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SPRING BARLEY YIELD PARAMETERS AFTER LIGNITE, SODIUM HUMATE AND NITROGEN UTILIZATION

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The existence of a small number of publications dealing with the impact of solid sodium humate and lignite on the quantity and quality of grown crops was the reason for establishing the field experiment. The objective of this experiment was to detect the impact of solid lignite and solid sodium humate on the quantity and quality of spring barley yield. These substances were applied into the soil either independently or along with nitrogen fertiliser. The next objective was to determine the impact of foliar application of sodium humate water solution applied either independently or along with nitrogen fertiliser on the quality and quantity of spring barley yield.The achieved results showed that the autumn application of solid lignite and the presowing application of solid sodium humate into the soil tended to decrease the yield of both grain and straw of spring barley, crude protein content in grain, proportion of the first-class grains and volume weight of grain, whereas the impact of humate was more negative. Lignite and sodium humate in the solid form should be used along with nitrogen fertiliser. The application of sodium humate in liquid form during the growth season of barley tended to increase the yield of both grain and straw. The joint application of nitrogen and liquid sodium humate during the growth season of barley increased the grain yield of barley significantly. A lower dose of nitrogen, applied during the growth season of barley (growth season BBCH 23), increased the grain yield of barley considerably more than a higher N dose, applied into the soil before barley sowing.

Key words: lignite, Lignofert, sodium humate, coal materials, spring barley

In Slovakia, the supply of humus creating material into soil (manure, postharvest residues, etc.) has fallen dramatically in the last 25 years. As a result, the yields of field crops have stagnated in many agricultural enterprises in the recent five or ten years, in spite of the growth of commercial fertiliser applications. The quality of cultivated products is also diminishing. One way to solve this unfavourable situation is to utilize the substances which are rich in carbon (sawdust particles, coal materials, etc.). However, their incorrect usage can lead to the inhibition of plant germination, growth retardation and yield decrease (Kováčik 2014). Therefore, the agricultural practice applies the materials which are rich in carbon only by foliar application, in particular, in gram and kilogram quantities per hectare. They have the stimulating effect on the plant growth, but they do not have any impact on carbon content in soil, which hereafter limits the yield quantity and quality. The usage of solid coal materials (dust of bituminous,

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sub-bituminous or lignite coal, sodium humate, potassium humate, etc.), which would supply soil with substantial quantities of carbon, does not appear. In spite of that, there are many facts that give evidence of their positive impact on the yield crop parameters and their quality. The experiments of Weismann *et al*. (1993), Valšíková and Viteková (2006) demonstrated that after the application of 5 t/ha lignite into soil, the yields of potatoes, sugar beet, maize, spring barley and lettuce increased. Similarly, the positive results after the application of 5 kg/ha sodium humate into the soil during spring barley cultivation were recorded by Kováčik and Fecenko (1996) and by Shalabey and Bízik (1998) during wheat cultivation. The significant yield growth after the application of the given materials into soil in solid form or dissolved in water during the cultivation of different crops, was also registered by other authors (Lee & Bartlett 1976; Lobartini *et al*. 1992; Patil *et al*. 2011).

We took into consideration a small number of publications dealing with the impact of usage of solid coal materials on the quantity and quality of yields of cultivated crops. Therefore, we started the experiment with the target to find out the impact of solid lignite and solid sodium humate applied into soil independently, or along with nitrogen fertiliser. The objective was also to guage the impact of foliar application of water solution of sodium humate on the quantity and quality of spring barley – when applied independently and when applied along with nitrogen fertiliser.

MATERIAL AND METHODS

The field small-plot trial was carried out during two farming years (2005 and 2006) at an altitude of 320 meters above sea level in the locality of town Sabinov (49°05´N and 21°04´E – eastern Slovakia) on Haplic Dystric Cambisol (WRB 2006), which was typical of high acidity to acid soil pH and low carbon content. The agrochemical parameters of Haplic Cambisol are given in the Table 1. They were determined by the following methods: $N\text{-}NH_4^+$ – colorimertically by Nessler`s agent, $N\text{-}NO_3^-$ – colorimetrically by the phenol 2.4 disulphonic acid, $N_{in} = N\text{-}NH_4^+ + N\text{-}NO_3^-$, P – colorimetrically (extract Mehlich 3 – Mehlich 1984), K and Ca – by flame photometry (extract Mehlich 3 – Mehlich 1984), Mg – by atomic absorption spectrophotometry (extract Mehlich 3 – Mehlich 1984), C_{ox} – oxidometrically by Ťjurin in modification of Nikitin (Dziadowiec & Gonet 1999), pH/KCl – potentiometrically (1.0 mol/dm^3 KCl).

In the first year of experiment, we detected acid soil reaction, and in the following year, high acidity of soil reaction was found. In both years, 2.5 t/ha 90% of $CaCO_3$ was applied before tillage in autumn.

Solid sodium humate (black flakes) was used in the experiment. This was produced in the Czech Republic by alkalic extraction from low-cal coal, which was carbonized imperfectly from undersurface coal, called Capuchin. Lignite was of Slovak origin, and it was sold with the trade mark Lignofert. It was produced by grinding, followed by sieving of lignite through sieves of 0.1–10.0 mm. The agrochemical and physical parameters of both tested materials are given in the Table 2 and 3.

The model crop was spring barley, malting cultivar Nitran, sown by 4.5 million seeds per hectare, cultivated within the sowing plan after potatoes. After the harvesting of potatoes, green fertilising was carried out using winter oilseed rape.

The experiment variants, doses of lignite, sodium humate and nitrogen are given in the Table 4. This table shows that in the control variant 1 neither coal materials nor nitrogen fertilisers were used. As the only tested material, lignite was applied in autumn because it is well known that this material is relatively stable (var. 2, 6 and 10). The dose of lignite 8 t/ha was in adherence with the knowledge about the application doses of processed coal mechanically, obtained by Weismann *et al*. (1993). In variant 2, lignite was applied individually, and in the variants 6 and 10, nitrogen was added to lignite in two different periods. Sodium humate was applied in two periods – before sowing and during growth season of spring barley in the same dose 10 kg/ha (var. 3, 5, 8 and 9). In the variants 3 and 8, lignite was applied individually, and in the variants 5 and 9, it was used along with N fertiliser. The dose 10 kg/ha originated from the dose of lignite (8 t/ha), and the knowledge presented by Kováčik and Jasiewicz (2009) that sodium humate contains about 800 times

more hot-water soluble carbon than Lignofert. The same quantity of hot-water soluble carbon was applied with the dose 8 t/ha of lignite as with the dose of 10 kg/ha sodium humate.

