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Factors affecting the processes of penetration of protectants into grain of seeds and increasing the yield of crops

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ABSTRACT

In our previous studies, were prepared by mechanical treatment and tested several formulations of plant protection products based on tebuconazole (TBC) with different delivery systems. As a result of these studies, polysaccharides showed high efficiency in increasing the solubility and the effectiveness of products based on these polymers. An important task in developing an effective seed treatment is to increase the efficiency of adhesion and penetration. However, the question arises, which factor is more important for plant protection or which factor plays the main role in the activity of the protectants - the amount of dressing agent on the surface of the seed or the amount penetrated into the grain? This question remained unanswered in previous experiments and the purpose of this study is to find an answer to this question. For this purpose, protectants of various compositions based on TBC and polysaccharides were prepared and spring wheat seeds were treated with them. At the same time, these seeds were divided into two variants - seeds treated with protectant (TBC on the surface and which managed to penetrate inside the seeds) and seeds whose surface was washed by water and so seeds were without sorbed tebuconazole (TBC only inside the seeds). So, seeds were prepared that contained only the adsorbed tebuconazole (SPrA) and seeds containing the drug that penetrated into the grain (SPrP). These two types of seeds were used in biological tests and the results obtained were compared. An analysis of seed germination and infection with the main pathogens showed that SPrA seeds had an advantage over SPrP seeds both in germination and in protection from diseases. Further research in this direction will help to understand the effect of the penetration of drugs into plants on the possibility of increasing their efficiency and the yield of grain crops.

Keywords: Tebuconazole, Polysaccharides, mechanical treatment, seed dressing, penetration, biological efficacy.

INTRODUCTION

Modern agriculture cannot be imagined without pesticides to protect plants from diseases and pests. At the same time, the chemical pesticides cause irreparable harm to the environment. One of the prospective approaches for their using are protectants for plant seed dressing. A protective coating could also contain essential nutrients for germinating plants. It allow crops to grow even in unproductive soils (Sharma *et al.,* 2015). Another approach, which could improve the effectiveness of protectants, is the use of nanotechnologies, in particular, nanocarriers based on different delivery systems (Pereira *et al.,* 2021). This approach is actively used in the medical chemistry, but the application of nanotechnology in agricultural sector is relatively new and in the early stages of development (Yadav and Yadav, 2016). Currently, scientists develop a new area of nanotechnology, which could revolutionize agriculture – nanopesticides: nanosized controlled release systems. According to recent studies, nanopesticides have a wide range of benefits, including increased stability and sustainability of yields of crops to pathogens, pests and weeds (An *et al.*, 2022).

The seed dressing is one of the targeted and cost-effective measures to protect plants from diseases and pests. In the process of treatment of pesticides are applied to the seeds to destroy not only external, but also internal infections of plant origin, protect both seeds and seedlings in the field from soil-dwelling phytopathogens and various pests (Vlasenko *et al.,* 2020). The environmental friendliness of this method of using chemical preparations is explained by the fact that fungicides are applied only where they are really needed. In all developed countries of the world, dressing is considered as a legally mandatory phytosanitary measure (Afzal *et al.*, 2020).

In our opinion, two important factors will affect the effectiveness of the protectants for seeds dressing. It is the adhesion of the drug to the surface of the seeds and the degree of its penetration into the seeds through plant membranes. Unfortunately, there is no data on the differentiation of these two factors on the biological activity of protectants. The important task in the development of effective product for seed dressing is the increase of the adhesion and penetration efficacy (Sharma *et al.,* 2015). In the present study, an attempt was made to determine the contribution of each of the factors: improved adhesion of the active substance to the surface, improved penetration of the active substance into the seed. Understanding the significance of each of these factors is a big step towards the rational development and use of plant protection products.

