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IN VITRO STUDIES OF CALLUS GROWTH OF  
*PINUS NIGRA* ARN.1. EFFECT OF SOME ANORGANIC AND  
ORGANIC NITROGEN SOURCES

BRANKA KOLEVSKA-PLETIKAPIĆ

(Department of Botany, Faculty of Science, University of Zagreb)

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In the reaction of the callus strain K of *Pinus nigra* Arn. to the source of anorganic nitrogen, the richer with nitrogen the medium was, the better the strain grew. Hence, it grew well on media with an increased concentration of  $\text{NH}_4\text{NO}_3$ , but also when this ammonium salt was substituted by  $(\text{NH}_4)_2\text{SO}_4$  or by  $(\text{NH}_4)_2\text{HPO}_4$ . The reaction of the strain K to the source of organic amino nitrogen was a very uneven growth. Best growth results were obtained on a medium containing glutamine on which the strain's otherwise yellowish tissue even turned green. Of amino acids, only lysine and arginine had a distinctly favourable effect on the strain growth, while all other amino acids, and also adenine and hypoxantine, had almost no or very little effect.

## Introduction

For many years callus tissues of some *Pinus* species were cultivated with various success. At first undefined media, containing natural extracts of unknown composition, were used giving very limited possibilities. After that defined media of known composition were used, having synthetic substances the quantity — and thus also the inductive power — of which could be modified.

Brown and Lawrence (1968) were the first to use and modify a defined medium, namely the MS-medium (Murashige and Skoog 1962), for the culture of a permanent callus in a *Pinus* species, i. e. in *Pinus palustris*. After that exclusively such media adapted to each species were used. So the MS-medium was used also by David (1970) for the culture of the permanent callus of *Pinus pinaster*, and by

Bonga and McInnis (1975) for the culture of the haploid callus of *Pinus resinosa* (obtained from mature pollen); the medium composed by Linsmaier and Skoog (1965) was used by Durzan et al. (1976) for the callus culture of *Pinus banksiana* etc. All these authors modified the media in a similar way: they left the composition of macro- and microelements unchanged, but varied the organic composition, mainly the growth regulators and the sources of nitrogen.

In her earlier investigations the author adapted the MS-medium to the callus culture conditions of *Pinus nigra* Arn. (Kolevska - Pletikapić 1974). In the present work the effect of various sources of nitrogen (as the component of the MS-medium) on the growth of callus tissue of *Pinus nigra* has been investigated in detail, since this component seemed to be exceptionally important.

## Material and Methods

As the material for the investigation the light yellowish K strain sufficiently firm for maintainance was chosen. It was grown and subcultivated on an induction medium — the modified MS-medium (Kolevska - Pletikapić 1974) — on which its growth is relatively fast, so that the necessary quantities of the tissue could be obtained in a reasonably short time. After the 19<sup>th</sup> and the 23<sup>rd</sup> subculture, it was transferred to media in which the content and the sources of anorganic and organic nitrogen were varied in certain ways.

The culture was grown in a culture room at a temperature of 299,5 K (26,5° C) under artificial light (fluorescent tubes IPR 40 W, 220 V, 4500 K; 16 hours illumination, 8 hours darkness daily at illuminating intensities of 1250—2500 lx).

## Experiments and Results

### Sources of anorganic nitrogen

The induction medium contained 1650 mg l<sup>-1</sup> NH<sub>4</sub>NO<sub>3</sub> (the content of KNO<sub>3</sub> was not been changed). In three kinds of media the concentration of NH<sub>4</sub>NO<sub>3</sub> was increased by 1/3, 1/2 and 2/3 of the amount mentioned. In three other kinds of media NH<sub>4</sub>NO<sub>3</sub> was replaced by either (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> or NH<sub>4</sub>Cl in the same total quantity as in the induction medium. In this way 7 media were obtained to which the strain K was transferred in 10 replications:

