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RESEARCH PAPERS

Synthesis and Biological Activities of Novel Pyridazine Derivatives[1]

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Abstract—A series of pyridazine derivatives was synthesized and some of them showed a growth stimulatory activity during preliminary screening. Their effects on germination, morphogenesis, peroxidase activity and lignan content were tested on common bean (*Phaseolus vulgaris* L.) plants. 2-[4-(6-ethoxy-pyridazin-3-ylsulfanyl)-6-ethylamino-[1,3,5]triazin-2-ylsulfanyl]-acetamide (**C8**) accelerated flowering and fruit production. In these plants, total lignan amount in the leaves correlated with the corresponding peroxidase activities. This compound can be recommended against the lodging of crops. Like IAA, 2-(6-ethoxy-pyridazin-3-yl)-isothiourea hydrochloride (**C3**) stimulated adventitious root growth with necrotic transformation of the bottom stem node. It promoted growth of leaves, the early reproductive development and pod formation. Preparations **C3** and **C8** can be recommended for the shortening of the juvenile time. In plants treated with each of these compounds, the highest content of lignans was recorded possibly relating to type I "non-host"—like plants resistance. 2-(6-chloro-pyridazin-3-yl)-isothiourea hydrochloride **(C2)** exerted a stimulatory effect on the growth of vegetative organs.

Keywords: Phaseolus vulgaris, growth stimulation, pyridazine derivatives, peroxidase lignan

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INTRODUCTION

From the last half of previous century, chloro-, methoxy-, fluoroalkyl-, methylthio-substituted triazines, triazinones and a large number of triazinylsulfonylureas are widely used as herbicides in agriculture [1]. Some triazine derivatives have a practical application also as fungicides (anilazine) [1] and plants growth stimulators [2]. Pyridazine derivatives represent one of the most active classes of compounds possessing a wide spectrum of biological activity, but the arsenal of pesticides in the series of pyridazine and pyridazone derivatives is less extensive in comparison with triazines. A few number of substances with herbicidal [1, 3] and fungicidal [4] activities can be mentioned.

In this regard, the significant practical interest presents the systems, in which the indicated heterocycles are combined in the same molecule. In the literature practically there are no data on the synthesis of such compounds. From this point of view, it is expedient to synthesize new systems with the combination of pyridazine and triazine cycles in the same molecule to search for new biological properties and physiologically active substances in the previously unexplored series of organic compounds.

Along with growth processes, it was necessary to characterize the biological activities of newly synthesized compounds by their action on peroxidase activity and content of lignans, in the synthesis of which peroxidase participates. At present, a general interest is attracted to peroxidase which is widespread in the plant kingdom and participates as individual peroxidase isoenzymes in tissue differentiation, lignin, IAA functioning, growth and protection against oxidative processes in plant cells [5–9]. Peroxidase is a multifunctional enzyme involved into many processes of plant life and highly sensitive to external influences [10].

Peroxidases may differ in specificities toward monolignols such as sinapyl alcohol or coniferyl alcohol [11]. Dehydrogenation of monolignols might be both via direct interaction with an electron-removing enzyme, and both by radical transfer. As a radical shuttle for lignification in grasses the *p*-coumarate moiety in sinapyl *p*-coumarate has been proposed is an excellent substrate for peroxidases, but the *p*-coumarate radical efficiently transfers its unpaired electron to sinapyl alcohol and, presumably, to lignin polymers

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Abbreviations: **C2**—2-(6-chloro-pyridazin-3-yl)-isothiourea hydrochloride; **C3**—2-(6-ethoxy-pyridazin-3-yl)-isothiourea hydrochloride; **C8**—2-[4-(6-ethoxy-pyridazin-3-ylsulfanyl)-6-ethylamino- [1, 3, 5]triazin-2-ylsulfanyl]-acetamide.