In the previous studies we have tested several compositions of plant protection products with water soluble delivery systems, polysaccharide arabinogalactan (AG) and saponin glycyrrhizin (GA) prepared by innovative "green" mechanochemical technology. As a result of these studies, both AG and GA have shown high efficiency in increase of solubility and permeability of some lipophilic fungicides (tebuconazole, imazalil, prochloraz) (Khalikov *et al.,* 2015; 2023). Previously, when studying the processes of fungicides penetration through cell membranes, which mainly consist of phospholipids forming bilayer structure, it was noted that any changes in its structure and dynamics could significantly affect the properties and functions of the cell membrane and associated proteins (Matsuoka *et al.*, 2015).

The properties of delivery systems based on glycyrrhizic acid (GA) were already studied in the models significant to medicinal chemistry (Selyutina *et al.,* 2017-a). Due to its amphiphilic properties, GA is capable to form micelles in water solution and "host-guest" complexes with hydrophobic molecule of tebuconazole (Vlasenko *et al.,* 2019). Given the physicochemical characteristics of GA and its disodium salt (Na_2GA), which forms solutions with a lower viscosity in contrast to GA, a synergistic effect could be expected from the use of Na_2GA as a drug delivery system (Selyutina *et al.,* 2020).

It was found that GA is able to interact with the cell membrane, affecting its properties, such as elasticity and permeability. GA penetrates into phospholipid bilayer and forms self-associates inside it, which lead also to changes in lipids mobility (Selyutina *et al.*, 2016).

Arabinogalactan is a natural polysaccharide consisting of arabinose and galactose fragments (Chauhan *et al.*, 2018) and demonstrates strong binding with different lipophilic biologically active molecules which results in a significant increase in their solubility (Selyutina *et al.,* 2020). In contrast to GA, AG is located on the surface of the lipid bilayer and do not penetrate inside it. However, AG is also able to affect lipid mobility and enhance membrane permeability (Selyutina *et al.,* 2017). Therefore, we tried to use these promising properties of GA and AG in the development of the nanopesticides (Khalikov *et al.,* 2015; 2023). An increase in the penetration of pesticides into seeds in the presence of both delivery vehicles was detected, but the greatest effect is observed in the presence of AG. Obtained results show that the effect of polysaccharides and oligosaccharides on the penetration efficacy of nanopesticides under the presowing seed treatment might be associated with the solubility enhancement and affinity of delivery systems to the surface of seeds, as well as due to modification of cell membranes by poly- and oligosaccharides (Selyutina *et al.,* 2017-b). However, it is still unclear, which factor is determinant in the increase of nanopesticide efficiency: solubility enchancement, improved adhesion to the seed surface or a better penetration of the pesticide into the seed.

In order to provide physicochemical and biological studies, the authors (Meteleva *et al.*, 2018) obtained solid dispersions (SD) of TBC with AG, GA, Na_2GA and licorice extract (LE), as substances that form supramolecular systems when the corresponding SD is dissolved in water. According to the results of these studies, it was found that the obtained SDs form supramolecular systems, which allow the transport of TBC through artificial and plant membranes. An attempt was made to establish the correlation "biological activity of the protectants- the degree of penetration of TBC". The expected result of biological activity was the sum of TBC on the surface of the seed and the drug that penetrated inside.

A continuation of these studies is the work (Vlasenko *et al.*, 2019) on reducing the TBC norm by obtaining its SDs with GA, Na_2GA, and LE. In this study, the consumption rates of TBC were reduced by 1.7-4.5 times, while maintaining biological efficiency. In field experiments, during pre-sowing seed treatment, wheat growth and development increased, its standing density, productive bushiness and yield increased by 0.52–0.69 t / ha. In this work, the biological effect was also the sum of the action of TBC on the surface of the treated seeds and TBC that penetrated into the seed (Selyutina *et al.*, 2020).

In order to differentiate the biological activity caused by these two sources (on the seed and inside the seed), in the present work we have compared the biological effect of so-called total amount of the protectants (on the surface of the seed plus inside the seed) and the activity of only part penetrated into the seed of wheat. To perform this work, protectants based on TBC and plant metabolites (AG, GA, Na_2GA, and LE) were obtained by mechanochemistry at their mass ratio of 1:9.

