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Influence of N-hydroxyurea on the growth of seedlings and the process of crown-gall tumour formation on sunflower plants

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Abstract

Hydroxyurea (HU) strongly inhibits formation of tumours induced with *Agrobacterium tumefaciens* in sunflower stems. This effect may partly be ascribed to the direct action of this substance on the bacterium. The course of the HU activity curve in the transformation process leads, however, to the supposition that it acts mainly on the host cells at the time corresponding to the induction phase. Maximal plant cell susceptibility to HU coincides with the wave of DNA synthesis induced by injury to the plant.
Under the experimental conditions the time of HU activity in the tissues of the control test plant was limited, and the effects receded during its growth and development.

INTRODUCTION

Investigations on the process of plant tumour formation under the influence of *Agrobacterium tumefaciens* have been carried on for more than 20 years. The mechanism of the phenomenon, has not, however, been definitively elucidated. In the process of normal plant cell transformation into a tumour cell, of decisive significance is, on the one hand, the DNA of the virulent bacterium, and on the other, DNA synthesis occuring in the cells of the injured host plant (Rasch, Swift, Klein, 1959; Kupila, Therman, 1971; Broekaert, Van Parijs, 1973). Therefore the factors acting on any one of these DNA's cause disturbances in the transformation process. This leads to reduction of the size of the tumours formed (Braun, 1958; Klein, 1957) or of their number (Beiderbeck, 1970; Lippincott, Heberlein, 1965) and makes possible investigation of the transformation process itself.

Hydroxyurea (HU) is a specific inhibitor of DNA synthesis. Its mode

of action differs widely from that of the known DNA inhibitors, such as MC or FUdR (Rosenkranz, Levy, 1965; Rosenkranz, Carr, 1966). Owing to its properties, HU has been utilized as anticancer agent for animals and humans (Yu, Van Scott, 1974). No data, however, been found on the effect of HU on tumour formation in plants. The present author's earlier studies demonstrated that this compound has a characteristic influence on the growth and metabolism of sunflower tumour tissue in sterile culture (Rennert, 1977).

The purpose of the present study was to investigate the influence of HU on the course of tumour formation on the stems of sunflower plants. This entailed information on the degree of noxiousness of HU for the test plants during their growth and development.

MATERIAL AND METHODS

Material. *Helianthus annuus* L. var. Borowski prążkowany seeds from the 1969 harvest and *Agrobacterium tumefaciens* (Smith a. Towns. Conn), virulent strain CCM 1937, received from the Microorganism Stock of the Czechoslovak Academy of Sciences in Brno were used. It was cultured on a potato-agar medium (Izrailski, 1962).

Germination. Sterilized ($HgCl_2$ 0.1% 10 min) equal-sized seeds were selected and placed on a layer of lignin imbibed with water or aqueous solutions of N-hydroxyurea (Schuchardt, München), in closed crystallizers. The seeds were germinated at 25°C in darkness for 12 days. The length of the growing radicles was measured on an underlying millimeter scale. The results concerning HU influence on seed germination and root growth are means from 3 series of determinations. One variant of each series comprised 120 determinations.

Growth of seedlings treated with hydroxyurea. Seedlings which germinated on HU solution for various time periods were selected according to the root length, and, in the case of the highest HU concentrations (3.8 mg/cm³, 9 and 10 days), seedlings were also eliminated which exhibited external symptoms of poisoning (darkening of root apexes). The selected seedlings in groups of twenty from each variant were transferred to the soil in the garden or to boxes with soil in the glasshouse, according to the season. Then the number of seedlings resuming growth was counted and further observations were made on them. The final height of the plants was measured after 3 months. Plants growing from seedlings germinating in water for a corresponding time or obtained from seeds sown directly into the soil were used as controls. Three series of experiments were performed. Two in the garden (May — — August, and June — September, 1972) and one in the glasshouse (February — May, 1973). The diagram gives the standard deviations.