1. MS — medium with the normal quantity of NH<sub>4</sub>NO<sub>3</sub> (= induction medium = control medium).
2. MS 1/3 — medium with 2200 mg l<sup>-1</sup> NH<sub>4</sub>NO<sub>3</sub>; other components unchanged, i. e. as in the induction medium (abbreviation: o. c. u.).
3. MS 1/2 — medium with 2475 mg l<sup>-1</sup> NH<sub>4</sub>NO<sub>3</sub>, o. c. u.
4. MS 2/3 — medium with 2750 mg l<sup>-1</sup> NH<sub>4</sub>NO<sub>3</sub>, o. c. u.
5. MS (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> — medium with 1650 mg l<sup>-1</sup> of ammonium sulfate instead of NH<sub>4</sub>NO<sub>3</sub>, o. c. u.
6. MS (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> — medium with 1650 mg l<sup>-1</sup> of ammonium phosphate instead of NH<sub>4</sub>NO<sub>3</sub>, o. c. u.
7. MS NH<sub>4</sub>Cl — medium with 1650 mg l<sup>-1</sup> of ammonium chloride instead of NH<sub>4</sub>NO<sub>3</sub>, o. c. u.

After 6 weeks of culturing the callus tissue K the growth differences were determined on each of the seven types of media. The results have shown that the changes of the concentration of ammonium nitrate as well as of the change of ammonium salt considerably influenced the growth of the callus strain K (Figs 1 and 2). The data on the increase of fresh and dry weight (Fig. 3) show that the callus grew the better the higher the concentration of  $\text{NH}_4\text{NO}_3$  in the medium. On media, in which the ammonium nitrate had been replaced by other ammonium salts, the growth was considerably different. On the MS  $(\text{NH}_4)_2\text{SO}_4$ -medium the growth rate was maximal, the average increase per explant being in fresh weight  $1028 \pm 197$  mg, and in dry weight  $28 \pm 7$  mg. On MS  $(\text{NH}_4)_2\text{HPO}_4$ -medium the K callus grew considerably more slowly but still better than on the induction medium. On MS  $\text{NH}_4\text{Cl}$ -medium the growth of the callus K was the smallest and lesser than on the induction medium. Therefore we may say that the strain K grows the better the higher the concentration of the ammonium ion in the medium.

### *Sources of organic nitrogen*

The induction medium contained  $100 \text{ mg l}^{-1}$  asparagine. In 4 types of media instead of asparagine glutamine, glutamic acid, aspartic acid and urea were taken in the same amount. In 11 other types of culture media amino acids and some purine derivatives, combined in 5 groups, were added to the complete induction medium (Table 1). These substances were taken each in concentration  $10 \text{ mg l}^{-1}$ ; at first group per group were added, that all were added and finally in the same way subtracted. So 16 types of media were obtained, to which the callus strain K was transferred. The types of media were as follows:

1. MS-medium with asparagine (=induction medium = control medium).
2. MS Glut.-medium (glutamine instead of asparagine, o. c. u.).
3. MS Glut. acid-medium (glutaminic acid instead of asparagine, o. c. u.).
4. MS Asp. acid-medium (aspartic acid instead of asparagine, o. c. u.).
5. MS urea-medium (urea instead of asparagine, o. c. u.).
6. MS (A)-medium (inductive medium with substances of group A).
7. MS (B)-medium, o.c.u.
8. MS (C)-medium, o. c. u.
9. MS (D)-medium, o. c. u.
10. MS (E)-medium, o. c. u.
11. MS (A, B, C, D, E)-medium, o. c. u.
12. MS (B, C, D, E)-medium, o. c. u.
13. MS (A, C, D, E)-medium, o. c. u.
14. MS (A, B, D, E)-medium, o. c. u.
15. MS (A, B, C, E)-medium, o. c. u.
16. MS (A, B, C, D)-medium, o. c. u.

After 6 weeks culturing the callus tissues, grown on each of the 16 types of media, were compared. The results show that the various sources