MATERIALS AND METHODS

Materials

Tebuconazole (TBC) – (RS)-1p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-yl-methyl) pentane-3-il is a systemic fungicide for the treatment of grain seeds in the fight against phytopathogens transmitted with seeds, such as *Helminthosporium* spp., *Fusarium* spp., *Bipolaris* *sorokiniana*, *Penicillum* spp. It is also used for the treatment of vegetative plants of rapeseed and cereals as an integral component of combined preparations. Solubility (25 ^{0}C) in water is 32.0 mg/l (Paranjape *et al.*, 2014). Production of Shanshen Sunrising Industry Co., Ltd. (China), content of active ingredient ≥ 97.84 %, powder.

Arabinogalactan (AG) is a polysaccharide consisting of arabinose and galactose units with molecular weight 15 kDa (97 %, Ametis JSC, Russia). It is derived from Siberian larch *Larix sibirica* and differs from AG from other suppliers (Khalikov *et al.*, 2015).

Glycyrrhizic acid (GA) – triterpene glycoside from licorice root extract. Its chemical name is 20 β-Carboxy-11-oxo-30-norolean-12-en-3β-yl-2-O-β-D-glucopyranuronosyl-alpha-D-glucopyranosiduronic acid (Selyutina et al., 2016). Production of Shaanxi Pioneer Biotech Co., Ltd, China; content of active ingredient ≥ 98.14 %.

Disodium salt of glycyrrhizic acid (Na_2GA) (Selyutina et al., 2014). Production of Shaanxi Pioneer Biotech Co., Ltd, China- content of active ingredient ≥ 91.14 %.

Licorice extract (LE) is a dry fine powder of light to dark brown color with a content of 25 % GA. Production of “Visterra", Altai Territory, Russia.

Methods

Mechanochemical method for obtaining of tebuconazole composition

Dressing agents were obtained in the form of solid dispersions (SD) from TBC and polysaccharides (mass ratio of 1:9) by their joint mechanical processing in a roller mill LE-101. These SDs are finely dispersed powders that have increased solubility in water and form supramolecular complexes in water with high biological efficiency (Khalikov, 2021).

Analysis of solubility of tebuconazole composition

The solubility of TBC in various compositions was determined by HPLC on an Agilent 1100 liquid chromatograph (Germany) on a Hypersil HyPURITY Elite C18 analytical column (150×4.6 mm, 5 microns) in isocratic mode (Khalikov *et al.*, 2023). The temperature of the column thermostat is 30° C. Eluent-acetate buffer pH 3.4/acetonitrile in a ratio of 1:1. Eluent consumption – 1 ml/min. UV detection at a wavelength of 220 nm. Data on the measurement of the solubility of the studied solid dispersions are presented in Table 1.

Biological tests of tebuconazole composition

Seed dressing of wheat seeds Novosibirsk 31 was carried out (Vlasenko *et al.,* 2020) as follows: wheat seeds (20.0 g) were placed in 150 ml round-bottomed glass flask. For seeds dressing 5.0 g of 1% suspension of all compositions was used. The flask was rotated on the rotor (with speed of 100 rpm) for 15 minutes until the seeds completely poured out from the flask walls. After that, the seeds were placed in a Petri dish in the humid atmosphere of the desiccator for 3 days (with periodic spraying of the seeds with distilled water). Then the seeds will dried in air at room temperature 24-25^{0}C for 3 days and divided into three parts:

A) 7.5 g of seeds submit for biological testing (to study the effect of TBC adsorbed on the surface of the seed and trapped inside the seed).

B) 7.5 g of seeds leave to find the amount of TBC adsorption on wheat seed.

C) 5.0 g of seeds leave to learn the degree of penetration of TBC into wheat seed.

