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Effects of Medium Components and Growth Regulators on Callus Development and Shoot Regeneration from Shoot Explants of Black Locust (*Robinia pseudoacacia*)

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Abstract Effects of growth regulators and medium components were tested for shoot multiplication and callus growth from shoot explants of black locust.

During shoot multiplication, callus growth at the cut end of shoot explants proceeded shoot development. The basal callus growth seemed to be a function of both mineral components and cytokinin supplemented in the medium. Maximum callus growth was induced by 0.5 μM BAP and the callus growth decreased as the level of BAP increased. Positive correlations were found between basal callus growth, and shoot multiplication and growth. Shoot multiplication was greatest on BSM medium (black locust shoot culture medium) supplemented with 1 μM BAP. With medium containing high nitrogen content, both shoot multiplication and growth were significantly enhanced. A new BRM medium was the most effective for rooting of black locust among three rooting media tested.

Key words: *Black locust, callus, shoot multiplication*

Introduction

Black locust (*Robinia pseudoacacia*), a nitrogen fixing and stress tolerant leguminous tree, is one of the most promising species due to its diverse use and short generation interval [1-9]. Its amenability to tissue culture and *Agrobacterium*-mediated transformation [6] makes the species suitable as a test organism in genetic engineering of tree species

Micropropagation provides a means for both the clonal multiplication of selected or manipulated genotypes, and providing highly uniform plant materials for the application of biotechnology. In black locust, most tissue culture systems have been established.

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Shoot regeneration from seedlings, cambial tissues and embryogenesis from immature seeds have been reported [2,3,7,10]. During *in vitro* shoot multiplication, formation of callus at the cut end of shoots has been noticed [3,10]. This could be problematic in determining the source of shoot multiplication. Shoots could be derived either via organogenesis from callus tissues or direct adventitious shoot formation. The competition between growing callus and shoot bud for uptaking nutrients in the medium may affect the efficiency of shoot multiplication from the explant.

In this paper, we describe effects of medium components and growth regulator concentrations to improve previously reported micropropagation methods, as well as to find corelation between callus formation and shoot multiplication from shoot explants of black locust as affected by medium components.

Materials and Methods

Shoot multiplication

Shoot cultures, which had been regenerated previously from internodal segments of in vitro shoot cultures [7], were used as starting explants. A shoot segment about 2 cm in length was transferred to a 10 ml of culture medium supplemented with 1 μM BAP in 25 × 150 mm culture tube. Four different concentrations (0.1, 0.5, 1.0 and 5.0 μM) of BAP combined with BSM (black locust shoot culture medium) were used for testing the effects of BAP concentration. Two black locust media (BSM and BRM; Table 1) were selected empirically by modifying salt concentrations of either MS [10] or WPM medium [11].

After 4 weeks of culture on shoot multiplication medium, the number of shoot multiplied, shoot dry weight, and diameter of callus at the base of shoot explants were measured. For measuring shoot dry weight, shoots were excised

Table 1. Composition of nutirent media used in *in vitro* culture of black locust

Reagents	MS	WPM	BSM	BRM
NH_4NO_3	1650	400	800	400
KMO_3	1900	556	1011	
$Ca(NO_3)_24H_2O$				556
KCl				750
K_2SO_4	370	990	505	
$MgSO_47H_2O$	440	370	370	250
$CaCl_22H_2O$	170	96	440	96
$KH_2PO_4H_2O$		170	96.5	
				37.25
Na_2EDTA	37.25	37.25	37.25	27.85
$FeSO47H_2O$	27.85	27.85	27.85	
$MnSO_44H_2O$	22.3		22.3	
$MnSO_4H_2O$		22.3		22.3
$ZnSO47H_2O$	8.6	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2	6.2
KJ	0.83		0.83	0.83
$NaMoO_42H_2O$	0.25	0.25	0.25	0.25
$CuSO_45H_2O$	0.025	0.25	0.025	0.25
$CoCl_26H_2O$	0.025		0.025	0.025
Thiamine HCl	0.1	1.0	0.1	0.1
Nicotinic acid	0.5	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5	0.5
Glycine	2.0	2.0	2.0	2.0
Myo inositol	100	100	100	100
Sucrose	20,000	20,000	20,000	20,000

1) MS ; Murashiege and Skoog (1962)
WPM (Woody Plant Medium) ; Lloyd and McCown (1980)
BSM (Black locust Shoot culture Medium)
BRM (Black locust Rooting Medium)

from callus clump and dryed at 80℃ for 72 hr.

Culture were maintained in a growth chamber at 26℃ under 16hr cool white fluorescent light (50~75 umol $m^{-2}s^{-1}$).

