mm in diameter) under a regulated light and temperature regime (specific for every experiment). Four explants were placed in each Petri dish 90 mm in diameter and three explants in each Petri dish 55 mm in diameter. Modified MS nutrient medium [15] containing 30 $g \cdot l^{-1}$ sucrose (pH 5.5 before autoclaving) and 9 $g \cdot l^{-1}$ phytoagar enriched with GA_3 or other plant growth regulators (obtained from ICN Biochemicals GmbH, Germany) was used for the cultivation of explants. GA_3 (soluted in ethyl alcohol and diluted with distilled water), ABA and BAP (both diluted in sodium hydroxide and with distilled water) were added to the medium after autoclaving before the sterilized medium congealed. Syringe-driven Millex filters (pores 0.22 μM) were used for sterilization of plant growth regulators.

Growth conditions for explants collected in the first decade of April

European larch axillary buds collected in the first decade of April were divided into four experimental sets:

1. Nutrient medium without plant growth regulators (PGR-free). Photoperiod 16 h.
2. Nutrient medium enriched with 0.2 μM (0.07 $mg \cdot l^{-1}$) GA_3. Photoperiod 16 h.
3. Nutrient medium without plant growth regulators (PGR-free). Photoperiod 12 h.
4. Nutrient medium enriched with 0.2 μM (0.07 $mg \cdot l^{-1}$) GA_3. Photoperiod 12 h.

Each experimental set on PGR-free nutrient medium contained 100 explants and each experimental set on medium enriched with GA_3 contained 32 explants. Auto-matically regulated white-light illumination was used to maintain a certain photoperiod (brightness 2200–3600 luxes) in the growth room. The temperature was partially dependent on illumination: when the lights were switched on it rose to 25 °C and was maintained at 18 °C during the dark period.

Growth conditions for explants collected in the second decade of October

European larch axillary buds collected in the second decade of October were divided into eight experimental sets:

1. Nutrient medium without plant growth regulators (PGR-free). Photoperiod 16 h.
2. PGR-free nutrient medium. Photoperiod 8 h.
3. PGR-free nutrient medium. Photoperiod 16 h. Temperature regime (cyclic): 12 h – 25 °C, 4 h – 18 °C, 4 h – 4 °C, 4 h – 18 °C.
4. Nutrient medium enriched with 0.2 μM (0.07 $mg \cdot l^{-1}$) GA_3. Photoperiod 16 h.
5. Nutrient medium enriched with 0.2 μM (0.07 $mg \cdot l^{-1}$) GA_3 and 10 μM (2.64 $mg \cdot l^{-1}$) ABA. Photoperiod 16 h.
6. Nutrient medium enriched with 0.2 μM (0.07 $mg \cdot l^{-1}$) GA_3 and 1 μM (0.22 $mg \cdot l^{-1}$) BAP. Photoperiod 16 h.
7. Nutrient medium enriched with 0.2 μM (0.07 $mg \cdot l^{-1}$) GA_3. Photoperiod 8 h.
8. Nutrient medium enriched with 0.2 μM (0.07 $mg \cdot l^{-1}$) GA_3. Photoperiod 16 h. Temperature regime (cyclic): 12 h – 25 °C, 4 h – 18 °C, 4 h – 4 °C, 4 h – 18 °C.

The BINDER APT.Line KBW growth chamber was used for maintenance of specific cultivation conditions (8 h photoperiod and cyclic temperature regime). Illumination (white-light) inside the chamber during the "daytime" was 2600 luxes. A 16-h photoperiod without specifically regulated temperature variations was maintained in the growth room (described at the characterization of growth conditions for explants collected in the second decade of October). Each experimental set cultivated in the growth room contained 39 explants and every set in the growth chamber contained 30 explants.

Processing of results and statistics

Effects of certain nutrient medium or cultivation conditions were estimated after 20 days from the onset of cultivation of axillary buds *in vitro*. Following morphological features were assessed: development of needles (in April explants with fully developed needles from half or more of needle primordia were counted and in October explants with visibly developing needles from

half or more of needle primordia were counted), development of needles in the axial zone, formation of clusters of green non-developing needles around the axial zone and general viability of explants. All these mor-phologic features were estimated in percentage (the portion of explants with certain feature was assessed). Because of very low viability of explants harvested in October and cultivated on nutrient medium containing gibberellin, only the percent of explants with greenish base of bud axis was estimated in these samples.