Leaf weight determination. Seeds of equal size were sown in the garden soil (May, 1973). The successively forming pairs of young leaves were sprayed with water or HU in 3.8 mg/cm³ concentration in the amount of 0.38 mg/leaf. The leaves of each pair (1-4) were collected from the particular plant groups 7 days after spraying or at the end of the experiment. In the latter case the time of leaf collection fell to the 15th day after spraying of the last (4th) pair of leaves. Each pair without petioles was weighed on analytic scales immediately after harvesting. The number of leaf pairs examined in each combination was twenty (Table 1). Similar experiments were performed with plants treated with HU by spraying the terminal buds. More details are given in table 2, next to the results are given standard deviations.

Influence of HU on tumour formation. The plants were infected by punction of the stems with a needle immersed in a 24-h bacterial culture. As measure of the degree of inhibition served the size of the tumours weighed about 2 months after infection. Hydroxyurea was applied in two ways: (1) terminal buds of the plants were repeatedly sprayed with the solution or water at various time intervals. In this case the site of infection was at a certain distance from the site of HU application (for details see tables); (2) bands saturated with HU solution or water were placed on the stems over the sites of inoculation (above the 1st pair of leaves). The bands were applied at various time periods before and after inoculation. The time of tissue contact with HU depended on the amount of solution imbibed in the band and lasted until the latter was completely dry. This time could not be much longer than the time of action of the solution applied in the form of a single spraying of a definite surface of the plant. All bands were removed (quite dry) 24 hrs after the last treatment of the stems (6 days after plant infection). The number of plants in one experimental combination was about 30. Each combination was repeated 2-3 times. These experiments were carried out in two vegetation seasons in the years 1974 and 1976. The plants were always harvested after inflorescence formation. In all experiments a 3.8 mg/cm³ concentration of HU was used.

Influence of HU on bacterial growth. Appropriate amounts of HU were added to the potato-agar medium before its sterilization in the autoclave. The medium was poured into Petri dishes and *A. tumefaciens* was placed from 48-h cultures on them. Bacterial growth was estimated visually after 18, 24, 48, 72 and 120 hrs of culture at 26°C. Partial inhibition occurred at a HU concentration of 1.52 mg/cm³, and complete at 2.66 mg/cm³.

RESULTS

HU did not affect the process of sunflower seed germination (Fig. 1), whereas it strongly inhibited radicle growth (Fig. 2). With lapse of time

after germination the differences between the control seedlings (on water) and those treated with HU became more and more pronounced. With increase of inhibitor concentration the roots became shorter and