Root initiation

Shoots about 2 cm in length were placed in 25×150 mm culture tubes with 10 ml of BRM (black locust rooting medium), 1/10 strength MS with no sucrose but 1 μM IBM, or half strength WPM without growth regulator. After four weeks of culture on rooting medium, rooting percentage, number of root produced, root dry weight and shoot dry weight were scored.

Plantlets were potted in a peat moss / perlite / vermiculite mix (1/1/1), and watered with a fertilizer solution (1 gl^{-1} N/P/K, 20/20/20 in tap water as described previously [7].

Statistical analysis

Minimum number of 22 replications per treatment was used for the experiments. The data for shoot multiplication and rooting were analyzed separately for each treatment by a general linear model analysis of variance/covariance (GLM - ANOVA) [12]. The data for each treatment were separated by Duncan's New Multiple Range Test [13].

Result and Discussion

Basal callus growth

Callus tissues were developed at the cut end of shoot explants before shoot multiplication took place. Diameter growth of the callus was function of both mineral medium and cytokinin. The media used in the present studies can be grouped into two categories, that is, high (MS and BSM) and low (WPM and BRM) nitrogen media. Media with higher nitrogen source produced bigger callus (Fig. 1). With same level of nitrogen, BRM medium (total I = 46.2 meq/1) was more effective for callus growth than WPM (total I = 53.2 meq/1) which has higher total ionic stregth.

It seems that the nitrogen effect override the effects of other nutrients on the basal callus growth. A readily available supply of nitrogen has been speculated to be important to maintain cultured cells in an undifferentiated state [14]. By using the medium with lower nitrogen level, callus growth can be reduced significantly. However, there were strong positive correlations between callus growth, shoot multiplication (Pearson's r = 0.41, alpha = 0.01) and shoot dry weight (Pearson's r = 0.46, alpha = 0.01). Therefore, reducing macronutrient will also decrease the number of shoot multiplied and shoot growth. Callus growth was decreased as BAP level increased (Fig 2). However, it is notable that no callus was developed when BAP was not added (data not shown). Maximum callus growth occurred on the medium containing 0.5 μM BAP. On the contrary to the effects of BAP, NAA did not affect the growth of basal callus growth. Since the callus formation and growth is controlled by auxin/cytokinin balance, the data may suggest that black locust has relatively high endogenous auxin level. The addition of either auxin synthesis inhibitor or auxin did not affect basal callus growth [3]. However, it is possible that the relatively high level of endogenous auxin, which has been already synthesized during shoot cultures, still active and plays a role for callus growth regardless of the auxin

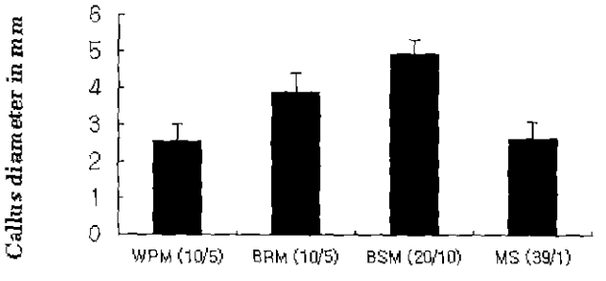

Fig. 1. Medium effects on callus growth during shoot culture.

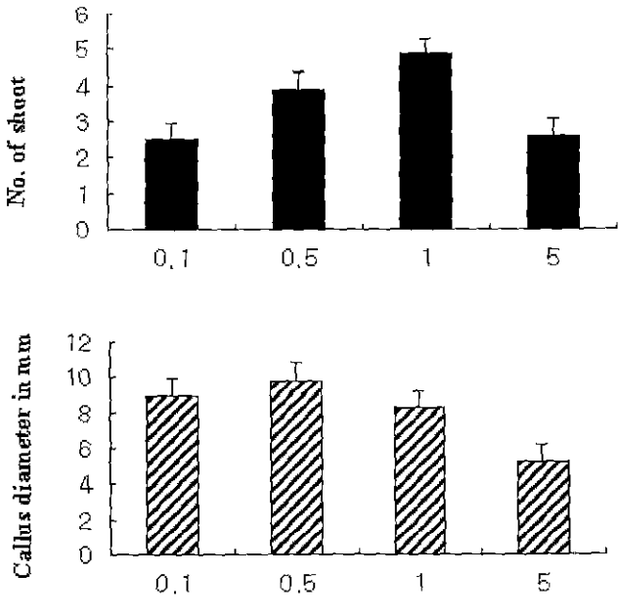

Fig. 2. BAP effects on callus and subsequent shoot induction from black locust.

synthesis inhibitor or exogenous auxin supplementation. To test this possibility, either the application of an inhibitor for auxin action or the direct assay of endogenous auixin could be a next step.