Analysis of penetration process of tebuconazole composition

The amount of the TBC adsorption on the seed was determined as follows: 7.5 g of treated seeds was immersed in a conical flask for 50 ml and, after adding 10 ml of acetone, stirred on a magnetic stirrer for 10 minutes. Acetone filtrates were analyzed for the content of TBC and it is an indicator of the drug's adsorption on the seed (see Table 2).The remainder of wheat seeds were transferred to biological tests (to study the effect of TBC that penetrated into the seed).

The amount of TBC penetrated from various SDs was determined as follows: 5.0 g of treated seeds was placed in a conical flask for 25 ml and, after adding 10 ml of acetone, the system was stirred for 10 minutes. The seeds were separated by decanting the solvent and were dried in the air for 2 days at a temperature of 24-25^o C. The dried seeds were crushed in a mortar and, after being loaded into a 50 ml conical flask, 10 ml of acetone was poured and the system was stirred on a magnetic stirrer for 1 h. The acetone filtrate was analyzed for the content of TBC and it is an indicator of the penetration of TBC into the seed (see Table 2).

The biological effect was studied based on the development of spring wheat seedlings of the Novosibirsk 31 variety and their infection with diseases after treating their seeds with preparations in the form of SD based on TBC and polysaccharides (AG, GA, Na_2GA and LE). In the laboratory experiment, the effect of drugs on the development of seed diseases (Vlasenko *et al.,* 2020) and on the length of the seedling, the length and number of roots was evaluated by the "roll" method. As a control, untreated wheat seeds were used in comparison with treated seeds, and in the experiment with seeds washed from protectants, the control seed material was also washed with acetone. The results of biological investigations are presented in Tables 3 – 6.

Statistical analysis. All experiments were repeated thrice and resulted values were calculated as an arithmetic mean, and resulted error was calculated as mean value of least squares errors, the differences were considered statistically significant at $p < 0.01$ using a t-test (Dospekhov, 2012).

RESULTS AND DISCUSSION

Tebuconazole is the active substance of the most widely used fungicidal drugs used both for seed treatment and for plant treatment during the growing season (Vlasenko *et al.*, 2020). Due to the poor solubility in water, various formulations of TBC have been developed, which to some extent improve its solubility and bioavailability. Despite this, the consumption rates of TBC in these preparations are quite high, namely, in the preparations Raxil SC 60 or Raxil Ultra the TBC content is 60 and 120 g/l, respectively. Solid dispersions obtained by machinery of TBC and polysaccharides had 100% efficiency with a reduction in the flow rate to 10 – 15 g / l. These results were explained by a significant increase in the solubility of the obtained SDs (see Table 1).

Table 1. Solubility* of tebuconazole compositions obtains after 3 h machinery.

Preparation	Solubility	
	Absolute, mg/l	**Increase, times**
TBC, initial	32.0	-
SD of composition TBC : AG (1:9)	185.6	5.8
SD of composition TBC : GA (1:9)	601.6	18.8
SD of composition TBC : Na_2GA (1:9)	144.0	4.5
SD of composition TBC : EL (1:9)	403.2	12.6

*- Value of standard error ±3%.

To understand the difference in biological activity caused by two sources (on the seed and inside the seed), the amount of TBC on the surface and inside the treated seeds have been measured. The results are shown in Table 2.

Table 2. Adsorption of TBC-based compositions on the seed and its penetration into the seed (All experiments were repeated thrice and resulted values were calculated as an arithmetic mean).

N	composition	TBC* content, %	
		On the grain (adsorption)	Inside the grain (penetration)
1	TBC : AG	61	39
2	TBC : GA	57	43
3	TBC : Na_2GA	71	29
4	TBC : EL	68	32

*- Value of standard error ±3%.

Data analysis of Table 2 indicates that the sorption of TBC on the seed surface slightly depends on the adhesive properties of polysaccharides, and the amount of TBC on the surface is almost two times higher than inside the seed. Also, all compositions show effective penetration of TBC into the seed. This effect might be explained by the similar mechanism of influence of these carriers on the plant membranes and the earlier studied lipid membranes (Selyutina *et al.,* 2017-a).