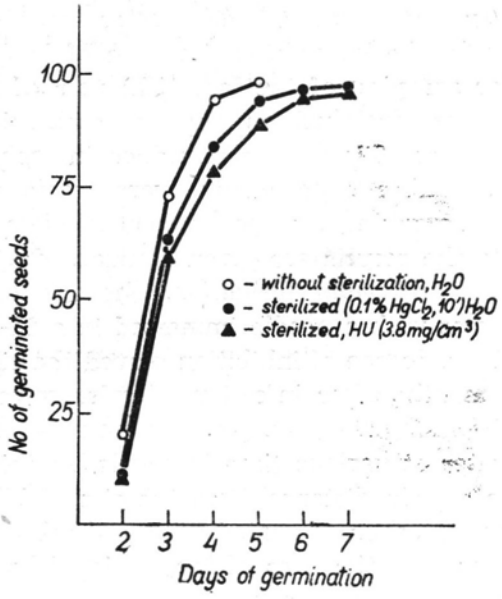

Fig. 1. Sunflower seed germination: unsterilized, sterilized and treated with HU

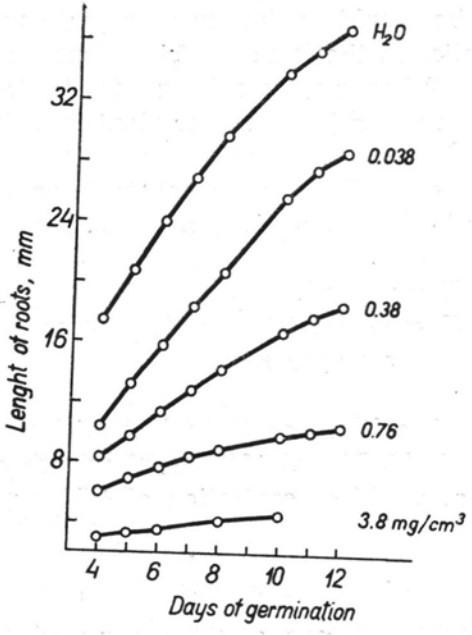

Fig. 2. Influence of various HU concentrations on root elongation in sunflower seedlings. The figures denote increasing HU concentrations in mg/cm³

formation of root hairs and lateral roots was also inhibited. At highest HU concentration (3.8 mg/cm³), 10 days after germination, the seedlings exhibited an abnormal appearance. The greatly shortened roots and hypocotyls were distinctly thicker than all the others at any phase of their development between the 3rd and 12th day, and the cotyledons usually remained hidden in the sead coat. About 30 per cent of these abnormal seedlings showed symptoms of poisoning such as necrotization of root apexes and, sometimes, their lysis.

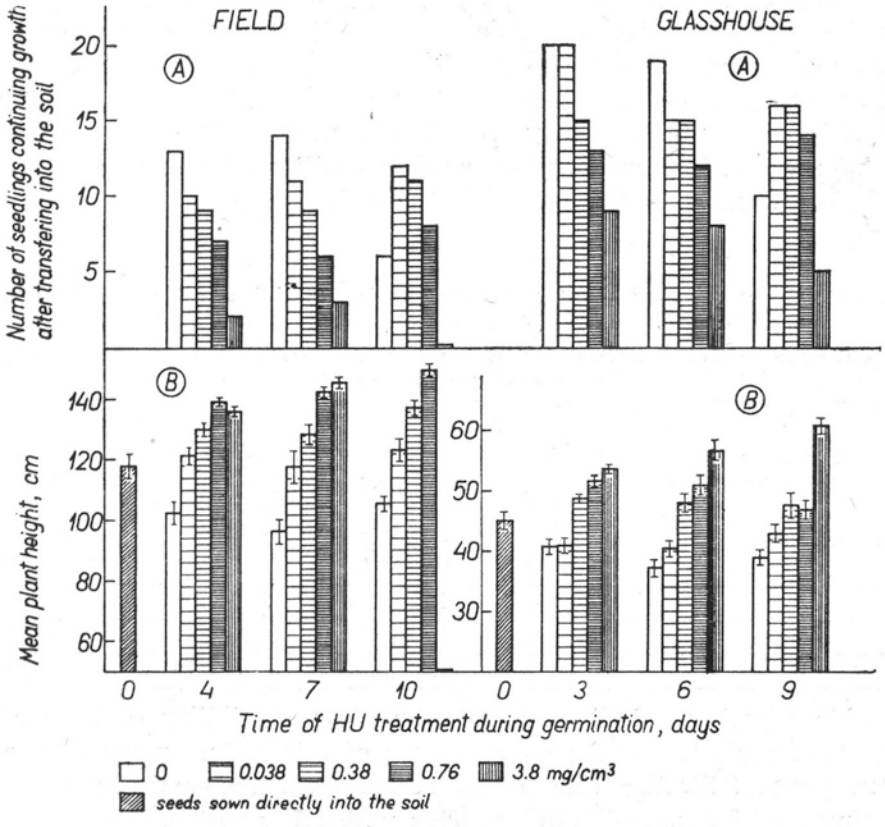

Fig. 3. Reduction of number of seedlings continuing growth after various periods of germination on HU solution (A) and height of plants which grew from these seedlings (B)