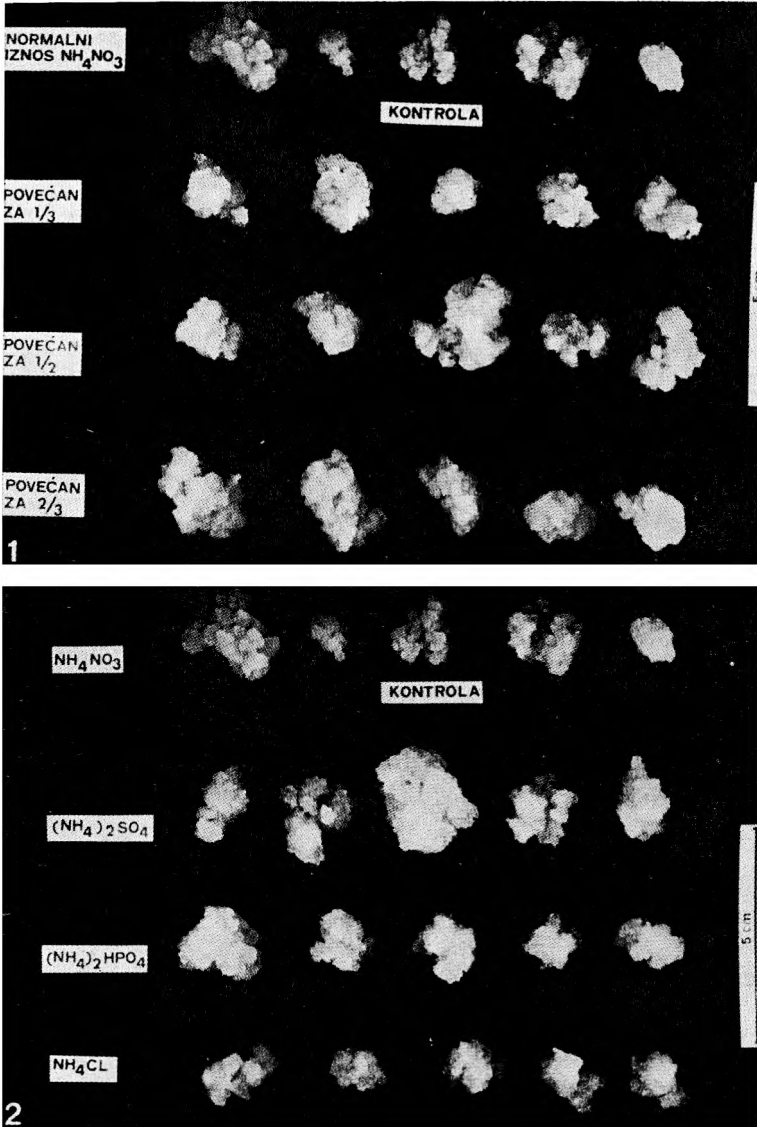
Table 1.

Group:	A	B	C	D	E
	alanine aspartic acid cysteine glutaminic acid glycine histidine isoleucine proline serine	leucine methionine phenyl- alanine threonine tyrosine valine	arginine lysine	tryptophane	adenine hypoxan- thine

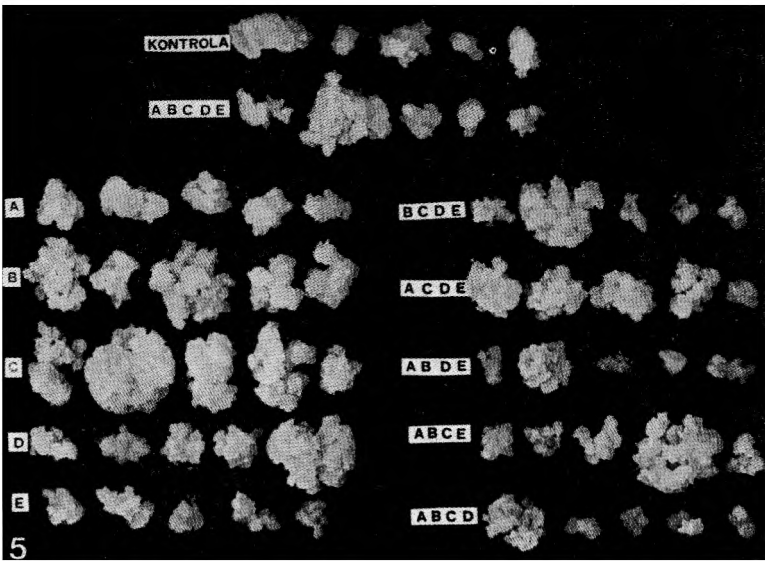
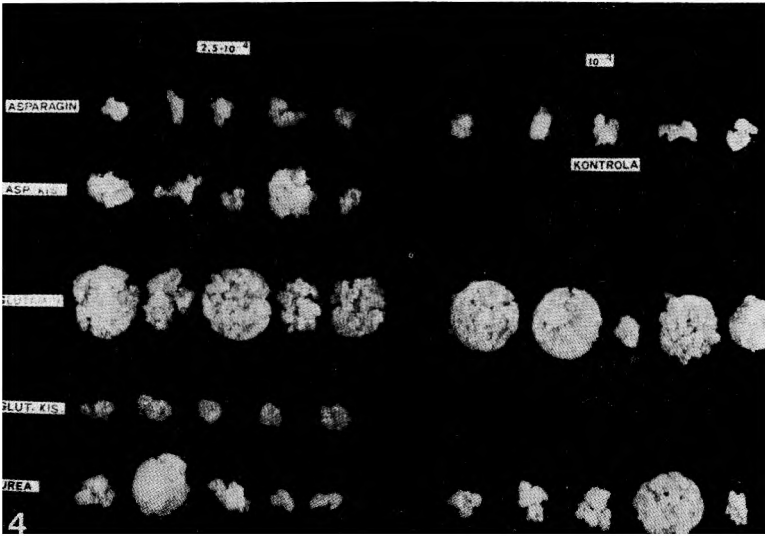
Groups of amino acids added to the medium as accessory sources of organic amino nitrogen.