As a next step, we have compared the biological effectiveness of total TBC adsorbed on the seed surface and entered into the seed ("treated seeds"= *TS*) with the biological effectiveness of the part of TBC penetrated into the grain ("seeds treated and washed" =*STW*).

As a result of the conducted studies, it was found that *TS* increased germination by 4 – 17%, and *STW* - by 13 –21% relative to the control variants (62 and 55%) (Table 3). The maximum increase in the percentage of germination in both cases was observed from the treatment of seeds with TBC with GA and TBC with EL: 14 and 17% - in the first case, 19 and 21% - in the second. It should be noted that the percentage of germination of seeds when germinating in rolls is low, due to high humidity conditions.

Table 3. Effect of treatment of spring wheat seeds with compositions of TBC on the percentage of germination and infection with the main pathogens (All experiments were repeated thrice and resulted values were calculated as an arithmetic mean).

Composion	**Seed germination rate, %**	**Infection with diseases, %**			
		Fusarium spp.	*Bipolaris sorokiniana*	*Penicil-lium* spp.	*Alternaria* spp
Treated seeds (TS)					
Control (non-treated)	62	6	4	1	78
TBC : AG	66	3	0	1	30
TBC : GA	76	2	2	0	32
TBC : Na_2GA	73	1	0	0	33
TBC : EL	79	3	3	0	40
Seeds treated and washed (STW)					
Control (only washed)	55	2	6	1	56
TBC : AG	68	1	0	1	8
TBC : GA	74	3	2	0	16
TBC : Na_2GA	68	0	2	0	15
TBC : EL	76	0	1	0	18

Phytoanalysis of seeds sprouted in rolls showed the best results in reducing the infection rate of their main root rot pathogens (*Fusarium spp* and *Bipolaris sorokiniana*) from the protectants TBC : AG, TBC: GA and TBC : Na_2GA in 3.3, 2.5 and 10 times. When analyzing the washed seeds, the best results were shown by the compositions TBC : AG, TBC : Na_2GA and TBC : EL, where the percentage of infection of seeds with these pathogens in relation to the control variant decreased by 8, 4, and 8 times, respectively. It should be also noted that the germination of washed seeds significantly reduces the level of damage to seedlings of *Alternaria spp*.

All the studied preparations showed their growth-stimulating abilities to a greater or lesser extent already on the 7th day of plant growth, increasing the root length by 0.8 – 4.1 cm (8.8 – 45.3%) relative to the control (9.05 cm) when germinating seeds treated with protectants and by 0.25 – 3.4 cm (2.2 – 29.3%) when washing off the protectants from the seed surface (in the control – 11.6 cm). Due to the retardant effect of chemicals (in this case, tebuconazole) on plant height, the length of sprouts in most variants was lower than the control ones (Table 4). The number of roots in the variants increased relative to the control (2.85 pcs.) by 10.5 – 28.1% from the surface treatment of seeds and by 5.5 – 6.8% from the treatment with the flushing of the drug (in the control, 3.65 pcs.).

Table 4. Effect of treatment of spring wheat seeds with compositions of TBC on the biometrics of spring wheat seedlings, day 7.

Composition	**Biometrics**		
	Sprout length, cm	Number of roots, pcs	Root length, cm
Treated seeds (*TS*)			
Control	8,9±5,05	2,85±1,0	9,05±4,25
TBC : AG	7,2±2,4	3,65±1,0	11,35±2,3
TBC : GA	9,3±3,45	3,5±0,8	13,15±3,45
TBC : Na_2GA	8,15±3,45	3,55±1,0	10,85±4,0
TBC : EL	7,65±3,35	3,15±1,15	9,85±4,15
Seeds treated and washed (*STW)*			
Control	9,95±2,6	3,65±0,9	11,6±2,85
TBC : AG	10,2±2,0	3,9±0,85	15,0±2,0
TBC : GA	8,8±2,2	3,9±0,8	11,35±2,7
TBC : Na_2GA	9,3±2,25	3,85±0,85	11,85±2,85
TBC : EL	8,95±2,2	3,6±0,75	12,5±2,6