Nitrogen was applied through the fertiliser DAM-390 containing 30% N in three forms: 7.5% N in nitrate form, 7.5% in ammonium form and 15% in amidic form. The pH value of the fertiliser was 7.5. In the variants 4, 5 and 6, N was applied before sowing, and in the variants 7, 9 and 10, N was applied during barley growing season in the growth phase BBCH 23 (tillering). The dose of nitrogen fertilisers (D_N) in the variants 4, 5, and 6 was calculated on the basis of respecting level N_{in} (inorganic nitrogen) in the soil sample taken from the layer 0.0–0.6 m in spring (Table 1) and the necessity N for the planned yield (P_N). According to the facts presented by Kováčik and Fecenko (1992), who have given 6 dif-

T a b l e 1

Agrochemical parameters of Haplic Cambisol (dry matter)

Layer [m]	Year of experiment	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	N_{in}	P	K	Ca	Mg	pH/KCl	C_{ox} [%]	N_{an} at BBCH 22 [mg/kg]
		[mg/kg]									
0.0 – 0.3	first	9.8	5.2	15.0	138	275	700	138	5.41	1.08	12.4
0.3 – 0.6		8.4	3.9	12.1	95	290	800	165	5.64	0.73	10.1
0.0 – 0.3	second	9.8	4.1	13.9	123	353	1,000	163	4.63	1.14	13.3
0.3 – 0.6		8.3	4.0	12.3	81	203	1,225	198	4.98	0.82	9.5

T a b l e 2

Agrochemical parameters of sodium humate and lignite (dry matter)

Material	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	N_{in}	P	K	Ca	Mg	pH/KCl	C_{ox} [%]	EC [mS/cm]
	[mg/kg]									
Sodium humate	3,125	6.5	3,132	165	27,250	52,000	2,840	9.66	45.0	13.87
Lignite	11.1	1.6	12.7	traces	75	2,750	908	5.35	30.7	2.62

T a b l e 3

Size fractions of sodium humate and lignite (Lignofert)

Size fraction [mm]	Sodium humate	Lignite
	[%]	
>7	0.19	4.80
>5	0.38	9.66
>3	2.67	17.68
>1	23.87	20.97
>0.5	33.67	14.65
>0.25	19.09	9.43
<0.25	20.12	22.80

ferent effective approaches for the calculation of N dose for spring barley, it was calculated for 100% usage of nitrogen, which occurs in the layer 0.0–0.6 m, and the requirement of 24 kg of N per 1 tonne of the main product and the respective quantity of a by-product. The planned yield was 6 t/ha. N dose was calculated using the formula given below:

$$D_N = P_N - N_{in} \times 9,$$

where: P_N = necessity of N for the planned yield, N_{in} = the content of inorganic nitrogen in the soil sample taken from the layer 0.0–0.6 m in spring. The number 9 is the coefficient of recount from unit mg/kg to unit kg/ha. The coefficient was determined by soil layer (0.6 m) and volume weight of soil in the particular layer (1.5 g/cm^3).

In the variants 7, 8, 9 and 10, the soil samples were taken from the layer 0.0–0.6 m in the growth phase of spring barley BBCH 22 (tillering) and N_{in} content was determined. At the same time the aboveground phytomass was taken in order to specify N content in it. The quantity of nitrogen (in kg/ha) taken in by vegetation was calculated. Based on the data obtained, the N dose was calculated according to the following formula:

$$D_N = P_N - N_s - N_p,$$

where: D_N is N dose, P_N is N needed for planned yield and N_s is nitrogen (kg/ha) in soil in the layer 0.0 – 0.6 m (N_{an} as mg/kg × 9).

We assumed 100% utilization of nitrogen and N_p meant nitrogen in a plant. In the growth phase, there was 22.7 kg/ha N of BBCH 22 in the barley plants in the first year of the experiment and 21.3 kg/ha N in the second year. The analyses and calculation were accomplished in the course of five days because it was necessary to apply N fertilisers after sampling as soon as possible.

The experiment was established using the split-plot design as fully randomised blocks in three replications. The area of one plot was 21 m^2 (1.5 m × 14 m). The harvest was carried out manually in the following way – the whole aboveground phytomass was taken from the area 1m^2 in the period of technological ripeness of barley from each plot. The yield of grain and straw were evaluated. In grain,

Table 4

Variants of experiment and dosages of tested materials

Variant		First year of experiment					Second year of experiment				
		before growing season			at the time of growing season		before growing season			at the time of growing season	
		lignite [t/ha]	sodium humate	N	humate	N	lignite [t/ha]	sodium humate	N	sodium humate	N
designation	number	[kg/ha]					[kg/ha]				
0	1	–	–	–	–	–	–	–	–	–	–
Lig_{Aut}	2	8	–	–	–	–	8	–	–	–	–
Hum_{Sow}	3	–	10	–	–	–	–	10	–	–	–
N_{Sow}	4	–	–	22	–	–	–	–	26	–	–
$Hum_{Sow} + N_{Sow}$	5	–	10	22	–	–	–	10	26	–	–
$Lig_{Aut} + N_{Sow}$	6	8	–	22	–	–	8	–	26	–	–
N_{BBCH23}	7	–	–	–	–	20	–	–	–	–	20
Hum_{BBCH23}	8	–	–	–	10	–	–	–	–	10	–
$Hum_{BBCH23} + N_{BBCH23}$	9	–	–	–	10	20	–	–	–	10	20
$Lig_{Aut} + N_{BBCH23}$	10	8	–	–	–	20	8	–	–	–	20

0 – control; Lig_{Aut} – lignite in autumn; Hum_{Sow} – humate before of sowing; N_{Sow} – nitrogen before sowing; $Hum_{Sow} + N_{Sow}$ – humate and nitrogen before sowing; $Lig_{Aut} + N_{Sow}$ – lignite in autumn and nitroben before sowing; N_{BBCH23} – nitrogen in growth phase of BBCH 23; Hum_{BBCH23} – humate in growth phase of BBCH 23; $Hum_{BBCH23} + N_{BBCH23}$ – humate and nitrogen in growth phase of BBCH 23; $Lig_{Aut} + N_{BBCH23}$ – lignite in autumn and nitrogen in growth phase of BBCH 23

the content of crude protein and weight of one thousand grains (TKW – thousand kernel weight), the proportion of the first-class grain and the volume weight and nutrient content (N, P, K, Ca) was determined. In order to determine the nutrient content, the methods published in Kováčik (1997) were used. Crude protein content was calculated according to the formula 6.25 × % N. The obtained results were processed using the mathematical and statistical methods, namely, analysis of variance (ANOVA) and linear regression analysis using Statgraphics PC software, version 5.0.

