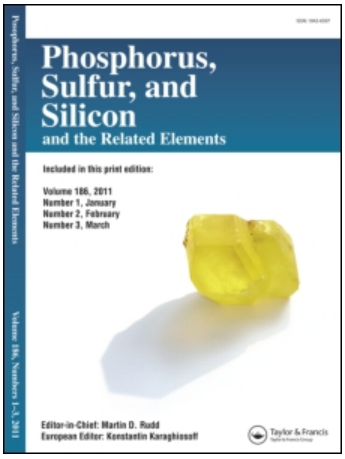

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STUDIES ABOUT SYNTHESIS, CHARACTERIZATION, AND THE EFFECT OF 2-*n*-PROPYL-3-ETHYL-3-METHYL-1,4,2-BENZOXAZA-PHOSPHORINE-2-OXIDE ON GROWTH PARAMETERS AND CHLOROPHYLL CONTENT OF WHEAT

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*This article presents the synthesis and characterization of a new phosphorus heterocycle, namely 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide, by a Mannich-type reaction between* n*-propyl-dichlorophosphine, methyl ethyl ketone, and* o*-aminophenol.*

*Taking into consideration that chlorophyll contents in plants were revealed to have connection with nitrogen and phosphorus concentrations at early growth stage, and because the above-mentioned heterocyclic compound can be considered as a phosphonic analogous of naturally occuring α-aminoacids, it was expected to develop biological activity from it. That is the reason why 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide was subjected for biological tests on wheat.*

*The present study was also undertaken to investigate the interrelationship between chlorophyll content, dry matter, and other growth parameters of wheat, after treatment with different concentrations of 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide.*

Keywords: Chlorophyll a and b; Mannich reaction; *n*-propyldichlorophosphine; phosphorus heterocycle

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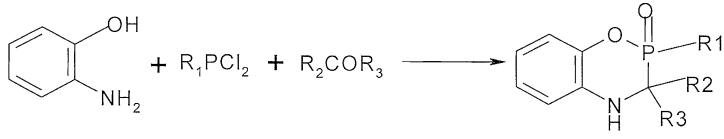
INTRODUCTION

Plants are subjected to a great variety of environmental factors such as light, temperature, water conditions, level of nutrient substances, and microelements. All of these factors produce different changes in the photosynthesis process of plants.[1]

Chlorophyll contents were revealed to have connection with nitrogen and phosphorus concentrations at growth early stage. Nitrogen (N) is a component of protein. Therefore, forages and grain must be adequately fertilized with N or protein content will be low. N is also part of the chlorophyll molecule that gives green color to plants. Deficient N results in pale (yellowish) leafy vegetables. Phosphorus (P) is important to seed quality, playing a direct role in genetic transfer. Phytin, an organic compound which contains P, stores important dietary minerals such as calcium (Ca), zinc (Zn), and iron (Fe).

The present study was developed to investigate the correlative effects exhibited by different concentrations of a new synthesized phosphorus heterocycle, which also contains nitrogen, on growth parameters, chlorophyll a and b contents, as well as on chlorophyll a/b ratio of wheat (Alex cultivar) treated with the above-mentioned compound.

For this purpose, and in connection with previous researches concerning the involvement of alkyl- and aryl-dichlorophosphines in cyclization reactions,[2–5] 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxazaphosphorine-2-oxide was synthesized by a Mannich-type reaction[6] between *n*-propyldichlorophosphine, methyl ethyl ketone and *o*-aminophenol, according to Scheme 1.



$R_1 = n\text{-}C_3H_7$; $R_2 = CH_3$; $R_3C_2H_5$

SCHEME 1

To establish the auxinic effect activity of 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide, the Tsibulskaya-Vassilev general biotest[7,8] was used.

For quantitative determination of chlorophyll a and b, the spectroscopic methods were chosen because they are suitable for quick, routine analysis of the pigment content. The precision of the spectroscopic method depends on the type of device used, the ability to determine the absorbance maxima with precision, and the accuracy of the absorption coefficient used for the calculation.[9–11]

Chlorophyll concentrations were determined by analysis of the ultraviolet (UV)–visible spectrum of the extracted pigments in 80% acetone. For calculations, the standard extinction equations reported by Lichtenthaler[12] were used.