- Fig. 1. Callus strain K grown on media with various concentrations of  $\text{NH}_4\text{NO}_3$ .
- Fig. 2. Callus strain K grown on media in which  $\text{NH}_4\text{NO}_3$  was substituted by other ammonium salts. These were:  $(\text{NH}_4)_2\text{SO}_4$ ,  $(\text{NH}_4)_2\text{HPO}_4$  and  $\text{NH}_4\text{Cl}$ . MS = induction medium = control.
- Fig. 3. Callus growth (average increase in fresh and dry weight) on media with increased concentrations of  $\text{NH}_4\text{NO}_3$  (by 1/3, 1/2 and 2/3 of the normal quantity), and media with other ammonium salts —  $(\text{NH}_4)_2\text{SO}_4$ ,  $(\text{NH}_4)_2\text{HPO}_4$  and  $\text{NH}_4\text{Cl}$ . Control = induction medium = MS.
- Fig. 4. Strain K grown on media in which asparagine was substituted by other sources of organic nitrogen — aspartic acid, glutamine, glutamic acid and urea. Control = induction medium = MS.
- Fig. 5. Callus strain K grown on media with amino acids and purine derivatives. The substances A, B, C, D and E were added separately and all together, and then taken away in the same way. Control = induction medium = MS.
- Fig. 6. Callus growth (average increase in fresh and dry weight) on media to which instead of asparagine, glutamine, glutamic acid, aspartic acid and urea were added.
- Fig. 7. Callus growth (average increase in fresh and dry weight) on media in which amino acid groups A, B, C and D as well as adenine and hypoxanthine (E) were added to the complete induction medium. Control = medium = MS.

EFFECT OF NITROGEN SOURCES ON *PINUS NIGRA* CALLUS GROWTH



Figs. 1—2.



Figs. 4—5.

EFFECT OF NITROGEN SOURCES ON *PINUS NIGRA* CALLUS GROWTH

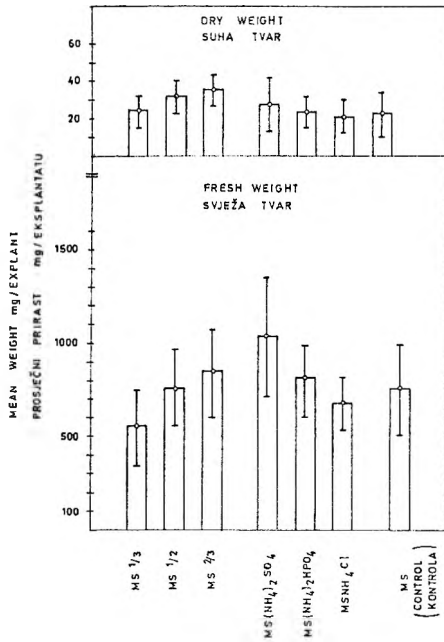


Fig. 3.