RESULTS AND DISCUSSION

The autumn application of lignite and the pre-sowing application of sodium humate tended to decrease the yield of grain and straw of spring barley. Here the impact of humate was more negative, and it was statistically insignificant (var. 2 and 3, Table 5). In the variant (var. 3) where sodium humate was used, the absolutely lowest yield of grain and straw was achieved out of the 10 variants in the experiment. The negative impact of sodium humate and lignite on the yield of barley grain and straw, which was recorded in the experiment, did not correspond with the facts published by Halčínová and Kováčik (2011), who recorded 12.6 and 11% increase of grain yield after the application of lignite and humate in the doses 900 kg/ha and 300 kg/ha.

The presowing N application in the dose, which took cognizance of N_{in} in soil, increased the yield of grain and straw as compared to the control variant, however, only insignificantly (Table 5, var. 4). The unimportant yield growth after the application of N fertilisers did not correspond to the opinion of many authors who claimed that after presowing, the fertilisation of spring barley yields increased significantly, often by 30% or more (Užík *et al*. 2009; Šrek & Kunzová 2011; Chen *et al*. 2016). On the contrary, N application carried out during barley growth season (BBCH 23) evidently resulted in the highest yields of grain and straw out of all the variants (var. 7). The recorded higher growth of spring barley yield after N application during the growth season as compared with presowing N fertilisation is identical with the findings of several authors (Kováčik *et al*. 2006; Škarpa 2006; Candráková *et al*. 2009). Because of the frequent significant increase of contents of crude

T a b l e 5

The impact of lignite, sodium humate and nitrogen on the spring barley yield parameters (average of two years)

Variant		Grain	Straw	Crude protein	Grain of the 1st class	Volume weight [g/l]
designation	number	[t/ha]		[%]		
0	1	4.63^{ab}	3.64^{ab}	9.51^{b}	78.87^{abc}	741.00^{ab}
Lig_{Aut}	2	4.54^{ab}	3.57^{ab}	9.31^{ab}	77.57^{ab}	737.33^{a}
Hum_{Sow}	3	4.35^{a}	3.44^{a}	9.10^{ab}	77.30^{a}	738.50^{ab}
N_{Sow}	4	4.82^{b}	3.72^{abc}	9.34^{ab}	79.76^{cd}	741.11^{ab}
$Hum_{Sow} + N_{Sow}$	5	4.80^{bc}	3.66^{ab}	9.26^{ab}	80.17^{cd}	742.09^{ab}
$Lig_{Aut} + N_{Sow}$	6	4.88^{bc}	3.66^{ab}	9.27^{ab}	81.30^{d}	742.83^{ab}
N_{BBCH23}	7	5.14^{c}	3.98^{c}	9.56^{b}	80.47^{cd}	744.17^{b}
Hum_{BBCH23}	8	4.71^{ab}	3.67^{a}	8.96^{a}	78.93^{abc}	741.09^{ab}
$Hum_{BBCH23}+ N_{BBCH23}$	9	5.06^{c}	3.83^{bc}	9.37^{ab}	80.43^{cd}	742.65^{ab}
$Lig_{Aut} + N_{BBCH23}$	10	5.09^{c}	3.85^{bc}	9.41^{ab}	79.37^{bc}	741.83^{ab}
$LSD_{0.05}$		0.368	0.295	0.465	1.864	6.409

$LSD_{0.05}$ – least significant difference at the level a = 0.05 (*LSD* test); different letter behind a numerical value indicate statistically significant difference at the level 95.0%

Explanation: See Table 4

proteins in spring barley grain after the N application during growth season, several authors do not recommend fertilisation of spring barley during the growth season (Cook 1982; Kandera 1994). Užík and Žofajová (2006) pointed out that it is important to adapt spring barley fertilisation to the cultivar requirements.

The solo application of sodium humate in the form of water solution (var. 8) carried out in the growth phase of barley BBCH 23, unlike the presowing application of the solid humate (var. 3), did not have a negative impact on the yield of grain and straw (Table 4). This fact refers to the sensitivity of germinating barley plants, or young barley plants to the presowing application of the solid sodium humate, which is typical of a high value of electrical conductivity (Table 2). Thus, as the autumn lignite application is recommended, similarly, the application of the solid humates should be carried out much in advance, before the crop sowing.

The presowing nitrogen application into soil, which contained coal materials (var. 5 and 6), had a positive impact on the grain and straw yield in comparison with the control variant. However, the difference was not significant. In spite of the fact that the solo applications of lignite and humate did not increase grain yield (var. 2 and 3), a higher grain yield was achieved by the joint application of lignite and nitrogen (var. 6), as compared to the solo nitrogen application (var. 4). This finding proved the negative impact of solid sodium humate application into soil on the barley grain yield recorded in the variant 3.

The pre-sowing addition of nitrogen to the coal materials did not bring a higher straw yield in relation to the N application itself (var. 5 and 6 versus var. 4).

The nitrogen fertilisation in the growth phase BBCH 23 (var. 7, 9 and 10) resulted in a higher grain and straw yield, regardless of the application of N on the plants cultivated on soil treated or untreated by lignite or sodium humate. The comparison of the grain and straw yields between variant 7 and the variants 9 and 10 confirmed the negative impact of coal materials on the quantity of spring barley yield, where humate had more negative effect than lignite.

The evaluation of impact of tested materials and studying the crude protein content in spring barley grain, detected that the lowest content of crude protein was recorded in variants where only sodium humate had been applied (var. 8 and 3). Amongst all the nine treated variants of the experiment, the content of nitrogen substances decreased in all but one variant, where nitrogen was applied on barley leaves during the growth season (var. 7). The detected negative impact of coal material on the content of crude protein in grain can be evaluated as a positive effect if barley is grown on soils rich in total and organic nitrogen because barley, which is grown on these soils, usually achieves excess content of crude protein. The drop of crude protein level as a positive effect of humate was also presented by Fecenko *et al*. (1995).