RESULTS AND DISCUSSIONS

From the data presented in Table I it can be observed that for wheat, which is treated with 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxazaphosphorine-2-oxide; all the biometric measurements reached their best value at the concentration of 100 ppm.

Dry substance has recorded important growths at all the treated variants (the best value of 13% has been achieved also at the concentration of 100 ppm), demonstrating that the new tested substance has stimulated the metabolism and thus the accumulation of protein substances. In very good agreement with the general evolution of the wheat treated with 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxazaphosphorine-2-oxide, the chlorophyll a and b concentrations jump over the values of control at the same concentration value, that of 100 ppm, as can be seen in Table II. This concentration of 100 ppm proved to be the most benefical for all the plant evolution, probably because of the higher chlorophyll content.

TABLE I The Effect of 2-*n*-Propyl-3-ethyl-3-methyl-1,4,2-benzoxazaphosphorine-2-oxide Concentrations on Growth Parameters of Wheat

	Average length of the seedling		Average length of the roots		Average number of roots		Dry substance	
Variant	cm	%	cm	%	No.	%	g	%
Water control	10.32	100	9.17	100	3.88	100	0.0119	100
2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide								
10 ppm	9.49	92	7.89	86	4.15	107	0.0123	103
20 ppm	10.52	102	8.44	92	4.31	111	0.0130	109
50 ppm	10.93	106	8.53	93	4.54	117	0.0133	112
100 ppm	11.25	109	9.21	100.4	4.58	118	0.0135	113
200 ppm	10.01	97	8.16	89	4.31	111	0.0125	105

TABLE II Wheat Leaf Chlorophyll Concentrations in Control and After Treatment with Different Concentrations of 2-*n*-Propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide

Wheat	A_{663}	A_{647}	Chlorophyll a ($mg \cdot l^{-1}$)	Chlorophyll b ($mg \cdot l^{-1}$)	Chlorophylls a + b ($mg \cdot l^{-1}$)	Chlorophyll a/b ratio
Water control	0.612	0.284	6.7046	2.9848	9.6894	2.24626
2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide						
10 ppm	0.599	0.271	6.5816	2.7716	9.3532	2.37467
20 ppm	0.635	0.289	6.9724	2.9750	9.9474	2.34367
50 ppm	0.678	0.316	7.4238	3.3362	10.7600	2.22524
100 ppm	0.704	0.323	7.7228	3.3541	11.0769	2.30250
200 ppm	0.643	0.299	7.0425	3.1492	10.1917	2.23629

The changes in chlorophyll content in wheat were also accompanied by changes in stem elongation and development (dry matter, average number of roots, average length of the seedlings), as can be seen in Figure 1.

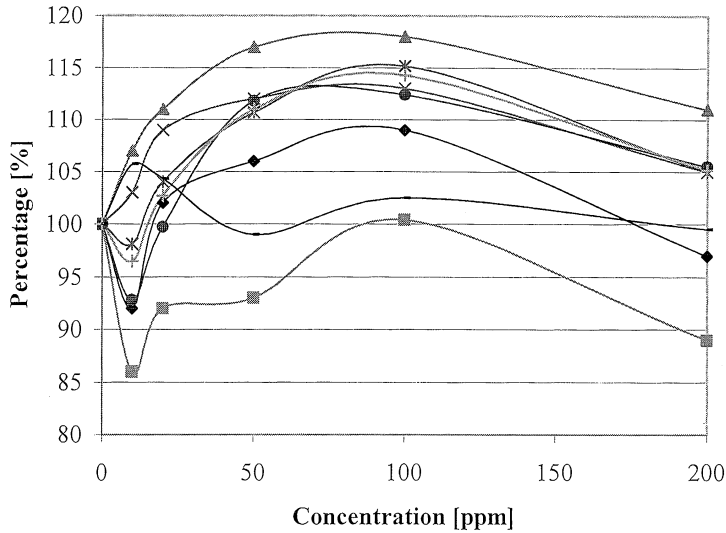


FIGURE 1 Effect of different concentrations of 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide on growth parameters and chlorophyll content of wheat seedlings. —♦—, Average length of the seedling; —■—, Average length of the roots; —▲—, Average number of roots; —×—, Dry substance; —✳—, Chlorophyll a; —●—, Chlorophyll b; —+—, Chlorophyll a+b; ——, Chlorophyll a/b ratio.