Bias of value expressed in percentage (S_p) was used for statistical verification of reliability of obtained results. It was calculated by the formula:

$$S_p = \pm \sqrt{(p(100-p) / n)},$$

here p is the value of the parameter expressed in percentage, and n is sample size.

RESULTS

Development of axillary buds harvested in April

The nutrient medium enriched with GA_3 had a slight negative effect on the viability of larch explants cultivated under long-day conditions (photoperiod 16 h) and a slight positive effect on the viability of explants cultivated under short-day conditions (photoperiod 12 h) compared to the medium without gibberellin (Table 1). Gibberellin had no effect on formation of the clusters of green non-sprouting needles around the axial zone, if explants were cultivated under a 16-h photoperiod. By contrast, under a 12-h photoperiod the formation of such clusters did not increase in the medium with GA_3, while gibberellin-free grown explants under short-day conditions increased almost fourfold the formation of clusters of non-sprouting needles around the axial zone. The effect of gibberellin on sprouting needles was strongly related with the length of the photoperiod. Under long-day conditions, GA_3 slightly decreased the number of explants with intensively developing needles, but under the shorter photoperiod needle development was more significantly promoted by gibberellin treatment. Interestingly, the rate of needle development was almost the same under both photoperiods if explants were cultivated on the medium with gibberelin, but on the PGR-free nutrient medium the development of needles was 2.5 times increased by the longer photoperiod.

The most pronounced effect of gibberellin was observed in the development of needles in the axial zone. This effect was also photoperiod-dependent. GA_3 significantly delayed development of the axial zone under both photoperiods, but under short-day conditions this effect was not so strong: development of needles in the axial zone decreased only 1.5 times as compared to the PGR-free grown explants. Meanwhile an almost tenfold decrease in the development of axial needles caused by exogenous gibberellin was observed in the group of explants cultivated under the 16-h photoperiod. The effect of daylength was totally reverse for larch explants developing on different media (without gibberellin and with GA_3). On the PGR-free medium, development of axial needles was strongly increased by the 16-h photoperiod (but not so strongly as the general needle development). By contrast, a fourfold decrease in the development of needles in the axial zone was caused by the longer photoperiod.

Development of axillary buds harvested in October

The total majority of axillary larch buds harvested in October and put on the nutrient medium enriched with 0.2 μM GA_3 did not develop at all. Several explants that initiated needle development broke it shortly and kept browning too. In that case the sample of explants on the medium with gibberellin contained no developing fully viable buds that could be compared with explants developing on the PGR-free medium. Only the number of explants with not fully atrophied base of the bud axis or not fully brown needle fascicles was assessed (Figure). Assessment of explants with greenish base of the bud axis exposed the role of abscisic acid (ABA) in maintaining partial viability of larch buds treated with GA_3. The number of explants that maintained greenish base of the bud axis increased 2.4 times when nutrient medium containing 0.2 μM GA_3 was supplemented with 10 μM ABA. Supplementing GA_3-containing medium with 1 μM 6-benzylaminopurine (BAP) also caused an increase in the partial viability of larch explants (1.7 times). Cytokinin positively influenced chlorophyll maintenance: a more significant number of non-developing small needles remained green for a somewhat longer time. Short-day conditions (8 h photoperiod) and low temperature (4 hours of chilling at 4 °C in every 24-hour cycle) had a similar effect on the maintenance of partial viability for gibberellin-treated larch buds as did cytokinin treatment.

Table 1. **Development of larch axillary buds (%) harvested in the first decade of April**

	PGR-free medium, photoperiod 16 h	PGR-free medium, photoperiod 12 h	GA_3 0.2 μM, photoperiod 16 h	GA_3 0.2 μM, photoperiod 12 h
Browning explants	13.0 ± 3.4	7.0 ± 2.6	7.1 ± 4.5	12.5 ± 5.8
Green non-sprouting needles around the axial zone	11.0 ± 3.1	39.0 ± 4.9	10.7 ± 5.5	9.4 ± 5.2
Intensively developing needles	46.0 ± 5.0	18.0 ± 3.8	35.7 ± 8.5	34.4 ± 8.4
Developing needles in the axial zone	69.0 ± 4.6	41.0 ± 4.9	7.1 ± 4.5	28.1 ± 7.9