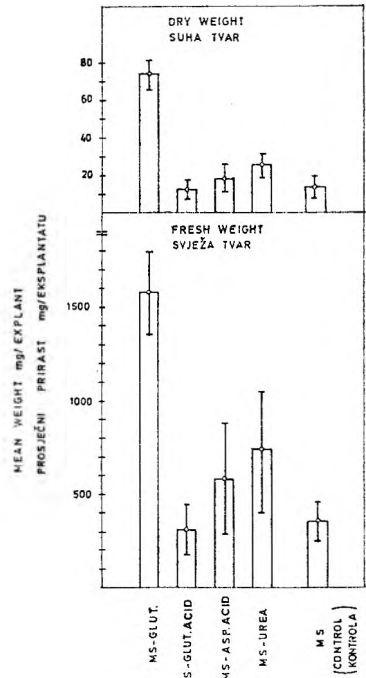


Fig. 6.

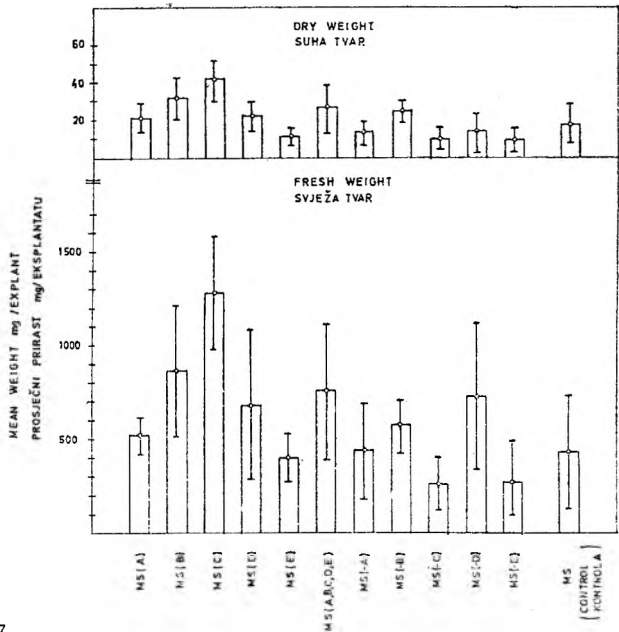


Fig. 7.

of organic nitrogen have a considerable effect on the growth of the callus strain K (Figs. 4 and 5). The average increase in fresh and dry weight (Fig. 6) shows that among the various sources of reduced organic nitrogen glutamine has the most favourable effect. The callus tissue K, grown on MS Glut.-medium shows an average increase per explants of  $1589 \pm 203$  mg in fresh, and  $57,0 \pm 10,3$  mg in dry weight per explant. In addition to the increase in fresh and dry weight glutamine still caused greening of this otherwise yellowish strain. On MS Urea-medium the strain K grows to some extent better than on the induction medium, while glutaminic acid and aspartic acid have a very unfavourable effect.

On media with amino acids and derivatives of purine the strain K grows also very inconsistently (Fig. 7). The growth was maximal on MS (C)-medium with an average increase in fresh weight of  $1286 \pm 288$  mg, and in dry weight of  $43 \pm 12,8$  mg per explant.

Therefore we may say that of all amino acids tested only arginine and lysine have a favourable effect, while all other amino acids as well as adenine and hypoxanthine show either inconsiderable or even unfavourable effects.

### Discussion

Any change in the composition of the nitrogen sources used has some influence on the growth of the callus strain K. So the higher content of  $\text{NH}_4\text{NO}_3$  favoured to some extent the growth of this strain and we may say that *Pinus nigra* has high demands of nitrogen content, just as *Pinus sylvestris* (Rogozinska 1970). However, the way in which this strain reacts to other ammonium salts differs from the results described by other authors. While the strain grows most on MS  $(\text{NH}_4)_2\text{SO}_4$ -medium in *Picea glauca* this happens on the medium with  $\text{NH}_4\text{Cl}$  (White and Gilbey 1966). These authors stated that ammonium ion reduced the meristem activity, but stimulated the differentiation of xyleme. In the case of *Pinus nigra* one could not say it because the callus strain K grows by cell divisions and their increase. In addition to that, in spite of the high content of ammonium ions in the medium the differentiation of tracheids is not induced in the callus strain K. The situation with the organic nitrogen component of the medium was similar. While the callus of *Picea glauca* grew equally on media with asparagine and glutamine, but was thoroughly inhibited on media with urea, the callus of the K strain in *Pinus nigra* grew best on the medium with glutamine, while the effect of urea on the growth was inconsiderable, but still stimulative.