CONCLUSIONS

- Comparing the data resulting from the treatment with 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide, we can conclude that this substance exhibits biological activity in wheat, especially at 100 ppm concentration, and also the chlorophyll a and b concentrations are significantly increased.
- It seems that the increase of chlorophyll a and b concentrations plays an important role for the development of the whole plant. The tested substance produced the metabolism acceleration and thus the accumulation of protein substances up to 13%.
- It is obvious that the new substance, which has been tested, exhibits biological activity, and a very good connection between chlorophyll content response and all the other changes concerning the general development of the monocotyledonous plant was registered.

EXPERIMENTAL

Synthesis of 2-*n*-Propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide

o-Aminophenol (5.45 g, 0.05 mol) was dissolved in 100 ml of anhydrous benzene, and 7.25 g (0.05 mol) of n-propyldichlorophosphine was added at room temperature with vigorous stirring. In both cases, with every drop of dichlorophosphine added a red color appeared, which transformed immediately into dark yellow. After an hour, methyl ethyl ketone was added dropwise to the well-stirred solution at the same molar ratio, 1:1:1. The stirring continued for an hour, then the mixture was heated under reflux at 80–100°C for 8 h to give the desired compound, a white solid, which was separated by filtering the cooled reaction mixture. Note that the reaction mixture included some dark yellow salt-like products that were insoluble in usual solvents. We assumed that they were mixed polyphosphine compounds.

After recrystallization from benzene the new obtained product was subjected to infrared (IR), GC, MS, and H^1-NMR analysis. The main results are given below:

2-*n*-Propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide

White crystals, m.p. 84–87°C, $\eta = 46\%$. IR (KBr, cm^{-1}): 3068 (ν_{CHAr}); 1216 (ν_{PO}); 1155 (ν_{POC}); 1123 (ν_{CN}); ^{1}H-NMR spectrum ($CDCl_3$), δ: 0.96 (t, 6H, CH_3, $^3J_{HH} = 7.1$ Hz); 1.36 (d, 3H, CH_3, $^3J_{PH} = 14.7$ Hz); 1.6–1.9

(m, 6H, CH_2); 5.1 (brs, 1H, NH); 6.88–7.23 (m, 4H, C_6H_4). MS (m/z): 253 ($M^{+}\cdot$). Elemental analysis for $C_{13}H_{20}NO_2P$ (253) (%): calc. C, 61.66; H, 7.90; P, 12.25. Found C, 61.88; H, 8.03; P, 12.04.

The IR spectrum showed a band at 3440 cm^{-1}, characteristic of the NH stretching frequency and arround 3070 cm^{-1} for C—H aromatic. The IR spectra also showed P=O group existence at 1216 cm^{-1} and a C—N group band between 1090–1140 cm^{-1}.

The ^{1}H-NMR spectrum was in agreement with the assigned structure.

Based on the retention data obtained in gas chromatographic analysis performed for the new synthesized compound, the corresponding retention indices on the two silicone stationary phases having different polarities (OV-1 and OV-17) were determined using the well-known calculating formula of Kovats.[13] The compound ($I_{OV\text{-}1} = 2238$ and $I_{OV\text{-}17} = 2581$) proved to have great stability, because during the elution time there has been obtained only one symmetric peak.

The Method for Testing Growth Regulating Activity

To establish the auxinic effect activity of 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide, the Tsibulskaya-Vassilev general biotest[7,8] was used.