The experiments whith bacteria indicate that the effect of HU is reversible, this depends, however, on its concentration and time of action (Rosenkranz et al., 1966). It was, therefore, necessary to check (1) in how far the changes produced in sunflower seedlings by prolonged HU action are reversible and (2) whether the deformed seedlings are capable of further growth. For this purpose particular seedling groups, after selection, were transferred to the soil and observed. After 3, 4, 6 and 7 days of incubation less plants grew from the treated seedlings;

the higher the HU concentration the less numerous were the plants. After prolonged germination (9 and 10 days), however, there were more treated seedlings than controls (water). There were also more seedlings than after short treatment (Fig. 3A) only seedlings germinating for 10 days at the highest HU concentration did not grow at all. It is characteristic that plants derived from seeds germinating in HU solution grew always higher and more exuberantly than those germinating in water or directly in soil (Fig. 3B). This result is rather unexpected, but two probable explanations may be suggested: (1) too long keeping of the seedlings under nonphysiological conditions of growth (water, darkness) caused their gradual loss of viability. HU by inhibiting growth of these seedlings prevented the changes causing their loss of ability to continue growth; (2) HU played the role of an additional selection factor by damaging the weaker seedlings. These were discarded before transfer of the plants to the soil.

Table 1

Temporary decrease in fresh weight of sunflower leaves after their spraying with HU solution (0.38 mg/leaf)

Young leaves were measured between 3rd and 8th week of plant growth, mature leaves were measured in 11-week plants. Remaining details in Methods

Successive leaf pairs	Young leaves 7 days after spraying		Mature leaves at the end of experiment	
	Mean fresh weight in g/leaf pair			
	H_2O	HU	H_2O	HU
1	1.36±0.19	0.90±0.16	2.93±0.31	2.79±0.29
2	1.98±0.25	1.42±0.20	5.65±0.38	5.59±0.56
3	2.30±0.26	1.59±0.21	7.88±0.47	8.10±0.60
4	1.52±0.18	1.08±0.18	4.05±0.29	3.90±0.39

This experiment indicates the complete reversibility of HU action and full liquidation of the consequences of this action in the course of plant growth. Similar conclusions result from the experiments with treatment of leaves and terminal buds with HU solution.

A single spraying of young leaves with inhibitor solution caused a transient growth inhibition. Seven days after exposure to HU the weight of the experimental leaves in the particular pairs was about 30 per cent less than of the control ones, however, when the leaves reached maturity the difference disappeared completely (Table 1). Neither did repeated spraying with HU solution of terminal buds during their development affects the final height of the plants or the final leaf weight (Table 2).

Development of tumours on sunflower stems is strongly inhibited by HU. This occurred not only when the sites of infection were directly treated with HU, but also when the inhibitor was introduced at a site

distant from the point of inoculation. When terminal buds of plants were sprayed several times with HU solution, and the particular doses were given at short intervals, the tumours formed were much smaller than

Table 2

No effect of HU on growth of plant, leaves and tumours after spraying of terminal buds at longer time intervals

Seeds sown on May 12. After formation of first leaf pair the terminal buds were sprayed with HU (3.8 mg/cm³) 4 times during one month. The quantity of HU applied in each treatment was 0.38 mg per bud. First treatment was applied 17 and last 44 days after sowing. Stems infected on 37th day, 3 h after 3rd spraying of bud between the 2nd and 3rd leaf pair

Teatment	Number of plants	Plant age, days	Height, cm	Fresh weight of leaf pairs (g)			Tumour fresh matter
				2	3	4	
H_2O	29	58	100.4 ±7.8	6.08 ±0.49	8.93 ±0.57	5.18 ±0.30	—
HU	30	58	93.2 ±8.4	6.81 ±0.63	8.39 ±0.50	3.59 ±0.27	—
H_2O	26	93	141.8 ±10.1	—	—	—	0
H_2O+A. tumef.	28	93	128.5 ±9.3	—	—	—	4.98 ±0.52
HU+A. tumef.	31	93	136.9 ±7.5	—	—	—	4.06 ±0.63

on the controls (Table 3). Tumour inhibition was much stronger when the inhibitor was applied after infection and not before it (Table 3). Since the treatment was in both cases identical, it would seem that the process of transformation is more sensitive to HU than the bacteria. These results are also evidence that HU is translocated deeper into the