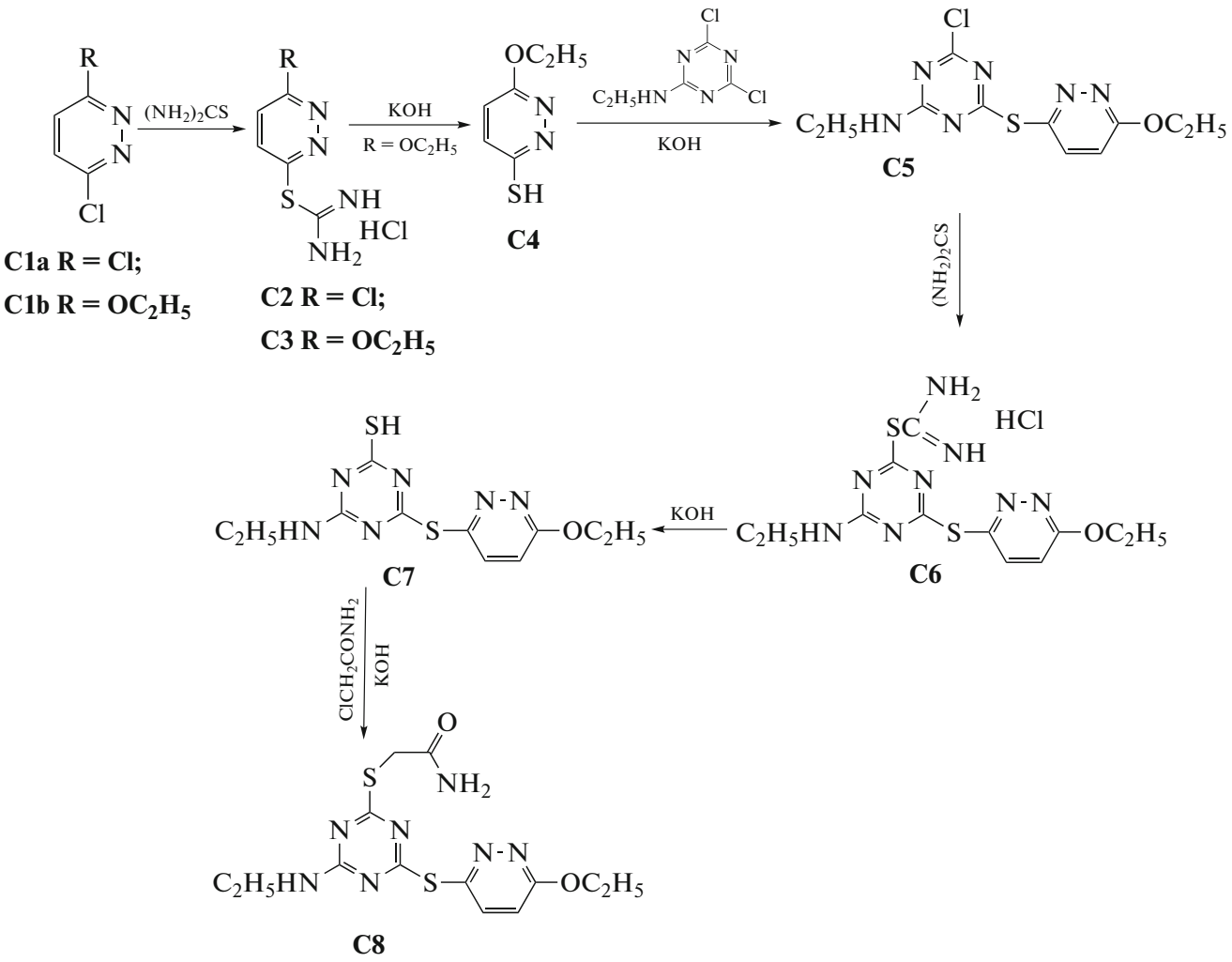

Fig. 1. Synthesis and specification of pyridazine derivatives.

[12]. One-electron oxidation of coniferyl alcohol monomers with peroxidase followed by free radical resonance distribution, leads to oxidative phenol coupling products such as lignans [13].

The aim of the present research was to study the influence of three most active preparations from the synthesized series of pyridazine derivatives: 2-(6-chloro-pyridazin-3-yl)-isothiourea hydrochloride (**C2**), 2-(6-ethoxy-pyridazin-3-yl)-isothiourea hydrochloride (**C3**) and 2-[4-(6-ethoxy-pyridazin-3-ylsulfanyl)-6-ethylamino- [1, 3, 5]triazin-2-ylsulfanyl]-acetamide (**C8**) (called **C2, C3** and **C8** hereafter, correspondingly) [14, 15] on germination, morphogenesis, peroxidase activity of leaves and lignan content in *Phaseolus vulgaris*.

