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THE EFFECT OF 2,4-D AND KINETIN ON SOYBEAN CALLUS CULTURE*

RENATA PAVLINA

(Faculty of Agriculture, University of Zagreb)

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Sections of different explants (cotyledon, hypocotyl or meristem-type), 2,4-D (0.3 and 1.0 mgl^{-1} and kinetin (0 and 1.0 mgl^{-1}) were examined in MS medium which was solidified with 0.8% Bacto-agar for callus induction of soybean.

2,4-D at a concentration of 0.3 mgl^{-1} with 1.0 mgl^{-1} kinetin was considerably better in all the explants for optimal callus growth than other hormonal concentrations.

Introduction

One of the main problems of in vitro plant induction has been the finding of medium (Boulter and Crocomo 1980) for each genotype of leguminous members separately (Gresshopp and Mohaporta 1981). According to Reinert's theory of organogenesis (Boulter and Crocomo 1980), minimal quantitative changes in relations to determined components in media are a determining factor in stimulating the development and differentiation of some organs.

Depending on the genotype examined scientists do not agree on the medium compositions used, the sources and quantites of carbon, the source of nitrogen (of organic and inorganic origin), as these differences are expressed even in organic and inorganic additions to media. There are also numberless differences in the use of explants.

Growth of soybean callus tissue has been tried on media of different micro- and macro-elements composition. Polacco (1976) reports a successful cultivation of soya on MS basic media. Nutritive B_5 medium has stimulated soya cells proliferation in experiments of many authors (Gamborg and Shyluk 1970, Bayley et al. 1972, Tresshoff and Mohaporta 1981, etc.). On modified Miller's medium Fosket and Torrey (1969) achieved callus induction, and Oswald et al. (1977) point out Phillip's medium as very favourable for callus growth.

The success and favourability of soya culture growth depended upon a just selection of organic additives, hormones particularly. Key hor-

* This paper is dedicated to prof. Z. Devidé on the occasion of his 65th birthday.

mones were primarily of auxine and cytokinine. 2,4-D solution concentration ratio varied from 1 to 22 μMl^{-1} (Fosket and Torrey 1969, Gamborg and Shyluk 1970, Bayley et al. 1972, Chu and Lark 1976, Collins et al. 1978, Gosal and Bajaj 1979, etc.). The variation grade of kinetin concentrations used ranged from 0.0 to 2.5 μMl^{-1} (Blaydes 1966, Witham 1968).

Quantity differences were evident in the source of carbon used (saccharose) in the breeding of legumes in vitro of 20 gl^{-1} (Bayley et al. 1972) to 60 gl^{-1} (Pevalek 1979).

Photoperiod lasted from 12 (Evans et al. 1979) to 24 hours (Witham 1968).

Since chemical and physical conditions differ in cultivation depending on the genotype used, the need for testing optimum culturing conditions for each genotype individually is becoming increasingly important.

The aim of the experiment was to select positive factors for callus in vitro of high protein soya genotype 146/82-5, by varying the breeding conditions. I have studied separately callus induction in relation to growth hormones (kind, concentration and combination) as well as explant sources.

Stimulation of soybean callus in vitro was the first step in creating conditions for determining urea metabolism in vitro in cell suspension culture. It was taken as the base for selection programme of urease-overproducing mutants separation. Since urease has higher methionine levels, concentration increase improves seed protein quality by plant cell culture technique.

Materials and Methods

Soybean (*Glycine max.* /L./ Merr., genotype 146/82-5) was left swelled in a knitted sack for 24 hours in running water. Subsequently, it was sterilized in 96% alcohol by a single washing. A 15-minute sterilization in blending machine followed (3% solution of IZOSAN — Pliva Zagreb). The treatment was finished by 4 successive 10-minute washings with sterilized destilled water.

The seeds were left germinating in Petri dishes on sterile moist cotton with 12-hour photoperiod, 1600 lx light intensity, at a temperature of 20 °C.