Table 3

Degree of tumour growth inhibition on sunflower stems after spraying of terminal buds with HU solution

Sowing on 7th and 12th June, measurement 98 days after seeding. Inoculation performed 40 days after seeding by punction of stem under terminal bud, above second leaf pair. Terminal buds were sprayed with HU solution (3.8 mg/cm³) or with water, in 3 portions of 0.2 ml, 4.5, 3 and 1.5 h before inoculation and 1.5, 3 and 4.5 h after inoculation of stem with bacteria

Treatment		H_2O	HU, 3×0.76 mg/bud	
			before infection	after infection
Number of plants		36	44	43
Height, cm		121,2 ±6.8	125.3 ±7.6	113.0 ±8.9
Tumor fresh matter	g	5.44 ±0.76	2.88 ±0.40	2.09 ±0.25
	%	100.0	53.0	38.5

plants and remains there for some time in unchanged form. The same way of HU application did not affect plant growth. In all the experimental combinations the plants reached the same height. Too short time intervals between the successive sprayings of young buds caused certain deformation of the leaf blades developing from the buds treated with inhibitor.

If spraying of buds was, however, extended in time (4 times monthly) no influence of HU on plants, leaves and tumour formation was noted (Table 2). This means that the time of HU activity in the plant is limited, probably owing to its degradation or transformation.

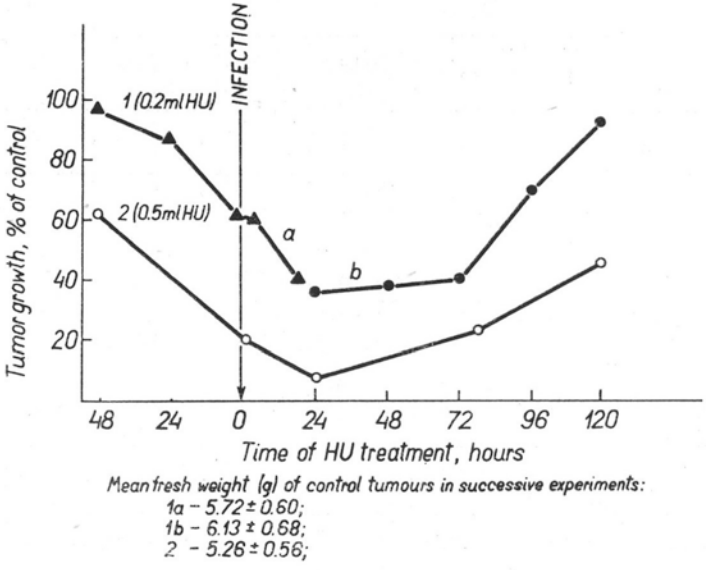

Fig. 4. Tumour growth inhibition on sunflower shoots by HU (3.8 mg/cm³) applied at various times before and after infection. The quantity of HU falling to each wound was in experiments 1a and 1b 0.76 and in exp. 2 — 1.9 mg