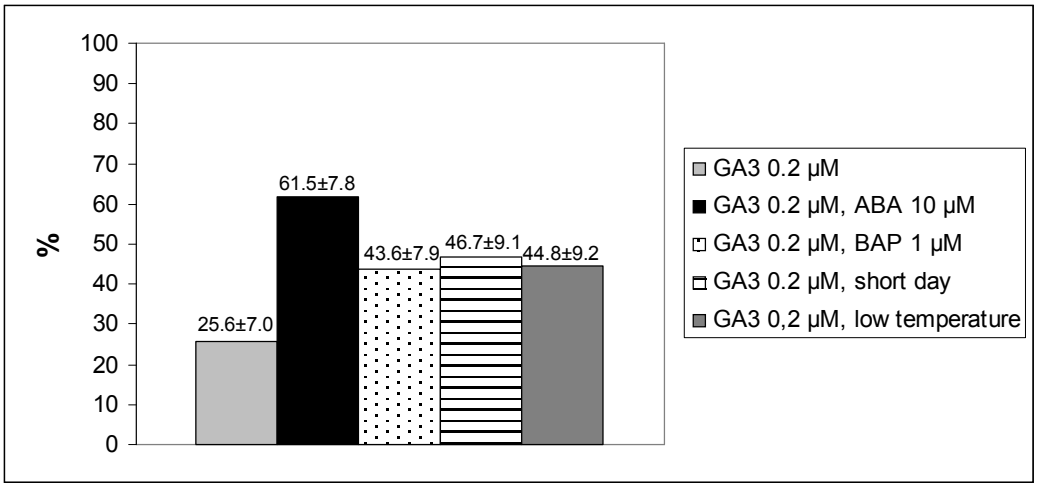

Figure. Larch explants collected in October and maintaining the greenish base of the bud axis

By contrast, larch explants cultivated on the PGR-free nutrient medium exhibited a nice viability and growth behaviour. The rate of needle elongation was not so high as in the sample of axillary buds collected in April, therefore not only explants with fully developed but also with elongating needles were registered in the sample collected in October to compare the development of larch axillary buds under different cultivation conditions (Table 2). Also the rate of the development of axial needles was relatively lower as compared to the development rate of axial needles in April. In spite of that, the general viability was very similar in samples of different seasons when cultivated on PGR-free medium. Only low temperature (4 °C) for 4 hours every 24 hours had a slight negative effect on the viability of larch explants harvested in October. Low temperature had a strong negative effect on the development of larch needles: it decreased 19 times. Meanwhile a short photoperiod (8 h) caused only a twofold decrease in needle development. Chilling strongly decreased the development of axial needles and increased the maintenance of the clusters of non-sprouting needles around the axial zone. Cultivation under short-day conditions had similar effects on the development of larch explants, but these effects, though very significant, were not so drastic as after everyday chilling.

DISCUSSION

Results obtained in this research show that an *in vitro* system can reveal a more diverse effect of gibberellins compared to an *in vivo* system. *In vivo* added gibberellins in most cases had a positive effect on the development of conifer shoots. Gibberellins were able to promote not only shoot elongation [11], but also expansion of terminal buds by increased mitotic activity [16]. Mean-while GA_3 had a contradictory effect on *in vitro* developing of European larch shoots. One of the most interesting facts was the total blockage of development of isolated axillary buds observed in October but not in April. The possibility to observe changes in explants collected in different seasons is one of the main advantages of the *in vitro* system, because in most cases *in vivo* experiments are carried out only during the season of intensive shoot development. Even during the growth season several tests can give different results if they are carried out not at the same time. Timing of $GA_{4/7}$ application on larch shoots for flower induction gave a variety of effects that depended on time of gibberellin application during vegetative season [6, 7, 17], but these effects were not so contradictory. It is not easy to explain why GA_3, having no negative effect on bud viability in spring, caused the total lost of viability of larch explants collected in autumn. One of the possible explanations could be based on the action of gibberellin oxidases. This suggestion is supported by our observation that larch buds collected in April showed a different response to exogenously applied gibberellin under different illumination conditions. It has been proved that plants accumulate larger quantities of transcripts of the genes coding certain gibberellin oxidases (GA 20-oxidase and GA 3-oxidase) under long-day conditions [18, 19]. That could be the reason for a different response of

