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EFFECT OF N-NITROSO-N-METHYLUREA ON THE BIOSYNTHETIC ACTIVITY OF *Ajuga turkestanica* CALLUS TISSUE

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The effect of N-nitroso-N-methylurea on the biosynthesis of ecdysteroids and polysaccharides has been studied. The yield of ecdysterone and water-soluble polysaccharides increases at a concentration of 8 mM.

Key words: callus tissue, ecdysterone, polysaccharides.

The content of biologically active substances in cell and tissue cultures is often lower than in the intact plant and decreases with prolonged cultivation [1, 2]. Induced mutagenesis is used to increase the production.

The use of chemical mutagens offers great promise in this area. In particular, the mutagen N-nitroso-N-methylurea (N-NMU) has the same effect on cell and tissue cultures as on the intact plant. Mutagenic activity was demonstrated on cell cultures of *Dioscorea deltoidea* Wall. [3] and tobacco [4].

The yield of ecdysterone [5] and the carbohydrate composition [6] of *Ajuga turkestanica* callus tissue have previously been reported. We studied the effect of various doses of N-NMU that were formulated for a culture of *Dioscorea deltoidea* Wall. cells on the quantitative and qualitative composition of polysaccharides and ecdysteroids in calluses of this plant. The starting culture was obtained from a plant ovary and cultivated in Murashige—Skoog medium [7].

The callus tissue was placed aseptically for 1 h in N-NMU solutions of concentrations 1, 4, 8, and 10 mM. The change of growth indicators revealed that a concentration of 1 mM stimulates growth (Table 1). By the 14-th day of cultivation after treatment, the growth index of this strain was greater than that of other varieties. Growth inhibition was noted at a concentration of 4 mM. Concentrations of 8 and 10 mM were sublethal. The tissue darkened but viable cell colonies appeared on it after 2-3 weeks. During the baseline period certain strains characteristically formed morphogenic structures, roots, especially upon treatment with 1 mM N-NMU. The calluses also had different morphologies in the subsequent periods.

Qualitative analysis in the second period showed that, like in the intact plant, the dominant metabolite in all strains is ecdysterone. Turkesterone is present as a minor component. Furthermore, iridoids, harpagide and 8-O-acetylharpagide, were found in trace quantities (Table 2).

The isolation of carbohydrates and the analysis of their composition were performed as follows. Alcohol-soluble carbohydrates (ASC) and mono- and oligosaccharides were extracted by boiling ethanol (82°). Water-soluble polysaccharides (WSPS) were extracted with water; pectinic substances (PS), by a mixture of oxalic acid (0.5%) and ammonium oxalate.

The alcohol extracts of all samples, including the control, contain fructooligosaccharides, saccharose, and traces of fructose. Mainly fructose and an insignificant quantity of glucose were found by paper chromatography after hydrolysis of the ASC. The isolated WSPS are amorphous white powders that are very soluble in water. The aqueous solutions do not give a positive reaction for starch, which indicates that starch-like glucans are absent.

The hydrolyzed WSPS were chromatographed on paper. Acidic and neutral monosaccharides of galacturonic acid, galactose, glucose, arabinose, xylose, and rhamnose were found.

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TABLE 1. Effect of N-NMU on the Growth and Morphogenesis of *A. turkestanica* Callus Tissue

N-NMU conc., mM	Baseline period (14 days)		Second period (30 days)	
	fresh wt., % of control	morphogenesis	fresh wt., % of control	morphogenesis
Control	100	+	100	+
1	113.53	++	128	+
4	71.43	-	120	-
8	39.85	-	115	+
10	31.58	-	111	-

TABLE 2. Effect of N-NMU on Formation of Ecdysteroids and Iridoids

N-NMU conc., mM	Ecdysteroids		Iridoids	
	ecdysterone, %	turkesterone	harpagide	8-O-Ac-harpagide
Control	0.01	+	-	-
1	0.02	-	+	+
4	0.01	-	-	-
8	0.2	+	+	+
10	0.01	-	-	+

TABLE 3. Effect of N-NMU on Qualitative and Quantitative Composition of Carbohydrates