Tumour formation was most strongly inhibited when HU was applied at the site of inoculation. It was administered a single time in the form of a band imbibed with an appropriate quantity of the solution at various time intervals before and after inoculation. In this way an inhibition curve of characteristic shape (Fig. 4) was obtained. On it 3 distinct segments may be distinguished. The first comprising the time from 48 h before to 18 h after infection. This period is most probably connected with the direct action of HU on the bacteria. The second segment from 18 to 72 h after inoculation comprises maximal HU activity. In the third segment between 72 and 120 h after bacterium introduction HU action declines distinctly. Application of various HU amounts (Fig. 4) allowed further conclusions. The respective complete lack of effect and

only slight reduction of the tumour mass by HU when administered 48 and 24 h before infection (Fig. 4, 1a) seems to indicate that, after a single application of the inhibitor in the amount of 0.76 mg per lesion, the time of its action at the site of introduction is not much longer than 24 h. By increasing the amount of HU, complete inhibition of tumour formation may be achieved (Figs 4, 2). The most favourable moment for this treatment with a single application is the time between 18 and 24 h after inoculation of bacteria.

DISCUSSION

It was established on the basis of numerous physiological studies on crown-gall tumour formation in plants that it occurs in two phases: of inception when normal host cells are transformed into tumour cells and of stimulation or development when the transformed cells continue abnormal and autonomic proliferation. In this second phase neoplastic outgrowth develop independently of the presence of the pathogenic bacterium.

A condition for a normal course of the first phase is the occurrence of two independent phenomena — conditioning and induction. Conditioning is a process owing to which cells around the wound become susceptible to tumour induction. Induction comprises an interaction between the bacteria and the conditioned plant cells.

According to K l e i n (1957), in the course of tumour transformation in isolated discs of carrot root, the period of preparation takes 16-18 h, the period of induction additional 60-70 h, and the period of promotion, another 28-30 h. Thus for optimal transformation a total of 104-120 h is necessary. This agrees approximately the results of B r a u n (1947 1952) and L i p e t z (1965, 1966) obtained with *Vinca rosea* and *Kalanchoe daigremontiana* stems. These results indicate that there occurs between the moment of inoculation and that of initiation of plant cell transformation a lag of somewhat more than one day. Between the second and third day maximum susceptibility is reached and the process of transformation occurs mainly between the second and fourth day after inoculation and ends on the latter day.

If these data are confronted with the results of the present study, it is seen that the time at which HU inhibits most strongly tumour formation in sunflower plants is the period of induction. Since HU acts both on the host cells and those of *A. tumefaciens*, it is difficult to establish the mechanism of action of this substance. It is known, however, that HU concentrations necessary for bacterial DNA synthesis inhibition are by two orders of magnitude higher ($\geqslant 0.76$ mg/cm^3) than for Eucaryota cells ($\geqslant 7.6$ mg/cm^3). The growth of *A. tumefaciens* was

susceptible in the present study to 1.52-2.60 mg/cm³, whereas sunflower callus tissues in sterile culture were sensitive to 22.8-60.8 µg/cm³ (Rennert, 1977).

This is a clear indication that the HU amounts used in the present experiments act much stronger on plant tissues than on the bacterial cells. The same is confirmed by the characteristic course of the inhibition curve shown in Fig. 5. The HU concentration used in the experiments is sufficiently high to inactivate the bacteria, particularly in direct contact. In the plant tissues, however, it was certainly lower than the initial one, owing to dilution in the cells. Nevertheless the inhibition of tumour formation, due to the action of the HU applied simultaneously with the bacteria (direct contact), was much weaker than that elicited by HU administered 3 days after introduction of the bacteria.

It would seem, therefore, that inhibition by HU of tumour formation on the stems of sunflower plants is caused mainly by its influence on the processes occurring in plant cells. The specific action of this inhibitor allows the conclusion that the time of maximal HU activity, that is the period between 18 and 72 h after stem inoculation, is the period in which injured cells synthesize DNA responsible for tumour transformation.