In the experiment, in all the variants, crude protein contents were lower than 10%. Therefore, the decrease of crude protein level after the application of coal materials was evaluated as a negative impact leading to the worse qualitative parameter of spring barley grain grown for malty purposes.

No significant relation was determined between the crude protein level and grain yield, the crude protein and straw yield, the crude protein and the proportion of the first-class grain and the volume weight of grain (Table 6).

The smallest proportion of the first-class grains and also the lowest volume weight of grains were registered in the variants where the solo application of lignite and humate was carried out into soil (var. 2 and 3). These facts along with the impact of solo applications of coal materials recorded till date proves unambiguously that in this experiment, the usage of solid lignite and solid sodium humate tended to decrease the quantity as well as quality of spring barley.

The pre-sowing nitrogen applications and N applications in the growth phase BBCH 23 carried out independently, or the nitrogen applications on the variants treated by coal materials – all these measures increased the proportion of the first-class grains (var. 4, 5, 6, 8, 9, 10). The statistically significant increase of the proportion of first-class grains in comparison with the control variant was recorded only in variant 6. In this variant, lignite was applied into soil in autumn and nitrogen before sowing. Along with an increase in the proportion of first-class grains in the variants where nitrogen was

applied apart from coal materials, there was an increase of grain yield also recorded in those variants. This fact means it is necessary to simultaneously apply lignite or humate with the nitrogen fertiliser.

The impact of experiment variants on the bulk density of soil was comparable with the impact of variants on the proportion of first-class grains, which can be proved by the correlation coefficient $r = 0.9096^{++}$ between the first-class grains and the volume weight of grain.

The application of the soil additive substances, the different biostimulants can often result in a higher yield with a lower content of nutrients, and a lower quantity of the content substances (Bielek 1998). The Table 7 shows that the tested coal materials had a negative impact on the contents of phosphorus and potassium in grain of spring barley. The partially different findings were achieved by Halčínová and Kováčik (2011), who did not record a significant change after the application of solid lignite and sodium humate in the content of P and K in barley grain.

In the presented experiment, a decrease in phosphorus contents was also seen in the variants where only nitrogen was applied without coal materials (var. 4 and 7). This fact confirms the information provided by Vaneková and Vanek (1983), Gill and Lavender (1983), who claimed that along with an increase in the application dose of nitrogen, there usually is a falls in the phosphorus content in a plant.

T a b l e 6

Correlation coefficient *r* expressing the relationship between the crude protein content and some quantitative and qualitative parameters of barley yield

Dependent parameter	Independent parameter	Correlation coefficient (*r*)
Crude protein	grain	0.535 ^{ns}
Crude protein	straw	0.610 ^{ns}
Crude protein	grain of the 1st class	0.350 ^{ns}
Crude protein	volume weight	0.412 ^{ns}

^{ns} – non significant (n = 10)

T a b l e 7

The impact of lignite, sodium humate and nitrogen application on the content of P, K and Ca in spring barley grains (dry mater – average of two years)

Variant		P	K	Ca
designation	number	[mg/kg]		
0	1	4,206.3^{f}	5,242.7^{e}	564.0^{a}
Lig_{Aut}	2	3,923.7^{cd}	4,974.3^{abc}	562.0^{a}
Hum_{Sow}	3	3,956.0^{cd}	4,939.7^{ab}	568.0^{a}
N_{Sow}	4	4,100.0^{e}	5,248.0^{e}	520.0^{a}
$Hum_{Sow}+N_{Sow}$	5	3,886.0^{bc}	5,146.7^{de}	534.0^{a}
$Lig_{Aut}+N_{Sow}$	6	3,824.3^{ab}	5,066.3^{bcd}	528.0^{a}
N_{BBCH23}	7	3,768.0^{a}	5,094.0^{cd}	522.0^{a}
Hum_{BBCH23}	8	3,991.0^{d}	4,998.0^{abc}	542.0^{a}
$Hum_{BBCH23}+N_{BBCH23}$	9	3,803.0^{ab}	4,956.0^{ab}	551.0^{a}
$Lig_{Aut}+N_{BBCH23}$	10	3,794.0^{ab}	4,912.7^{a}	538.0^{a}
$LSD_{0.05}$		98.124	136.927	46.412

Explanation: see Table 4 and 5

The impact of application of lignite and sodium humate on the calcium content was not recorded, which is in accordance with the above mentioned authors Halčínová and Kováčik (2011).

The level of available phosphorus in soil is influenced positively by the quantity of humus acids, which create chelate bonds with mineral sorbents of the phosphoric ions, protecting in this way the water-soluble phosphorus against the reactions with calcium, iron, aluminium and heavy metals (Kováčik 2014). The data in Table 8 proves this fact completely. The application of coal materials into soil increased the content of available phosphorus in soil considerably (var. 2 and 3). The addition of nitrogen to these materials (var. 5 a 6) increased the content of movable P in the soil, which is related to the support of mineralization of organic compounds containing phosphorus after the application of the mineral nitrogen fertiliser into soil. The utilizations of only foliar treatments by sodium humate (var. 8, 9) did not have a significant impact on the content of available phosphorus in soil. Table 8 shows that the lowest content of available P was in the control variant. This implies that with the foliar application of sodium humate, a part of substance gets into soil and influences the soil chemism.

We have enriched the information about the existence of direct correlation between the quantity of applied manure and the quantity of available phosphorus in soil (Kováčik 2014) by the fact about positive correlation coefficient *r* between the quantity of available phosphorus in soil and the content of total carbon in soil ($r = 0.647^{+}$).

The application of coal materials into soil (var. 2 and 3) increased the carbon content in soil, however, the increase was not considerable. The addition of nitrogen to lignite, or to sodium humate (var. 5 and 6) increased the carbon content significantly. Similarly, the independent application of commercial nitrogen fertiliser (var. 4 and 7) increased the quantity of carbon in the soil after harvest, which is a result of the positive impact of N not only on the aboveground phytomass (Table 8) but also on underground phytomass. Šimanský *et al*. (2008) also claim that after the application of commercial nitrogen fertilisers, the C_{ox} content can be both decreased and increased in soil.