MATERIALS AND METHODS

Chemical synthesis. By boiling of 3.6-dichloropyridazine (**C1a**) or 3-chloro-6-ethoxy-pyridazine (**C1b**) with thiourea in anhydrous acetone in the presence of catalytic amounts of hydrochloric acid the thiouronium salts (**C2,** C**3**) were obtained [14]. From **C3** in an alkaline medium 6-ethoxy-pyridazine-3-thiol (**C4**) was obtained, which was transformed into the corresponding potassium salt. At interaction of this salt with 2,4-dichloro-6-ethylamino-[1, 3, 5]triazine the triazinyl thiopyridazin (**C5**) was formed, in which molecule triazine and pyridazine cycles are linked through a sulfur atom. By functionalization of this compound the corresponding thiouronium salt **6** and thiol **7** were obtained. The reaction of the latter with chloroacetamide in dimethylformamide medium afforded amide **C8** [15] (Fig. 1). The structure and purity of synthesized compounds were proved, using NMR and TLC methods. Compounds selected for biological studies were **C2**, **C3** and **C8**.

Plant Material. Seeds of common bean (*Phaseolus vulgaris* L.) were obtained from Syunik region of Armenia.

Growth regulatory activity. At a preliminary screening, the herbicidal, fungicidal and growth regulatory activities of synthesized compounds **C2, C3** and **C8** were studied. All preparations did not exert noticeable herbicidal or antifungal properties, but they showed high growth stimulatory activity and were selected for deeper study of their influence on germination, morphogenesis, peroxidase activity and lignan content in the common bean plants.

Experiments were performed according two schemes. By the first scheme, the effects of aqueous solutions of **C2**, **C3,** IAA (for comparison) and aqueous suspension (in the presence of emulgator OP7) of **C8** at the concentrations of 25 and 50 mg/L on the seed germination, morphogenesis, seedling growth, and peroxidase activity were studied. There were 20 seeds for each experiment 5 replicates. Seeds were incubated for 24 h in the dark at 25°C. Then the seeds were transplanted into soil and watered daily.

According to the second experimental scheme, bean seeds were sown in soil in small vessels. The soil was a universal mixture of peat (20%), fertile soil (70%) and the sand (10%). When the length of the stems reached 15–20 cm (10 days), plants were dug out. After 24 h, they were washed and dipped into the vessels with water. Roots were cut off. Series of 8–10 cuttings were immersed in prepared aqueous solutions of IAA, **C2**, and **C3** at 25 mg/L and aqueous suspension of **C8** at 50 mg/L. All solutions were prepared just before usage. Water in the vessels was changed every day. Formation of root system had been observed on 8–9 day. The average number of roots, their length, and their fresh and dry masses of each series of plants were determined in 20–25 days. The results were compared with those of plants placed in IAA solutions, the activities of preparations were determined as percent of water control.

In both schemes, the dynamics of morphological and biochemical parameters was monitored within a month.

Field trials. The common bean seeds were treated with clean water, solutions of tested substances and IAA at the concentrations of 25 mg/L and 50 mg/L for 24 h, and then every group of seeds was planted out on total experimental plot of 30 m^2 at a distance of 20–25 cm from each other. Irrigation was done every 3 days. In 20 days, the number of shoots in each series was calculated. After two months, during the next 30 days week by week the yield of green pods was recorded and the masses were determined for each series. The overall yield and the average yield per plant were determined at the end of collection for each series. As the control, seeds treated with clean water were used.