Table 2. **Development of larch axillary buds (%) harvested in the second decade of October**

	PGR-free medium, photoperiod 16 h	PGR-free medium, photoperiod 8 h	PGR-free medium, photoperiod 16 h, low temperature
Browning explants	12.8 ± 5.4	13.3 ± 6.2	17.2 ± 7.0
Green non-sprouting needles around the axial zone	12.8 ± 5.4	40.0 ± 8.9	55.2 ± 9.2
Developing needles	64.1 ± 7.7	30.0 ± 8.4	3.4 ± 3.4
Developing needles in the axial zone	59,0 ± 7.9	26.7 ± 8.1	13.8 ± 6.4

larch buds cultivated under different photoperiods. Various gibberellin oxidases are responsible for both formation of bioactive gibberellins in plants and their conversion to inactive compounds [2, 20]. GA 20-oxidase and GA 3-oxidase, which can be induced by prolonged photoperiod in light-demanding plants, catalyze the last steps of synthesis of gibberellin-like compounds that are able to act as plant growth regulators [2]. Interestingly, the axial and basal zones of larch buds showed different responses to GA_3. Data of other researches indicate that the synthesis of gibberellins around the shoot apex is developmentally regulated in a strict way [21, 22]. This regulation occurs in the early phase of gibberellin biosynthesis before the steps that are catalysed by gibberellin oxidases. Induction of gibberellin oxidases depends on the compounds that could serve as substrates for these enzymes, namely, on appearance of certain gibberellin-like substances [2, 23]. The expression of genes encoding gibberellin oxidases can be increased suddenly in response to an increase of certain gibberellins [24]. Researches based on the genetic molecular analysis of gibberellin biosynthesis suggest that this regulation could occur in a very specific way because of the great variability of genes encoding for gibberellin oxidases [20, 25]. This variability was assessed in some herbaceous plants, but it is not quite clear how specifically a certain form of gibberellin oxidase is induced by a certain form of gibberellin. Gibberellin treatment *in vitro* may have caused unequal response of gibberellin oxidases in buds collected in different seasons, but it is difficult to conclude whether the ability of induction of gibberellin oxidases is decreased or increased in autumn. One of the possible explanations is based on the second variant. Exogenously applied gibberellin is first is transported to the needles [26], and needles are the main source of gibberellins in conifers [8] but gibberellins are shortly transported from the needles to the stem (shoot axis) [26]. Research of gibberellin content in evergreen conifers (Picea) showed that during the period of active growth the major gibberellin activity was found in a less polar fraction (as GA_4), whilst during the winter a more polar fraction (as GA_3) was predominant [27]. The primal effect of GA_3 on larch buds collected in October was indicated as the browning of needle primordia. This suggests that under cultivation conditions, unsuitable for growth conifers synthesize more GA_3-like gibberellins and the suddenly increased activity of gibberellin oxidases negatively affects the viability of needles. Abscisic acic (ABA), plant growth regulator acting as a gibberellin antagonist [5, 23, 28], can play some role in maintaining the viability of larch buds under GA_3 treatment. Cultivation conditions that are suitable for ABA accumulation (short photoperiod, low temperature [29]) also have a similar effect. However, the understanding of the system of specific response to gibberellin treatment in larch should be improved in the future.