The application of sodium humate, lignite and nitrogen fertiliser into soil and also the application of sodium humate and N fertiliser on leaves had an impact on the value of soil reaction; they increased it (Table 8, var. 2 – 10). The solo applications of sodium humate and DAM-390 into soil increased the pH value more than the application on leaves (var. 3 and 4 versus var. 7 and 8). The increase in pH values after the usage of sodium humate and also DAM-

T a b l e 8

The impact of lignite, sodium humate and nitrogen application on some soil parameters detected after finisching experiment in layer 0.0–0.3 m (dry mater – average of two years)

Variant		P [mg/kg]	C_{ox} [%]	pH/KCl
designation	number			
0	1	55.00^{a}	1.30^{a}	5.39^{a}
Lig_{Aut}	2	72.00^{bc}	1.40^{ab}	5.42^{ab}
Hum_{Sow}	3	79.50^{bc}	1.34^{ab}	5.60^{abc}
N_{Sow}	4	68.83^{ab}	1.43^{ab}	5.48^{ab}
$Hum_{Sow} + N_{Sow}$	5	86.75^{c}	1.47^{b}	5.73^{c}
$Lig_{Aut} + N_{Sow}$	6	82.00^{bc}	1.50^{b}	5.52^{abc}
N_{BBCH23}	7	57.75^{ab}	1.41^{ab}	5.43^{ab}
Hum_{BBCH23}	8	55.17^{a}	1.30^{a}	5.58^{abc}
$Hum_{BBCH23} + N_{BBCH23}$	9	59.83^{ab}	1.31^{a}	5.64^{bc}
$Lig_{Aut} + N_{BBCH23}$	10	73.50^{bc}	1.42^{ab}	5.54^{abc}
$LSD_{0.05}$		16.727	0.157	0.249

Explanation: see Table 4 and 5

390 were expected as these materials are alkalic. In the process of lignite application, the change was not expected because its pH value was almost the same as soil pH value. From a statistical viewpoint, the change did not happen. The difference was not significant. The considerable change of pH value appeared only in two variants, particularly in the case when apart from sodium humate, nitrogen was also applied (var. 5 and 9). This means that the significant change of soil pH value requires an abundant quantity of alkalic substances.

CONCLUSIONS

The autumn application of solid lignite and the pre-sowing application of solid sodium humate into soil tended to decrease the grain and straw yield of spring barley, content of crude protein in grain, proportion of the first-class grains and volume weight of grain. The impact of humate was more on the negative side; however, it was not significant statistically.

Lignite and sodium humate in solid form should be used along with nitrogen fertiliser.

The application of coal materials into soil increased the content of available phosphorus in soil significantly, and the carbon content insignificantly. The addition of nitrogen to these materials further increased the content of movable phosphorus and the total carbon in soil. We have detected a positive relationship between the quantity of the available phosphorus in soil and the content of total carbon in soil.

The application of sodium humate in liquid form during the growth season of spring barley tended to increase the grain and straw yield. The joint application of nitrogen and liquid sodium humate during the barley growth season increased the yield of barley grain significantly.

A lower dose of nitrogen, applied on barley leaves in growth phase BBCH 23, increased the yield of barley grain considerably more than a higher N dose applied into the soil before barley sowing.

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THE LONGEVITY OF CROP SEEDS STORED UNDER LONG-TERM CONDITION IN THE NATIONAL GENE BANK OF BULGARIA

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DESHEVA, G.: The longevity of crop seeds stored under long-term condition in the National gene bank of Bulgaria. Agriculture (Poľnohospodárstvo), vol. *62*, 2016, no. 3, p. 90–100.

Seed accessions from 7 plant families and 28 species stored for above 20 years in the National gene bank of Bulgaria were evaluated. All seed accessions were maintained as base collection under long-term storage conditions with low moisture contents (5±2%) in hermetically closed containers at –18°C. On the basis of experimental data, the seed storage characters σ (standard deviation of seed death in storage), $P_{50\%}$ (the time for viability to fall to 50%) and $P_{10\%}$ (the time for viability reduction of 10%) were determined allowing the prediction of seed storage life and the regeneration needs. The results showed significant differences in loss of seed viability among species and within the species. After 20–24 years of storage, eleven crops showed minimal viability decline under 5% as compared to the initial viability (oats, barley, maize, bread wheat, durum wheat, smooth brome grass, faba bean, chickpea, sunflower, cucumber and pepper). For the same storage time, another group of crops (sorghum, triticale, orchard grass, tall fescue, common vetch, grass pea, lentil, common bean, rapeseed, tobacco, flax, cabbage and tomatoes) presented 5–10% reduction of seed viability. More significant changes in seed viability – above 10% – were detected for peanuts, lettuce, soybean and rye. The σ values varied from 20.41 years (*Arachis hypogaea* L.) to 500 years (for *Avena sativa* L. and *Triticum aestivum* L). There was wide variation across species, both in time taken for the viability to fall to 50% and in time taken for the seed viability reduction of 10%. The study illustrates the positive effect of both seed storability early monitoring and prediction of regeneration needs as a tool for limiting undesired losses.

Key words: gene bank, long-term storage, seed germination, seed longevity, seed viability

The *ex situ* conservation of plant genetic resources in seed gene banks play an important role for food security in the future. Seed gene banks maintain genetic resources of the seeds over decades or centuries. They are an efficient and cost-effective way of conserving large amounts of genetic diversity (Waldren *et al*. 2000) as many thousands of seed collections representing different populations or plant taxa can be housed in a small area (Walsh *et al*. 2003). The initial viability of the seeds, seed moisture content and its interaction with relative humidity of the air and the storage temperature have significant influence on seed longevity (Roberts 1973). Even in seeds stored under optimal conditions suitable for long-term storage, viability may decrease as a result of deterioration processes (Sastry *et al*. 2008). Seed viability declines slowly at first, and then rapidly as seeds age (Roberts & Ellis 1982). Accordingly, it is important to know when this decline occurs so that the accession can be regenerated by replacing the exiting seeds with ones having high-viability (Ho-Sun *et al*. 2013). The Genebank Standards for Plant Genetic Resources for Food and Agriculture (2014) recommended the initial viability test be conducted as early as possible, before the seeds are packaged and enter the storage, and subsequent tests are determined at certain intervals during the storage. Viability monitoring test intervals can be set at one-third of the time predicted for viability to fall to 85 percent of initial viability or lower, depending on the species or specific accessions, but no longer than 40 years. If this deterioration pe-

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riod cannot be estimated, the interval is ten years for species expected to be long-lived and five years or less for species expected to be short-lived. It is recognized that viability monitoring is an expensive activity, and that gene bank curators wish to seek cost-cutting procedures.