Chemical and histophotophotometric analysis of plant tissue in the induction phase demonstrated that in the course of the first 3 days after inoculation DNA synthesis is activated (Klein, 1952; Klein et al., 1953, Rasch et al., 1959). This is not, however, the initial act of tumour induction, but the result of punction. It occurs, therefore, in sterile lesions as well (Kupila, Stern, 1961). It was found by using ^{3}H-thymidine that the peak of the DNA synthesis wave, induced by injury in young *Vicia faba* seedlings occurs about 19 h after the injury (Kupila, Therman, 1971). At the same time maximal HU activity starts which might be the beginning of the induction phase. DNA duplication in the conditioned cells of *Pisum sativum* was also reported by Broekaert and Van Parijs (1973). Using tritiated thymidine and the method of preparative centrifugation of DNA extracts in a CsCl density gradient, these authors demonstrated that DNA synthesized on the first and second day after wounding forms a satellite fraction rich in G-C whereas DNA of the main fraction is synthesized on the second and third day.

According to the scheme of nuclear changes during tumour transformation in plants, suggested by Guille and Quetier (1970) the conditioning step consists in the process of synthesis of specific DNA (DNA Nh) by the wounded host cells. The latter in the induction phase form a temporary complex with the bacterial DNA. Part of this complex is integrated into the plant genome in the promotion phase. In this

situation the rate of tumour growth should be proportional to the amount of DNA Nh present at the moment of action of the bacteria. If HU inhibits DNA Nh synthesis, the probability of formation of a hybrid conditioning tumour transformation would diminish proportionally to the concentration of this inhibitor.

The effect of HU on tumour transformation declined drastically 72 h after inoculation. It is at this time that the specific transformation inhibition by FUdR, started. It was applied by Bopp (1965) in investigations on *Kalanchoe daigremontiana* leaves. This fact brings out the difference between the mechanism of action of these two DNA inhibitors. As far as HU inhibits synthesis of the satellite DNA fraction, making possible the formation of the complex specific for the transformation, it is possible that FUdR prevents synthesis of the DNA necessary for completion of the process of integration of this complex into the plant genome. According to the "loop" model (Glick, Majumdar, 1972), these would be segments of the host DNA adjacent to the integrated bacterial DNA.

The here advanced conclusions concerning the action of HU on the formation of plant tumours require further experiments with the application of a more precise method for investigation of tumour transformation and an *A. tumefaciens* mutant resistant to HU.

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Wpływ hydroksymocznika na proces indukcji tumora bakteryjnego u Helianthus annus L.

Streszczenie

Łodygi młodych roślin słonecznika odmiany Borowski prążkowany infekowano wirulentnym szczepem *Agrobacterium tumefaciens*. Na łodygi w miejscach infekcji nakładano opaski nasycone roztworem N-hydroksymocznika (HU). Czas założenia tamponów był różny w różnych wariantach doświadczenia. Wielkość tu-

morów oceniano dwa miesiące po inokulacji. Równocześnie prowadzono obserwacje fizjologicznych skutków działania inhibitora na rośliny nie infekowane.

Hu nie wpływa na kiełkowanie nasion, jednak silnie hamuje wzrost korzeni siewek. Działanie to jest odwracalne, gdyż rośliny wyrosłe z traktowanych siewek nie wykazały zaburzeń wzrostu i rozwoju. Również zraszanie liści i wierzchołków pędów roztworem HU nie wpłynęło na przebieg wzrostu całych roślin, choć obserwowano okresowy spadek wagi liści, a przy częstym dozowaniu występowały objawy deformacji i odbarwienia blaszek liściowych.

HU silnie hamuje proces formowania tumorów. Efekt ten częściowo można przypisać działaniu na samą bakterię. Jednakże czas maksymalnej aktywności inhibitora w procesie transformacji tumorowej przypada na okres między 18 a 72 godziną po inokulacji. Również hamowanie wzrostu tumorów przez HU podany 3 dni po infekcji jest znacznie silniejsze (75%) od hamowania wywołanego działaniem 2 dni przed infekcją (38%). Przebieg krzywej aktywności HU w czasie trwania procesu transformacji pozwala przypuszczać, że działa on głównie w fazie indukcji, a maksymalna wrażliwość komórek roślinnych na HU zbiega się z falą syntezy DNA indukowaną zranieniem.