On the 15th day of wheat plant growth in rolls, the indicators of biometrics of sprouts and roots and the number of roots retained the trends of the previous accounting (Table 5): the main decrease in the length of sprouts compared to the control, an increase in the length of roots in the variants by 3.1 – 4.6 cm (41.4-60.5%) and an increase in the number of roots by 18.2 – 34.8% in the first method of using drugs and an increase in their number by 8.2 – 14.2% in the second.

Table 5. Effect of treatment of spring wheat seeds compositions of TBC on the biometrics of spring wheat seedlings, 15 days.

Composition	**Biometrics**		
	Sprout length, cm	Number of roots, pcs	Sprout length, cm
Treated seeds (TS)			
Control	14,65±7,2	3,3±1,05	7,6±4,05
TBC : AG	11,15±3,5	4,4±0,9	11,4±2,3

TBC : GA	14,65±4,4	4,35±0,85	12,2±3,7
TBC : Na_2GA	11,75±5,0	4,45±1,0	11,0±3,6
TBC : EL	11,6±4,9	3,9±1,05	10,75±3,2
Seeds treated and washed (STW)			
Control	18,5±5,55	3,65±1,05	11,9±2,9
TBC : AG	15,4±3,75	4,05±0,8	13,9±2,35
TBC : GA	15,3±3,35	4,15±0,85	11,3±2,45
TBC : Na_2GA	15,9±3,9	4,17±0,8	11,9±2,6
TBC : EL	15,6±3,35	3,95±0,75	11,9±2,55

When studying the new protectants, a significant growth-stimulating effect was also observed on the air-dry biomass of sprouts and roots (Table 6). The level of growth of aboveground biomass in the first case varied in the variants from 1.0 to 21.5% (the weight of sprouts in the control was 0.404 g) and root – from 62.9 to 90.3% (the weight of roots – 0.197 g). When the preparations were washed off, the accumulation of biomass by sprouts was slightly more intense than the control indicator (0.459 g) only in the variants of compositions TBC: GA (by 2.4%) and TBC: Na_2GA (by 1.1%), and the root mass – when using TBC: AG, by 14.3% (in the control – 0.384 g).

Table 6. Effect of treatment of spring wheat seeds with compositions of TBC on the air-dry biomass of sprouts and roots, g/50 plants (All experiments were repeated thrice and resulted values were calculated as an arithmetic mean).

Composition	Aboveground (sprouts)	Underground (roots)
Treated seeds (TS)		
Control	0,404	0,197
TBC : AG	0,481	0,375
TBC : GA	0,491	0,361
TBC : Na_2GA	0,455	0,329
TBC : EL	0,408	0,321
Seeds treated and washed (STW)		
Control	0,459	0,384
TBC : AG	0,436	0,439
TBC : GA	0,470	0,370
TBC : Na_2GA	0,464	0,372
TBC : EL	0,459	0,368

CONCLUSIONS

For effective treatment of wheat seeds, solid dispersions of tebuconazole with plant metabolites were prepared, forming supramolecular complexes when dissolved in water. These compositions have increased solubility and effectiveness while reducing the dose of TBC by 10 times or more. These preparations in water formed stable working solutions, convenient for the process of treating soft spring wheat seeds Novosibirsk 31. Studies conducted using spring wheat seeds treated with

TBC compositions showed its high (from 29 to 43%) penetrating ability into the seeds. This value was maximum in the case of the composition with GA. Comparison of the biological effectiveness of treated seeds with treated and washed seeds did not reveal differences in their effect on pathogens of common root rot. Approximately the same results were observed when comparing the growth processes of spring wheat seedlings. Such results can be explained by the fact that during the initial treatment of seeds, the foci of diseases on the seeds are already suppressed and further washing of the surface of the seeds from drug residues no longer affects the biological results.

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