Seed longevity is defined as seed viability after seed dry storage (storability), and therefore, describes the total seed life span (Rajjou & Debeaujon 2008). This storability period includes both dormant and non-dormant stages. During seed storage, seeds deteriorate, lose vigour, and as a result, become more sensitive to stresses during germination, and ultimately die. The rate of this aging depends on the seed moisture content, temperature, and initial seed quality (Walters 1998; Walters *et al*. 2005a). Seed longevity is a quantitative trait for which variation is present among naturally occurring accessions (Thu-Phuong *et al*. 2012). Understanding the differences in seed longevity in various species is therefore crucial to the effective management of seed conservation collections because it underpins the selection of viability re-test intervals, and hence regeneration or re-collection strategies (Probert *et al*. 2009; Groot *et al*. 2015).

The National Gene bank of Bulgaria was built in 1984 and carries out a scientific program for the long-term preservation of germplasm with seeds under controlled conditions, in accordance with the standards developed by FAO (1994/2014). The gene bank facilities are designed both for long-term storage and medium-term storage. There are 59,292 preserved seed accessions from 63,713 totally registered plant germplasms in the country (http://eurisco.ecpgr.org), where 72% are under long-term storage conditions (42,699 accessions).

The aim of this study was to evaluate the effect of the long-term gene bank storage from a practical point of view for the 28 plant species maintained for more than 20 years according to the recommended dry conditions and cold temperature.

MATERIALS AND METHODS

Seed material

A total of 5,876 seed accessions from 7 plant families and 28 species (**Poaceae** – *Avena sativa* L., *Bromus inermis* L., *Festuca arundinacea* Schreb., *Hordeum vulgare* L., *Secale cereale* L., *Sorghum vulgare* L., *X Triticosecale* Wittm., *Zea mays* L., *Triticum aestivum* L., *Triticum durum* Desf., *Dactylis glomerata* L., **Fabaceae** – *Vicia faba* L., *Vicia sativa* L., *Lathyrus sativus* L., *Cicer arietinum* L., *Arachis hypogaea* L., *Lens esculenta* L., *Phaseolus vulgaris* L., *Glycine max* Merr., **Solanaceae** – *Capsicum annuum* L., *Lycopersicon esculentum* Miller, *Nicotiana tabacum* L., **Brassicaceae** – *Brassica napus* L., *Brassica oleracea* L., **Asteraceae** – *Helianthus annuus* L., *Lactuca sativa* L., **Cucurbitaceae** – *Cucumis sativus* L., **Linaceae** – *Linum usitatissimum* L.) stored since 1981–1991 in the National Genebank of Bulgaria were evaluated. All seed accessions were maintained as base collections under long-term storage conditions with low moisture contents (5±2%) in hermetically closed containers (glass jars or three laminated aluminium foil packets) at –18°C.

Seed viability

Seed viability was detected on the basis of germination rate of accessions in storage. The recurring seed germination was determined at regular intervals: just before the storage, and periodically every following 10 years (i.e., 10 and 20 years, respectively). The germination tests of target plant species were carried out according to ISTA rules (ISTA 1985). The recommendations for work in the gene banks (Ellis *et al*. 1985a; 1985b; Hanson 1985) were also implemented. Seeds stored at –18°C for about 10 and 20 years, respectively, were pre-conditioned before these were set to germinate: equilibration of seed containers at room temperature for 24 hours was followed by re-humidification of seeds, as described earlier (Stoyanova 2001).

Seed moisture content

The moisture content of seed accessions, both before and after the time of storage, was determined using oven methods of ISTA (1985) for reduced working sample (about 1–3 g per accession).

Data analysis

The *Probit* analysis for modelling of data from seed storage experiments was used according to the models first described by Roberts (1973). It was based on a straight line relationship between viabil-

ity and storage period. The slope of this line was the value of σ and the intercept was the (theoretical) initial viability of seeds K_i (Ellis & Roberts 1980). The relationship used for calculation was:

$$\nu = K_i - p/\sigma,$$

where: ν was the viability in *Probit* after p years in storage.

Seed longevity is described by storage constants P_{50} and P_{10} according to Ellis and Roberts (1980), where P_{50} is the time for viability to fall to 50% and P_{10} is the time for viability reduction of 10%.

The information for seed accessions in storage was maintained as ACCESS-database. The raw data files were used for statistical analysis by analysis of variance (ANOVA) and *LSD* test using *IBM SPSS Statistics 19*.

RESULTS

Maintenance of seed germination in different crops

Cereals

In the group of cereals, 4,138 seed accessions from eight species were evaluated (Table 1). Minimal changes of seed quality under storage were detected for four species: barley, oats, maize and wheat. Here, no significant differences were examined between seed germinations at the beginning of storage and after a significant time of storage, in both first control test (with storage time of 10–14 years) and second control test (with storage time of 20–24 years). However, it should be pointed out that as presented in a previous research, the rate of difference before and after the storage influenced the mean value of seed germination, and also had a stronger effect on the standard deviation (Stoyanova 2001).

Another group of cereals was more significantly affected by storage: rye, sorghum and triticale. As presented, the decline of mean seed germination for rye accessions was from 94.57% to 85.51% after 12.94 years and to 83.68% after 23.48 years. The mean germination percentages of sorghum and triticale after 10–14 years of storage dropped by about 10% in comparison with the mean initial germination values. The seed germination rate of these three species after the second control test remained about 82–83%.

The rate of variation between species was illustrated by differences of standard deviations calculated for the mean values of germination after storage. But the difference was more significant in the cases with larger variations between minimum and maximum values and a considerable reduction in seed germinability.

Cereal grasses

Grass seeds included in the study belong to three plant species were *Bromus inermis* L., *Dactylis glomerata* L. and *Festuca arundinacea* Schreb. An inappreciable loss of viability occurred after 10–14 years of storage for *Dactylis glomerata* L. and *Festuca arundinacea* Schreb., and an unnoticeable increase in seed germination for *Bromus inermis* L. After 20–24 years of storage, the mean germination values of *Dactylis glomerata* L. and *Festuca arundinacea* Schreb. decreased more than 5% as compared to the initial germination percentages. Their SDs increased significantly from ±6.39 to ±12.18% and from ±5.29 to ±10.30%, respectively (Table 1).