Shoot multiplication

Shoot multiplication was greatest on BSM medium supplemented with 1 μM BAP (Fig. 2 and 3). With high concentrations of BAP, shoot multiplication was significantly decreased as well as callus diameter growth did (Fig. 2).

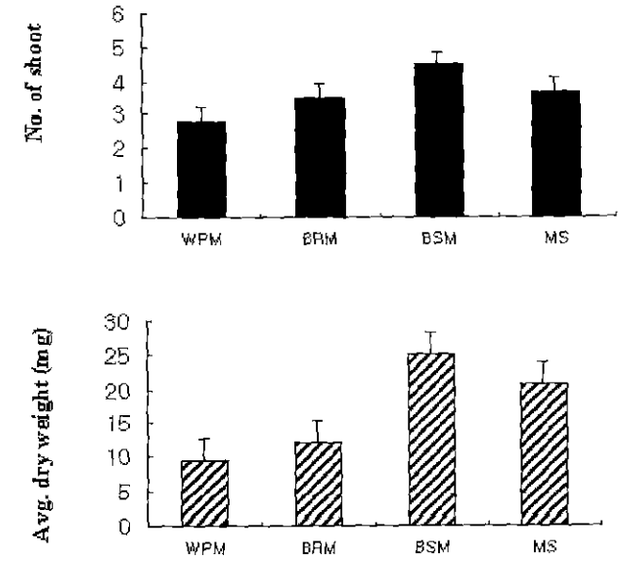

Fig. 3. Effects of mineral medium on black locust shoot culture.

Similar responses to BAP levels were reported *in vitro* shoot cultures derived from cuttings of a mature black locust [3]. BSM medium was modified from MS medium in two major respects: (1) total ionic strength (lowered from 94.25 mM to 58.72 mM); (2) concentration of several components (0.5 times less ammonium and nitrate, 0.7 times less potassium, and 2 times more sulfate). Media specially devised for tree species have consistently lower ionic concentration but higher amount of sulphate than those in other media [14]. With media contating higher nitrogen content (BSM and MS), both shoot multiplication and shoot growth were enhanced. However, in the media with lower nitrogen (BRM and WPM), shoot growth was lagging behind the shoot multiplication (Fig. 3). Although shoot multiplication was significantly affected by cytokinin concentrations, defining the best medium for the micropropagation of the species is of interest because of two reasons; (1) no such medium has been developed for tree legume, and (2) type of medium has strong positive correlation with both callus diameter and shoot growth as well as shoot multiplication from shoot explants. By modifying MS medium to make BSM containing reduced ionic strength, we could improve the methods from previous works [2,4,7,8,10], but were not able to solve the problem of callus formation at the base of shoot explant except reducing the growth of the callus. Interestingly, shoot multiplication and growth were not reduced by the growth of basal callus.

Root initation and growth

High rooting percentage was readily achieved following shoot multiplication. The composition and salt concentration of medium had significant effects on rooting (Table 2). One-tenth strength of MS medium (total macronutrient I=9.9 meq/l) containing 1 μM IBA and no sucrose and one-half strength of WPM medium (total macronutrient I =26.6 meq/l) without growth regulator (K. Gruber, personal communication) have proven to be successful for the rooting of the species. The new rooting medium (BRM; total macronutrient I =42.2 meq/l) produced significantly improved results from the two other media (Table 2). BRM medium was derived from WPM medium by mainly lowering sulfate 7 times but increasing chloride content 8 times.

Diluted media formulations for the root initiation of most plant species have been used commonly in the range of

Table 2. Effects of medium on *in vitro* rooting of black locust

Parameters	BRM	1/10 MS	1/2 WPM
Rooting percentage(%)	76.0±9.2ab*	54.6±7.0a	81.8±9.8b
Average root#	3.6±1.8b	1.8±0.3a	2.0±0.3a
Root dry weight(mg)	12.4±0.9b	4.4±0.9a	5.4±0.9a
Shoot dry weight(mg)	18.2±1.0b	6.6±1.0a	5.0±1.0a

*Values followed by different letters are significantly different at 0.05 level by Duncan's NMR test.

1/4～1/2 MS (total macronutrient I =24.8～46.9 meq/l) [3,14]. However, considering the number of roots and root/shoot growth as well as rooting percentage, lower strength than total macronutrient of half-strength WPM medium (I =26.6 meq/l) seemed inappropriate for black locust. Furthermore, our data (Table 2) suggest that the effects of media formulation can override the effect of auxin which has been frequently incorporated into rooting media for counter-balance the carryover effects of cytokinin used in shoot multiplication [14].

In conclusion, these experiments show that level of nutrient components in medium is more important than that of growth regulators for micropropagation of black locust.

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