Aseptic technique was applied to explant in vitro culturing. Thirteen-day old seedlings were taken for the experiment (3x2x2); they arranged by block-method, 6 repetitions at 24 °C, 1500 lx and 16-hour photoperiod.

A combination of micro- and macro-elements (Murashige and Skoog 1962) was utilized, varying the following 3 factors:

1. Source of explant with 3 factor levels:
   ex_1 — shoot tips (4.0 ± 1.2 mg)
   ex_2 — hypocotyl sections (23.0 ± 5.9 mg)
   ex_3 — cotyledon sections (58.0 ± 12.4 mg)
2. Auxin (2,4-D) in concentrations 0.1 mgl^{-1} and 0.3 mgl^{-1}
3. Cytokinin (kinetin) in concentrations 0.0 mgl^{-1} and 1.0 mgl^{-1}.

A two-month growth of fresh matter (in grams), visual, descriptive and score estimation of callus by stereo-microscope inspection, were indicators of a determined and examined combination effectiveness.

The results of weight measurements were F and t-tested in relation to the analysis of variance of trifactorial experiment 3x2x2, and the block-method (Snedocor and Cochran 1967), though visual evaluations were given in scores scale of 1—4 (Table 1), was tested with non-parametral HI^2-test considering Yates' correction of measuring results (Petz 1981).

Results and Discussion

Hormonal combinations 2,4-D without kinetin especially in shoot-apical meristems, resulted in watery, granular, transparent and sporadic necrotic callus.

Well-grown, watery, granular, and greenish callus without necrosis developed in both auxin concentrations, in the presence of kinetin (1 mgl^{-1}), particularly in hypocotyl and cotyledon explants. This descriptive evaluation conforms with the estimation score, shown in Table 1.

The results shown in Table 1 were HI^2 tested with Yates' result correction (Petz 1981). In hormonal concentration combinations (HI^2 = = 33.158**) differences in score estimations of callus growth were significant. Partial HI^2-tests for each pair of hormonal combinations were separately calculated (for a total of 6 pairs — Table 2) for the determination of combinations showing significant difference.

Results of the experiment allow a conclusion of kinetin utilization feasibility. All combinations without kinetin were poor and insufficient in respect of the *score estimation* and *descriptive evaluation.*

Differences in estimations of callus growth between auxine concentrations (0.1 or 0.3 mgl^{-1} 2,4-D) are not statistically significant.

Analysis of callus weight growth measurements by the method of preparatory variance analysis, proved that the greatest part of dispersive processes were caused by the differences in combinations (F_{exp} = 27.59**).

Testing (F-test) of some component combinations (factors and interactions) showed that the fresh weight increase was independent of explant source. however, greatly dependent upon the presence of 2,4-D (factor B) kinetin (factor C). and their mutual presence in medium (emphasized by the justified interaction BxC — Table 3).

In order to determine differences in medium weight growth in view the combinations, t-test was carried (Table 4).

The medium composition of 0.3 mgl^{-1} 2,4-D and 1 mgl^{-1} kinetin showed evident advantages in comparison to other hormonal combinations.

Equal growing possibilities were provided for all explant sources. and the experiment response was callus growth equally sufficient in all explant sections regardless of the origin.

Therefore the utilization of any of the above mentioned parts of the plant is made possible with high prospects for positive (and equal) reactions in vitro. This is worth mentioning as it offers greater possibility of employment of a plant as the explant source, because it reduces the need for breeding area.

The results of my experiment are in conformity with Witham's results (1968), who succeeded, by cultivating cotyledon segments (cv. Acme), in inducing callus growth on Miller's medium. He found the combined effect of 2,4-D and kinetin as more effective than 2,4-D alone.