Legumes

Seed accessions from eight species were evaluated. Two species – *Vicia faba* L. and *Cicer arietinum* L. – preserved high mean germination values after the first and second control tests. Practically no significant difference in comparison to initial germination percentages was assessed for both species. The highest decrease of viability was recorded for *Arachis hypogaea* L. and *Glycine max* Merr. After 13.77 years of storage, the germination of peanut seeds declined from 91.96% to 69.96%, while after 22.35 years of storage, from 91.96% to 63.07%. The SDs increased from ±6.67% to ±16.56% and ±18.35%, respectively. The seed germination of soybean accessions also decreased significantly – above 10% in relation to initial value. Other legumes (*Vicia sativa* L., *Lathyrus sativus* L., *Lens esculenta* L. and *Phaseolus vulgaris* L.) showed slower reduction in mean value of germination (with 7.81%, 7.14%, 5.22% and 7.68%, respectively) after 21–24 years of storage (Table 1).

Industrial crops

Four species were tested in this group. The average germination rate of *Brassica napus* seeds in the

beginning of storage was 94.72%. Subsequently, at the first control test after 13.27 years, it decreased to 85.79% and retained almost the same until the second control test. The seeds of *Helianthus annuus* were preserved with minimal changes. A slow drop of seed germination of *Nicotiana tabacum* was observed after 13 years of storage but the second control test presented a more significant decrease to about 90–91%. The value of standard deviation in both test cases for tobacco seeds increased, indicating variation in a large scale between accessions. For *Linum* accessions, a decrease in seed germination from the beginning of storage up to first control test was observed, maintaining the value to the second test. The tested seed accessions were preserved with no significant changes for more than 20 years (Table 1).

Vegetables

The vegetable seeds included in the study belonged to five plant species. Cucumber and pepper seeds showed no significant change in germination percentage after more than 20 years of storage. In contrast, viability of lettuce seeds decreased from 85.18% to 75.83% after 13.40 years and to 72.40% after 22.37 years of storage. The SDs increased from ±8.81% to ±11.60% and ±14.20%, respectively. The mean germination percentage of tomato seeds dropped by 7.16% after 11.30 years of storage. There was no further significant decrease in the following years in the second control test. The value of SD between both the tests was in the same range. The viability of *Brassica oleracea* L. after 22.64 years of storage also decreased significantly by 7.15%.

Monitoring of seed viability and longevity

Results from the assessment of *Probit* longevity for 28 plant species under real long-term storage condition are presented in Table 2. The parameters estimated were K_i (*Probit* value of initial seed viability), $1/\sigma$ (measure of seed deterioration in storage), σ (standard deviation of seed death in storage), $P_{50\%}$ (seed half-life or measure of time to 50% seed viability in storage) and $P_{10\%}$ (time in years for seed viability reduction with 10%).

The highest K_i value (2.39) was recorded for *Cucumis sativus* L., while rate of seed deterioration ($1/\sigma$) was the highest for *Arachis hypogaea* L. (–0.049) and the lowest for *Avena sativa* L. and *Triticum aestivum* L. (–0.002). The σ values varied from 20.41 years (*Arachis hypogaea* L.) to 500 years for *Avena sativa* L. and *Triticum aestivum* L. Nineteen plant species had σ values under 100 years ($\sigma < 100$ years), five species between 100 and 300 years ($100 < \sigma < 300$) and only oats, barley, bread wheat and sunflower had values above 300 years ($300 < \sigma < 1000$). There was a wide variation across species in both the time taken for viability to fall to 50% and the time for seed viability reduction by 10%. $P_{50\%}$ ranged between 28.51 years for *Arachis hypogaea* L. to 880 years for *Avena sativa* L. (Table 2). The safe storage time ($P_{10\%}$) was between 9 to 20 years for peanut, soybean and lettuce, and 318.5 and 338.5 years for bread wheat and oats, respectively.

DISCUSSION

It is very important that seeds stored in the gene bank are capable of producing plants when sown in the field. They must have high viability at the start of the storage, and maintain it during storage. Seeds with a high initial viability will also survive longer in storage. The seed initial germination percentage in the National Genebank of Bulgaria should be above 80%, which is the lowest viability standard for seed accessions to be included in the long-term storage (Stoyanova 2001). Good seed storage conditions maintain germplasm viability, but even under excellent conditions, viability declines with storage duration (Walters *et al*. 2005b). Seed ageing or seed deterioration is commonly described as the loss of seed quality or viability over time (Coolbear 1995). Therefore, gene banks need to assess the viability periodically to detect the loss in viability during storage before it falls below the threshold for regeneration (Chin 1994). Seed viability is determined through germination tests before seeds are packaged and placed in the gene bank storage room, and this serves as a reference point for subsequent periodic viability checks during storage (Smith *et al*. 2003; FAO 2014).

In this study, the viability after 10–14 and 20–24 years of storage were assessed among 28 crop species. The results showed that there were signifi-

cant differences in the loss of seed viability among species and within the species. Eleven crops (oats, barley, maize, bread wheat, durum wheat, smooth brome grass, faba bean, chickpea, sunflower, cucumber and pepper) showed relatively stable storage after 20–24 years, as indicated by the respective declines in the viability under 5%. Sorghum, triticale, orchard grass, tall fescue, common vetch, grass pea, lentil, common bean, rapeseed, tobacco, flax, cabbage and tomatoes presented 5–10% declines in viability, while peanuts, lettuce, soybean and rye showed above 10% after 20–24 years of storage (Table 1).

From the germination results obtained after 10–14 years of storage, some species as *Avena sativa* L., *Hordeum vulgare* L., *Dactylis glomerata* L. and *Bromus inermis* L. showed slow increase in the germination percentages from initial values, and except for *Dactylis glomerata* L., an insignificant decrease in the rates after 20–24 years of storage. The increase in germination after storage relates to post-harvest dormancy (Ruiz *et al*. 1999; Stoyanova 2001; Walsh *et al*. 2003; Pérez-García *et al*. 2009).

When comparing the initial germination percentages with results from the first control test (10–14 years of storage), other crops as rye, triticale, rapeseeds, flax, lettuce and tomatoes showed a significant decrease in the germination rate ranging between 6 and 10%. However, when comparing the results between first and second control tests, there were no significant changes.

Seeds of species as peanut and soybean are characterized as short-lived (Copeland & McDonald 2001; Stoyanova 2001; Walters *et al*. 2005b). In this study, they showed the largest reductions in viability after first and second retests.

In some leguminous seeds, the second test exceeded the seed germination from the first test. The cause for this was that in the past, the hard seeds in the germination test were described as 'non-germinating'. Some changes in the viability may have also been the results of different degrees of operator error, as the staff performing the germination test changed over time.