The callus strain K grew most poorly on media containing glutamic and aspartic acid. Brown and Lawrence (1968) have shown by their work that the callus of *Pinus palustris* grows equally well on media with asparagin and aspartic acid. In connection with this fact it is possible to say that — as to the use of nitrogen from various sources — *Pinus nigra* Arn. reacted differently from some investigated *Pinus* species.

In contrast to this, amino acids, when added to the medium according to the system used by Winton (1970), had a similar effect to the growth of the callus strain K as described by other authors. The callus strain K showed the best growth on media with arginine (group C), similarly as did the callus of *Pinus sylvestris*



(Rogozinska 1970) and *Pinus monticola* (Harvey 1967). The reason for this is, according to Bollard (1959), that the amino-group from the guanidine part of this amino acid is very easily incorporated in to any other amino acid in the tissue. Aromatic amino acids according to the opinion of the same author, inhibit the growth by blocking many other metabolic processes in the tissue. It is probable that the callus strain K grew very badly on the medium to which, in addition to other amino acids, glycine, histidine and proline had also been added. Tryptophane is an exception, because it did not thoroughly inhibit the growth of the callus strain K. The same happened in callus of *Pinus sylvestris*, but Rogozinska (1970) considers that in higher concentrations this amino acid stimulates the growth of the callus mentioned.

### Conclusion

The callus of *Pinus nigra* Arn. — strain K — was grown on 23 media which differed by the sources of anorganic nitrogen, of reduced organic nitrogen, the content of the amino acids as well as by adenine and hypoxanthine. The results have shown that the callus strain K reacts:

1. to the composition of the origin of anorganic nitrogen the better, the higher the content of nitrogen. Therefore it grew well on media with an increased concentration of  $\text{NH}_4\text{NO}_3$ , but also when this salt was replaced by either  $(\text{NH}_4)_2\text{SO}_4$ , or  $(\text{NH}_4)_2\text{HPO}_4$ .
2. to the composition of the source of organic amino nitrogen by a very unbalanced growth. It grew best on media with glutamine, where its normally yellowish tissue became greenish. Among the amino acids only arginine and lysine had a distinct influence on the growth of this strain, while all others, including adenine and hypoxanthine were either slightly active, or not active at all.

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## SAŽETAK

IN-VITRO-ISTRAŽIVANJA RASTA KALUSA VRSTE *PINUS NIGRA* ARN.

1. DJELOVANJE ANORGANSKOG I ORGANSKOG DUŠIKA IZ RAZLIČITIH IZVORA

*Branka Kolevska-Pletikapić*

(Botanički zavod Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu)

Kalusna loza K vrste *Pinus nigra* Arn. reagirala je na sastav izvora anorganskog dušika tako da je rasla to bolje što je podloga bila bogatija dušikom. Stoga je dobro rasla na podlogama s povećanom koncentracijom  $\text{NH}_4\text{NO}_3$ , no također i onda kad je ta amonijeva sol bila zamijenjena s  $(\text{NH}_4)_2\text{SO}_4$  ili  $(\text{NH}_4)_2\text{HPO}_4$ .

Na sastav izvora organskog aminodušika ta je loza reagirala veoma neujednačenim rastom. Najbolje je rasla na podlozi s glutaminom, na kojoj je njeno, inače žučkasto, tkivo ozelenilo. Od aminokiselina samo su lizin i arginin djelovali izrazito povoljno na rast ove loze, a sve su ostale, također i adenin te hipoksantin, bile malo ili neznatno djelotvorne.

Dr. Branka Kolevska-Pletikapić  
Botanički zavod (IV)  
Prirodoslovno-matematički fakultet  
Rooseveltov trg 6/III, P.p. 933  
YU-41001 Zagreb (Jugoslavija)