Lignan determination. The sampling for HPLC analysis was from the leaf suspension. Dry material of leaf after lyophilization (0.2 g) was ground with a mortar and pestle, mixed with 2 mL 96% ethanol and homogenized twice for 30 s using a high-velocity MPW-302 homogenizer (Mechanika Prezycyljna, Poland) at constant cooling. Distilled water (6 mL) was added and pH of suspension was brought to 5.4 by 0.6% o-phosphoric acid. Then 0.1 mg of β-glucosidase (Sigma, United States) in 0.1 mL was added, the mixture was incubated for 1 h in a water bath at 35°C. To promote lignin solubilization, 12 mL of 96% ethanol was added, and the mixture was incubated at 70°C for 10 min. The suspension was centrifuged at 12000 *g* for 15 min, and the supernatant was stored at –20°C. An HPLC-Termo Quest analyzer (Germany) equipped with a Spherisorb ODS-2 column (Sigma, United States) was used to determine the relative content of podophyllotoxins (Ptox, 5-mPtox, dePtox), α- and β-peltatins [16] by using the following conditions of gradient elution: 0.1 mL/L 90% o-phosphoric acid (A) and methanol- acetonitryl (B) (1 : 1); with 5% (B) for 4 min and linear gradient up to 100% (B) for 40 min. The elution rate was 1 mL/min, and the sample volume was of 25 mL. Commercially available preparations from Roth (Germany) were used as references. All peaks with the retention times corresponding to podophyllotoxins and peltatins, were automatically subjected to spectral analysis by using a special program. The concentrations of lignans were determined at 290 nm using the formula [16] $C = S \times 350\varepsilon^{-1}$ where S is the peak area, and ε is the extinction coefficient equal to 29000.

Determination of peroxidase activity. Peroxidase activity in leaves and stems was determined by measuring optical density changes at 420 nm with a Specord M400 spectrophotometer (Germany) and was calculated as described in [17]. As a substrate, pyrogallol was used. The enzyme mixture contained 0.8 mL of buffer, 1.1 mL distilled water, 0.15 mL of 0.5% H_2O_2, 0.5 mL of 2 mM pyrogallol. Reaction was initiated by the addition of 0.12 mL of the supernatant. As a unit of peroxidase activity, formation of 1 mg purpurgallin from pyrogallol in 20 s was taken. Peroxidase activity was calculated using the formula:

$$A = \Delta DV \times 10^6/[t]d\varepsilon,$$

where A—enzyme activity in pkat/g protein, ΔD—optical density changes, V—reaction mixture volume in mL, t—time in s, d—cuvette thickness in cm, ε—extinction in mM/cm. Protein content was assayed by [18].

Statistical analysis was carried out with standard statistical methods. Data are expressed as mean (± standard deviation) of at least five determinations. Changes in variables were analyzed by a one-way ANOVA for multiple comparisons. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The pesticidal and growth regulatory activities of compounds **C2, C3** and **C8** were studied. At preliminary laboratory screening all preparations did not pos-

Table 1. Effect of pre-treatment on germination and biomass of pods in the field trials (mean ± SD of five independent experiments, $p < 0.05$)

Treatment	Germination of seeds on day 7, %	Pods, g/plant	Yield /plant after 2 months, %
Control (water)	95 ± 5.0	46.9 ± 1.2	100 ± 2.0
IAA (25 mg/L)	94 ± 5.0	54.5 ± 2.1	116 ± 2.0
IAA (50 mg/L)	98 ± 3.0	72.2 ± 2.0	153 ± 2.3
C2 (25 mg/L)	91 ± 4.0	68.9 ± 2.3	147 ± 2.2
C2 (50 mg/L)	88 ± 3.5	64.1 ± 2.2	137 ± 2.1
C3 (25 mg/L)	91 ± 4.5	65.6 ± 2.5	140 ± 2.2
C3 (50 mg/L)	89 ± 4.3	62.4 ± 2.1	133 ± 2.1
C8 (25 mg/L)	91 ± 4.8	55.2 ± 2.3	118 ± 2.0
C8 (50 mg/L)	92 ± 4.6	69.8 ± 2.0	149 ± 2.1

sess any herbicidal and fungicidal properties, but the preparations **C2, C3** and **C8** showed a high growth stimulatory activity on common bean plants.