Carbohydrate type	Dose	Yield, %	Monosaccharide composition					
			Gal	Glc	Ara	Xyl	Rha	GalUA
WSPS	Control	4.6	++	+	+	+	Tr.	++
WSPS	1	6.8	++	+	+	+	Tr.	++
WSPS	4	6.6	++	+	+	+	Tr.	++
WSPS	8	8.0	++	-	+	-	-	+
WSPS	10	3.2	++	Tr.	+	+	-	Tr.
PS	Control	16.4	++	+	+	Tr.	Tr.	++
PS	1	24.2	++	+	+	Tr.	Tr.	++
PS	4	8.8	++	++	++	Tr.	Tr.	++
PS	8	18.4	++	+	+	Tr.	Tr.	++
PS	10	23.2	++	+	+	Tr.	Tr.	++

Samples treated with 1 and 4 mM N-NMU and the control typically have an increased content of galacturonic acid and galactose relative to glucose, arabinose, and xylose. Trace quantities of rhamnose are present (Table 3). Callus tissue that was treated with a sublethal dose of 8 mM typically had galactose predominating over arabinose. Galactose dominated over arabinose and xylose in the sample treated with the 10-mM solution. Galcturonic acid and glucose were found in trace amounts.

Pectinic substances (PS) are amorphous white or cream-colored powders that are very soluble in water. Their aqueous solutions also do not give a positive reaction for starch. The monosaccharide composition of the PS is mainly galacturonic acid and galactose with some glucose and arabinose and trace quantities of xylose and rhamnose.

The results show that strains treated with N-NMU have qualitatively the same synthesized compounds as the control. The strain treated with the 8-mM solution produces more ecdysterone and WSPS. The PS content in all samples except for that treated with the 4-mM solution is greater than in the control.

EXPERIMENTAL

We grew a callus culture of *Ajuga turkestanica* on a nutrient medium of the following composition: mineral part according to Murashige—Skoog; inosite, 100 mg/l; thiamine·HCl, 0.4 mg/l; saccharose, 3%; agar-agar, 0.75%; α-naphthylacetic acid (NAA), 1 mg/l; and thidiazurone (defoliant Dropp), 0.002 mg/l. The culture grew under illumination of 6000-7000 lux with a 16-h lighting period at 26 ± 2°C. The mutagen N-NMU was dissolved in DMF, diluted with liquid nutrient medium, sterilized through a bacterial filter (3G5), and added aseptically to the cell mass. The culture was incubated in mutagen solution for 1 h and washed five times with an excess of fresh nutrient medium. The callus culture was innoculated into Petri dishes. Biomass was dried at 60°C before analysis (second period, 30 days). Methanol extraction was carried out twice with heating on a water bath. The combined extracts were evaporated to dryness. The solid was dissolved in methanol (1 ml). TLC was performed on silica-gel containing 7% gypsum and on Silufol plates using $CHCl_3$—CH_3OH (4:1) eluent with development by vanillin—H_2SO_4.

A semi-quantitative method is based on the use of a standard for monitoring pure medicinal substances [7]. The chromatographic sensitivity for ecdysterone and turkesterone is 0.1 μg.

Solutions were evaporated in a rotary evaporator at 40°C. Paper chromatography of sugars was performed in descending mode using 1-butanol—pyridine—water (6:4:3 by vol.) on FN-11,12 paper (Germany). The developer was aniline acid phthalate. For ketosugars, a 5% alcohol solution of urea was used. Fructooligosaccharides were hydrolyzed for 2 h by 0.5 N H_2SO_4; WSPS, 8 h by 1 N H_2SO_4; PS, 24 h by 2 N H_2SO_4 on a boiling water bath. The hydrolysates were neutralized with $BaCO_3$ and deionized by KU-2 (H^+) cation exchanger.

Alcohol Extraction. Ground samples were extracted twice by 82° ethanol (1:10) on a water bath. The alcohol extracts were evaporated. The ASC were chromatographed. Fructose, saccharose, fructooligosaccharides, and glucose were found.

WSPS Isolation. Material after alcohol extraction was dried and extracted with water (1:15, 1:10) with stirring. The extracts were combined, evaporated, and precipitated with ethanol (1:3). The solid was separated by centrifugation, washed with ethanol, and dried. The yields and monosaccharide compositions are listed in Table 3.

PS Isolation. The remaining material was extracted with a mixture of oxalic acid (5%) and ammonium oxalate (1:1) at 70°C (1:15 and 1:10). The extracts were evaporated and precipitated. The solid was washed with ethanol and dried. The data are listed in Table 3.

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