Table 1. Estimations of callus growth on MS-medium

Explant	2.4−D + kin mgl^{-1}	Estimation 0$^+$	1$^+$	2$^+$	3$^+$	Combinations
K cotyledon	0.3 + 1	0	0	1	5	6
	0.3 + 0	0	6	0	0	6
	0.1 + 1	0	0	5	1	6
	0.1 + 0	3	3	0	0	6
cotyledon callus:		3	9	6	6	24
H hypocotyl	0.3 + 1	3	0	2	1	6
	0.3 + 0	3	3	0	0	6
	0.1 + 1	1	1	0	4	6
	0.1 + 0	3	0	0	3	6
hypocotyl callus:		10	4	2	8	24
VV meristem tip	0.3 + 1	0	0	0	4	4
	0.3 + 0	0	4	0	0	4
	0.1 + 1	2	2	0	0	4
	0.1 + 0	1	2	0	1	4
meristem-tip callus:		3	8	0	5	16
ESTIMATIONS:		16	21	8	19	64

$HI^2_{(hormon\ combinations)} = 33.158^{++}$ $\quad n-1 = (4-1)(4-1) = 9$

$HI^2_{(explants)} = 8.948$ $\quad n-1 = (3-1)(4-1) = 6$

\+ = no growth

1 = callus dark brown, only very slight growth

2 = callus watery, glassy, greenish, good growth

3 = callus watery, glassy, greenish, very good growth

Table 2. Summary of all testing results on MS-medium (HI^2 for all pairs of hormonal combinations were tested on MS-medium)

(2.4−D + kin/mgl^{-1}) : (2.4−D + kin/mgl^{-1})	n − 1	$HI^2_{(experimental)}$
(0.3 + 1) : (0.3 + 0)	(2 − 1)(4 − 1) = 3	21.47^{++}
(0.3 + 1) : (0.1 + 1)	(2 − 1)(4 − 1) = 3	2.02
(0.3 + 1) : (0.1 + 0)	(2 − 1)(4 − 1) = 3	9.83^{+}
(0.3 + 0) : (0.1 + 1)	(2 − 1)(4 − 1) = 3	1242^{++}
(0.3 + 0) : (0.1 + 0)	(2 − 1)(4 − 1) = 3	5.51
(0.1 + 1) : (0.1 + 0)	(2 − 1)(4 − 1) = 3	8.26^{+}

$HI^2_{(theoretical)} = 11.341$ $\quad ++$ (p 0,01)

$HI^2_{(theoretical)} = 7.815$ $\quad +$ (p 0,05)

Table 3. Table of variance analysis of experiment

Source of variability	SQ	n−1		F_{exp}
Total	50.74	47		
Block	0.61	3		
Comb	45.22	11	4.111	27.59^{++}
Mistake	4.91	33	0.149	
Factor A	0.79	2	0.40	2.68
Factor B	3.89	1	3.89	26.10^{++}
Factor C	33.11	1	33.11	222.21^{++}
A × B	0.96	2	0.48	3.22
A × C	0.03	2	0.02	0.13
B × C	5.54	1	5.54	37.18^{++}
A × B × C	0.91	2	0.46	3.09

Table 4. Average growth of fresh weight for soybean calus, on MS-medium with three explants stock and four hormonal combinations 2,4−D and kinetin

Explants	2,4−D + kin (mgl^{-1})	X (g)	$\bar{X}$ (g)
K Cotyledon	0.3 + 1	13.20	2.20
	0.3 + 0	4.23	0.71
	0.1 + 1	7.01	1.17
	0.1 + 0	2.96	0.49
H Hypocotyl	0.3 + 1	12.18	2.03
	0.3 + 0	1.45	0.24
	0.1 + 1	5.88	0.98
	0.1 + 0	3.51	0.59
VV meristem shoot-tip	0.3 + 1	10.45	2.61
	0.3 + 0	2.06	0.52
	0.1 + 1	7.95	1.99
	0.1 + 0	2.59	0.65

GD_p 0,01 = 0.44
GD_p 0,05 = 0.32

The same effect could be achieved with a lower concentration of auxin in the presence of kinetin. However, high concentrations of 2,4-D could provoke kinetin to react as inhibitor. Optimal concentration ratio should be established henceforth, for callus induction in the presence of both hormones.