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References

1. Darginavičienė J, Novickienė L. Augimo problemos šiuolaikinėje augalų fiziologijoje. Vilnius, Lietuvos mokslų akademijos leidykla, 2002.
2. Olszewski N, Sun T, Gubler F. The Plant Cell 2002; Supplement: 61–80.
3. Richards DE, King KE, Ait-Ali T, Harberd NP. Annu Rev Plant Physiol Plant Mol Biol 2000; 52: 67–88.
4. Huttly AK, Phillips AL. Physiol Plantarum 1995; 95: 310–21.
5. Tanino KK. J of Crop Improv 2004; 19/20: 157–99.
6. Eysteinsson T, Greenwood MS. Tree Physiol 1995; 15: 467–9.
7. Smith RF, Greenwood MS. Tree Physiol 1997; 17: 407–14.
8. Odén PC, Wang Q, Högberg KA, Werner M. Tree Physiol 1995; 15: 451–6.
9. Pharis RP, Ross SD, McMullan E. Physiol Plantarum 1980; 50(2): 119–26.
10. Dunberg A. Physiol Plantarum 1973; 28 (2): 358–60.
11. Little CHA, MacDonald JE. Tree physiol 2003; 23: 73–83.
12. Ewald D, Kretzschmar U, Chen Y. Biol Plantarum 1997; 39(3): 321–9.
13. Kretschmar U. Silvae Genetica 1993; 42: 4–5.
14. Kretschmar U, Ewald D. Plant Physiol 1994; 144: 627–30.
15. Murashige T, Skoog F. Plant Physiol 1962; 15: 473–9.
16. MacDonald JE, Little CHA. Tree Physiol 2006; 26: 1271–6.
17. Aasamaa K, Sõber A, Hartung W, Niinemets Ü. Tree Physiol 2002; 22: 267–76.
18. Kamiya Y, García-Martinez JL. Curr Opin in Plant Biol 1999; 2: 398–403.
19. Wu K, Li L, Gage DA, Zeevaart JAD. Plant Physiol 1996; 110: 547–54.
20. Lange T. Planta 1998; 204: 409–19.
21. Silverstone AL, Chang C, Krol E, Sun T. Plant J 1997; 12: 9–19.
22. Smith MW, Yamaguchi S, Ait-Ali T, Kamiya Y. Plant Physiol 1998; 118: 1411–9.
23. Rademacher W. Annu Rev Plant Physiol Plant Mol Biol 2000; 51: 501–31.
24. Itoh H, Ueguchi-Tanaka M, Kawaide H et al. Plant J 1999; 20: 15–24.
25. Hedden P, Proebsting WM. Plant Physiol 1999; 119: 365–70.
26. Wang Q, Little CHA, Odén PC. Tree Physiol 1997; 17: 715–21.
27. Lorenzi R, Horgan R, Heald JK. Planta 1975; 126 (1): 75–82.
28. Heide OM. Tree Physiol 1986; 1: 79–83.
29. Li C, Welling A, Puhakainen T et al. Tree Physiol 2005; 25: 1563–9.

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NUO APŠVIETIMO PRIKLAUSANTIS GIBERELINO POVEIKIS EUROPINIO MAUMEDŽIO ŪGLIŲ VYSTYMUISI *IN VITRO*

Santrauka

Morfologiniai ypatumai, kuriais europinio maumedžio ūgliai (išsivystę iš izoliuotų pažastinių pumpurų, nuo motinmedžio surinktų balandžio pirmą dekadą), kultivuoti ant giberelinu GR_3 praturtintos maitinamosios terpės, skyrėsi nuo kontrolinės grupės eksplantų, buvo glaudžiai susiję su kultivavimo sąlygomis, ypač šviesos intensyvumu. Išoriškai pridėtas giberelinas turėjo nedidelį neigiamą poveikį spyglių vystymuisi ilgo fotoperiodo sąlygomis, tačiau esant trumpesniam fotoperiodui bei mažesniam šviesos stipriui giberelinas pastebimai skatino spyglių vystymąsi. Ant terpės be GR_3 maumedžio spygliai sparčiau skleidėsi intensyvesnio apšvietimo sąlygomis, tačiau šviesos intensyvumas neturėjo panašaus poveikio eksplantus kultivuojant ant terpės su giberelinu. Nesant maitinamojoje terpėje giberelino, intensyvus apšvietimas labai paskatino ilgų ašinių spyglių vystymąsi. Tuo tarpu pridėtas giberelinas, priešingai, lėmė intensyvesnį ašinių spyglių vystymąsi silpnesnio apšvietimo sąlygomis, tačiau apskritai giberelinas neigiamai veikė šių spyglių vystymąsi. Didelė dalis maumedžio eksplantų ant terpės be GR_3 trumpos dienos sąlygomis suformavo ilgųjų ūglių užuomazgas (vietoje ilgėjančių ašinių spyglių) besiskleidžiančių pumpurų viršūninėje zonoje, tačiau giberelinu paveikti eksplantai ilgųjų ūglių užuomazgų neformavo gausiau net ir esant mažam šviesos intensyvumui. Maumedžio pumpurų atsakui į gibereliną didelę reikšmę gali turėti ir sezoniškumas: spalio mėnesio antrą dekadą surinkti pažastiniai pumpurai ant terpės su GR_3 visiškai sunyko.

Raktažodžiai: giberelinas, maumedis, eksplantas, pumpuras, fotoperiodas