In the first scheme, no significant changes caused by the preparations in germination percentage were observed (Table 1) as compared to the control group (water). These compounds induced significant morphological changes of shoot organs after leaf formation and shortening of juvenile period, the acceleration of development of the reproductive organs (Fig. 2). After seed incubation with **C2**, the thickening of the stems was observed, which did not occur during the incubation with IAA or preparation **C8**. Compound **C2** reduced the growth of above-ground vegetative organs as compared with the control. After 7 days of germination the tested compounds can be ranged according the decrease of their influence on seedling development in the following way: **C2** (25 mg/L) > **C2** (50 mg/L) > IAA (25 mg/L) > IAA (50 mg/L) = **C8** (50 mg/L) > **C8** (25 mg/L). After 20 days, from seeds treated with C**8** preparation, shortening of the juvenile time and formation of flowers and fruits were observed, whereas after seed treatment with **C2** and IAA these processes were not accelerated (Fig. 2). Under influence of **C8**, common bean stems were shortened and thickened that is similar to ethylene action [19].

The results of the second scheme experiments showed that on the 7th day the marked changes in plants morphogenesis, juvenile development, acceleration of reproductive development were observed. IAA and preparation **C2** stimulated the adventitious root growth, but led stem bottom node and necrotic formation. Leaf growth was more pronounced at low concentrations, and experiments on bean cuttings were prolonged at 25 mg/L concentration (data non shown). Under the influence of **C3**, early reproductive developments and shortening of juvenile time were observed, whereas in control treatment reproductive organs did not develop even after 20 or more days. Actions of preparation **C3** and ethylene are very similar [20].

Under the influence of IAA and **C2** for 10 days, the flowers were formed while in the presence of **C3** pods were formed at this time. **C2** produced the highest leaf number in all treatments. It is known that plant hormones including IAA are involved in regulation of lectin synthesis in root meristems of common bean plants [21]. The action of tested preparations on the morphogenesis may be mediated by lectins.

Table 1 shows the results of pre-treatment effects of the tested compounds on common bean biomass of pods in the field trials. Under the influence of all compounds, an increase of the total yield and the average pod weight per plant was obtained. The highest pod yields were collected from the plants treated with IAA, then **C8** (50 mg/L) and **C3** (25 mg/L).

Peroxidase activity was determined in the leaves of common beans on 7 and 21 days after seed pre-incubation in the presence of tested growth regulators (Table 2). It was shown that the enzyme activity was much higher in the leaves than in the stems (data not shown). Depending on the treatments, there was no significant deviation of peroxidase activity in the stems or the leaves. After 21-day, the highest activity was observed in the samples previously incubated with **C3** and IAA, as compared to treatments **C2** and **C8,** but all were more active than in control (Table 2). In the second experimental scheme, the peroxidase activity greatly increased after 24-h treatment with abovementioned preparations. The highest activity of peroxidase (four times) compared to the control was observed in the presence of **C3**, which also was two times higher than enzyme activity in the presence of IAA. The enzyme activity increased only twice under influence of **C2** as compared to control.

After a week, there was a decline of the total peroxidase activity (Table 2). In long experiments with cutted plants in the second experimental scheme, the peroxidase activity of leaves, pre-treated with **C3** decreased

Fig. 2. Seedling growth of bean seeds by the first scheme, pre-incubated in the presence of preparations **C8** (50 mg/L), **C2** (25 mg/L) and IAA (25 mg/L) after 20 days germination (left to right). The frame is an enlarged view of the pod.

more than five times while in other cases significant decline was not observed (Fig 3). A possible reason for high peroxidase activity in the first 24 h after cutting of roots is the production of reactive oxygen species and oxidative stress stimulated by wounding of the stem, and this in turn can induce "wound lignin". Our results agree with the findings that wounding of plants and overproduction of IAA lead to the elevation of peroxidase activity leaves and hence also to lignin deposition [22]. Lignin is the generic term for a large group of aromatic polymers resulting from the oxidative combinatorial coupling of 4-hydroxyphenylpropanoids [23]. The main building blocks of lignin are monolignols. In the first step, the monolignol phenol is oxidized by peroxidases and/or laccases. The peroxidative cycle is predominant in creating monolignol radicals for lignin polymerization and uses H_2O_2 for oxidative power [12].