Since cytokinin with slight effect, as adenine, methyladenine or ethyladenine, requires the presence of auxin (Miller 1967, according to Witham 1968) in higher concentrations than in strong cytokinin, a synergistic effect of two hormonal groups could be expected.

In my experiment the interaction of auxin and cytokinin was statistically justified; it is one proof more of the exactness of Miller's observations.

The testing of differences in score estimations between pairs of combinations, proved lower concentrations of 2,4-D with kinetin, to be higher graded than the higher auxin concentrations without kinetin; however, there was no difference in the score estimation between two auxin concentrations without kinetin in the medium. This observation is in conformity with Miller's hypotesis of synergism between these two groups of hormones.

It seems that auxin reactions appear critical for the efficiency of cell division; therefore, to learn more about the possibilities of influencing the process has become very important.

The efficiency of cell division could be increased by metabolic modifications caused by cytokinins or by employment of more effective auxin (Witham 1968), but also by utilization of certain combinations of inorganic salts, which could induce a physiological process of auxin synthesis unknown to us, excluding the need for auxin addition to the medium (Braun and Wood 1962).

Scientific interpretations can be reduced to the sole conclusion, namely that a cell without auxin cannot have optimum mitotic activity, and that the efforts to find favourable auxin concentrations or synergists in this activity or activators in vitro auxin syntheses, lead to a common goal: provoking the cells to intensified multiplication.

These results may cotribute to a better understanding synergistic auxin and cytokinin relations.

Conclusion

The scope of the experiment was to find favourable auxin and cytokinin combinations, and differentiation of the value of explant sources for soybean callus induction.

The type of explant (cotyledon, hypocotyl or shoot-tip) has not revealed statistically significant difference in intensity and quality of callus induction. According to score estimations of the quality and fresh weight measurements, the optimum hormonal combination was the concentration of 0.3 mgl^{-1} 2,4-D and 1.0 mgl^{-1} kinetin. Presumably, a range-correlation between score estimations and callus measurements would be worth establishing, which would emphasize the prognostic value of visual evaluation, and since it is technically faster to perform, in case of good prognostic value, it could be readilly acceptable.

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SAŽETAK

UTJECAJ 2,4-D I KINETINA NA KALUSNU KULTURU SOJE

Renata Pavlina

(Fakultet poljoprivrednih znanosti, Zagreb)

Postavljen je trofaktorijelan pokus (2 x 2 x 3) u 6 ponavljanja u svrhu ispitivanja utjecaja 2,4-D (0,1 i 0,3 mgl^{-1}) i kinetina (0,0 i 1,0 mgl^{-1}) te eksplantatskih izvora (kotiledone, hipokotil ili vegetacijski vršci) na kvalitetu i težinski prirast kalusa soje (*Glycine max* /L./ Merr, linije 146/82-5). Bodovne procjene kvalitete kalusa testirane su HI^2-testom (Tab. 1). Ustanovljena je različitost bodovnih procjena po harmonskim kombinacijama u odnosu na pretpostavku da razlika u kvaliteti nema. Parcijalnim testiranjem parova (Tab. 2) izdvojen je kao kvalitetniji kalus na kombinacijama auksina s kinetinom nego bez kinetina. Istosmjerna ocjena je moguća analizom vrijednosti težinskih izmjera (Tab. 3) provođenjem F-testa i t-testa (Tab. 4). Optimalna kombinacija za težinski prirast kalusa je 0,3 mgl^{-1} 2,4-D i 1,0 mgl^{-1} kinetina. Nije uočena statistički značajna razlika između kultiviranih kotiledonskih ili hipokotilskih odsječaka ili pak vegetacijskih vršaka.

Renata Pavlina, mr. sci.
Fakultet poljoprivednih znanosti
Zavod za oplemenjivanje bilja,
genetiku i metodiku istraživanja
Šimunska 25
YU-4100 Zagreb (Jugoslavija)