For the determination of the biological activity of 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide, the biotest method was carried out on monocotyledonous wheat caryopses (*Alex variety*) comparatively with water control. The concentrations of 2-*n*-propyl-3-ethyl-3-methyl-1,4,2-benzoxaza-phosphorine-2-oxide were: 10 ppm, 20 ppm, 50 ppm, 100 ppm, and 200 ppm. The seeds, previously disinfected with calcium hypochlorite solution, were treated with bioactive compounds and were held in plastic Petri dishes ($\Phi = 90$ mm, 20 seeds/dish, 2 repetitions/concentration) on agar medium (5 g/l concentration) at 25°C for six days. Next, the biometrics measurements were carried out, watching for the average height of plants, the average number of the roots for one plant, the average length of the roots, and the dry matter. The obtained data were calculated in percentage and compared to the water control.

The Chlorophyll Content Determination

One gram of fresh leaf tissue was weighed and cut into small pieces (about 1 mm wide) with scissors or razor blade and ground with a mortar and pestle in the presence of a little sea sand, 0.2–0.5 g of $MgSO_4$, and ca 0.5 ml of 100% acetone. Two to five milliliters of 80% acetone were

added to the fine powder, and the solid compound elements were separated by centrifugation at 5000 rpm for 10 min.

The extracted solutions were kept in dark conditions and refrigerated for 30 min prior to measurement. The spectrophotometer was calibrated using 80% acetone in a quartz cuvette. Four ml of extract were placed in a 1 cm quartz cuvette, and absorption was measured at two different wavelength positions. The quartz cuvette was rinsed between samples with 80% acetone, and the spectrophotometer was recalibrated every 10 samples.

Extract solution absorbance was measured with a V-550 UV/VIS Jasco Spectrophotometer at 647 nm and 663 nm,these being the absorbance maxima in 80% acetone for chlorophyll a and b.

Absorbance values were used to calculate pigment concentrations using standard extinction equations reported by Lichtenthaler:[12]

$$\text{chlorophyll}\, a\,(\text{mg}\cdot\text{l}^{-1}) = (12.25 \cdot \text{A}_{663} - 2.79 \cdot \text{A}_{647}) \cdot \text{D}$$

$$\text{chlorophyll}\, b\,(\text{mg}\cdot\text{l}^{-1}) = (21.5 \cdot \text{A}_{647} - 5.1 \cdot \text{A}_{663}) \cdot \text{D}$$

$$\text{chlorophyll}\, a + b\,(\text{mg}\cdot\text{l}^{-1}) = (7.15 \cdot \text{A}_{663} + 18.71 \cdot \text{A}_{647}) \cdot \text{D}$$

where A is absorption at given wavelengths and D is the thickness of the used cuvette (cm).

Reactions, reagents, and all operations were carried out with protection from atmospheric moisture, using Schlenk glassware and purging inert gas.

n-Propyldichlorophosphine (97%) was obtained from Pierce (Lausanne, Switzerland); methyl ethyl ketone (97%) *o*-aminophenol (98%) and benzene were from Sigma Aldrich Division (Seelze, Germany). All chemicals used were dried and distilled from appropriate drying agents, according to Perrin.[14]

The melting point was determined on a Betius apparatus and is uncorrected.

The phosphorus content was determined by the Schöniger method on a Heraus Apparatus. Elemental analysis was carried out on a CARLO ERBA 1106 analyzer.

^{1}H-NMR spectra were determined in $CDCl_3$ solution with a Varian Gemini 300 apparatus. Chemical shifts (δ) are given in ppm downfield from internal TMS.

IR spectrum was determined on a SPECORD M80 JENA.

The mass spectrum was registered using a VARIAN FINNIGAN MAT 212 mass spectrometer.

The GC analysis was performed on Carlo Erba, Fractovap GT 200 gas chromatograph with double-column system and thermo conductibility detector, equipped with DP700 Fisons Instruments data station, by

using two Pyrex glass columns 2 m long, filled with two silicone stationary phases having different polarities (OV-1 and OV-17) on Gas Chrom Q (80–100 mesh) support, at a flow rate of 80 ml/min hydrogen, as carrier gas. The compound was investigated as a toluene solution. The temperatures were: 260°C for the columns, 290°C for the injector, and 275°C for the detector. The GC analyses certified the purity of the new product.

UV-VIS spectra were measured on V-530 UV/VIS Jasco Spectrophotometer.

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