Table 2. Peroxidase activity in common bean leaves after incubation in water (control), IAA (25mg/L), **C2** (25 mg/L), **C3** (25 mg/L) and **C8** (50 mg/L) according to the first and second schemes

Treatment	Peroxidase activity, pkat/g protein		
	II-d scheme 24 hours	I-st scheme on day 7	I-st scheme on day 21
Control (water)	1.20 ± 0.22	0.50 ± 0.03	0.55 ± 0.02
IAA (25 mg/L)	2.12 ± 0.30	0.48 ± 0.04	2.60 ± 0.50
C2 (25 mg/L)	2.64 ± 0.45	0.47 ± 0.05	0.82 ± 0.20
C3 (25 mg/L)	4.52 ± 0.60	0.58 ± 0.05	2.80 ± 0.50
C8 (50 mg/L)	1.95 ± 0.43	0.52 ± 0.05	1.81 ± 0.35

Because the structure of lignin depends on the availability of monolignol radicals, peroxidase specificity may determine in part the final lignin polymer structure, opening possibilities for altering lignin structure by modified expression of specific peroxidase isoforms [11]. We revealed some changes in the composition and electrophoretic mobility of peroxidase isoenzymes, which suggest that it associated with changes in the biosynthetic capacity (data not shown). On the other hand, in the control samples and plants treated with IAA and **C2** and grown by the second scheme, in the lower parts of the stems necrotic areas were observed, but not in the plants treated with **C3**. Elicitors are usually capable to induce various modes of plant defense including the hypersensitive response and the production of phytoalexins, i.e. antimicrobial secondary compounds [24–26]. We assume that at treating with **C3** the synthesis of lignans may be also activated. It can be also assumed that **C3** and **C8** compounds may have antinecrotic action. We determined the amount of total lignans in the common bean leaves after different treatments and revealed a correlative relationship when compared with the corresponding peroxidase activity (Fig. 3). Indeed, under influence of **C3** and **C8** the highest content of lignans was

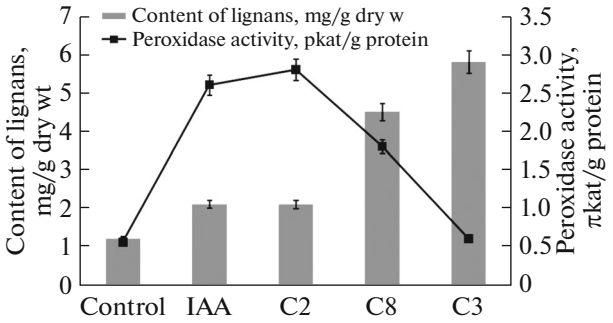

Fig. 3. Content of total lignans and peroxidase activity in common bean leaves in different conditions after 20 days of experiment: in water (control), IAA (25 mg/L), **C2** (25 mg/L), **C3** (25 mg/L), **C8** (50 mg/L).

observed while under influence of IAA and **C2** their amount is only slightly greater than in the control.

Along with the described actions of these preparations on bean plants, a type I "non-host"-like resistance, [27] (non-visible symptoms) was revealed. The same plants produced visible symptoms (such as necrosis) after treatment with IAA or **C2** have shown the "non-host"-like resistance type II [28, 29]. But we believe that the cause of the observed necrotic transformation under the influence of IAA and **C2** (second scheme of experiment) is an induction of ethylene synthesis, increased peroxidase activity and hence also lignin synthesis [22] that inhibit the cellulose synthesis which in turn can lead to necrotic transformation of cells [30].

Thus, the amount of total lignans in the leaves of plants after the described treatments correlated with the corresponding peroxidase activity. The results of experiments carried out according the second scheme showed that IAA and **C3** stimulated adventitious root growth, but led to transformation of stem bottom and necrotic formation, and **C3** promotes the growth of leaves. In plants treated with **C3** and C**8**, there was the highest content of lignin, perhaps these plants revealed a type I "non-host"-like resistance. Peroxidase activity increase can be very importance for the plant defense against stressors. Our preparations will be tested also for this purpose. Based on these results it can be concluded that for the shortening of juvenile period and activation of the reproductive development the preparations **C8** (50 mg/L) and **C3** (25 mg/L) can be recommended. For vegetative organs stimulation, **C2** (25 mg/L) can be used, and for antilodging of crops **C8** (50 mg/L) can be recommended.

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