According to Lu *et al*. (2004), the genetic characteristics of species and pre-storage environments are the main factors for seed viability decline. Adverse climate at the stages of seed ripening and harvesting, as well as the damage caused by seed-extraction, drying and transportation after harvest could affect the rate of seed viability decline during storage (Sai Babu *et al*. 1983).

From a practical point of view, it is important to be able to predict the appropriate frequency of control tests in the gene bank. The determination of the maximum storage period for each material in particular conditions of each seed bank is of great importance in designing management guidelines that minimize viability controls and regeneration/multiplication of the samples (Pita *et al*. 1998; Probert *et al*. 2009). Regeneration is a costly gene bank operation, and may also negatively affect the genetic integrity of an accession through exposure to the influence of genetic drift, selection, contamination and human error (Parzies *et al*. 2000; Benkova & Zakova 2008; Fu *et al*. 2015). Therefore, it is important to maximize seed longevity and keep operational costs and logistics manageable through the monitoring of seed deterioration, an essential task for managing stored germplasm (Engels & Visser 2003).

The measure of seed longevity in this study is based on the σ value (standard deviation of seed death in storage), defining the period during which the percentage viability is reduced by one *Probit* as described by Hong *et al.* (1998). According to Ellis and Roberts (1980), the life span of a seed-lot, the time until all the seeds have lost viability, depends on the value of σ and on the proportion of the seeds which are viable at the start of the storage, K_i (in *Probit*). The seed longevity varies among families, species, genotypes, seed lots, and even among individual seeds inside the same bag and depends on the storage conditions (Walters *et al*. 2005b; Nagel *et al*. 2009; Probert *et al*. 2009; Nagel & Börner 2010; Nagel *et al*. 2010; van Treuren *et al*. 2013; Ho-Sun *et al*. 2013). In our study σ value varied from 20.41 to 500 years. It was the lowest for *Arachis hypogaea* L. (20.41 years) and the highest for *Avena sativa* L. and *Triticum aestivum* L. (500 years). Grain legumes, except chickpea, were characterized with a short life span ($\sigma < 100$ years) (Stoyanova 2001). The seeds of bread wheat, oats, barley and sunflower were found to have the longest storability ($\sigma > 300$ years). The predicted longevity was 111.11 years for *Triticum durum* Desf., *Dactylis glomerata* L., *Cicer arietinum* L. and *Capsicum annuum*

T a b l e 1

Changes in the seed viability values of plant species during the storage period in the National Gene bank of Bulgaria

Crops/Species	Number of access.	Initial seed germination [%]	Storage period to the first control test [year]	Mean value of germination in the first control test ±SD [%]	Storage period from first to second control test [year]	Mean value of germination in the second control test ±SD [%]	Total storage period [year]
Cereals							
Avena sativa L.	457	95.76±6.08	12.94	96.59±5.74	10.18	95.28±7.34	23.12
Hordeum vulgare L.	1195	93.22±5.46	14.28	93.40±6.04	8.50	91.99±6.19	22.78
Secale cereale L.	35	94.57±5.18	12.94	85.51±4.84^{+++}	10.54	83.68±7.71^{+++}	23.48
Sorghum vulgare L.	99	91.39±6.37	10.72	80.41±18.85^{++}	13.41	83.63±17.35^{++}	24.13
X Triticosecale Wittm.	52	92.00±6.48	11.67	82.62±12.98^{++}	12.33	82.03±16.50^{++}	24.00
Zea mays L.	364	94.60±5.54	12.30	90.31±8.75	11.19	91.19±9.26	23.49
Triticum aestivum L.	1547	95.51±4.99	13.70	95.11±5.12	9.33	94.97±4.82	23.03
Triticum durum Desf.	389	95.37±5.54	11.42	94.12±7.10	15.87	92.58±7.22	27.29
Cereal grasses							
Dactylis glomerata L.	55	83.81±6.39	11.83	84.36±5.94	10.95	78.4±12.18^{++}	22.78
Bromus inermis L.	37	89.83±6.15	13.45	90.37±5.86	7.68	87.83±5.27	21.13
Festuca arundinacea Schreb.	34	96.23±5.29	13.23	92.26±10.41	9.74	89.52±10.30^{++}	22.97
Legumes							
Vicia faba L.	57	95.68±5.99	13.35	94.64±7.20	8.59	92.45±7.18	21.94
Vicia sativa L.	131	91.94±7.50	10.45	81.39±9.39^{+++}	13.71	84.12±12.47^{++}	24.16
Lathyrus sativus L.	65	94.81±4.40	11.24	85.30±7.33^{+++}	12.51	87.67±7.48^{+++}	23.75
Cicer arietinum L.	37	94.48±6.34	12.59	89.59±5.96	11.32	91.72±5.36	23.91
Arachis hypogaea L.	54	91.96±6.67	13.77	69.96±16.56^{++}	8.58	63.07±18.25^{++}	22.35
Lens esculenta L.	56	95.25±5.41	11.92	88.25±10.40^{++}	11.70	90.03±8.22^{++}	23.62
Phaseolus vulgaris L.	56	91.71±6.51	12.72	79.30±15.48^{++}	8.97	84.03±14.18^{++}	21.69
Glycine max Merr.	306	91.87±7.29	11.09	77.91±13.64^{++}	12.78	80.56±13.12^{++}	23.87
Industrial crops							
Brassica napus L.	44	94.72±6.08	13.27	85.79±8.93^{+++}	8.45	85.31±8.29^{+++}	21.72
Helianthus annuus L.	53	91.92±6.63	13.33	92.72±7.09	9.74	90.54±10.42	23.07
Nicotiana tabacum L.	117	97.02±5.38	13.00	93.64±12.00	8.82	90.76±13.29^{++}	21.82
Linum usitatissimum L.	67	96.71±4.71	12.22	90.86±5.19^{+++}	11.00	89.13±10.09^{++}	23.22
Vegetables							
Brassica oleracea L.	48	89.00±8.03	13.64	84.27±17.78	9.00	81.85±17.78^{+}	22.64
Lactuca sativa L.	177	85.18±8.81	13.40	75.83±11.60^{++}	8.97	72.40±14.20^{++}	22.37
Cucumis sativus L.	68	99.26±2.15	13.54	97.88±3.22	9.37	97.94±2.80	22.91
Lycopersicon esculentum Miller	63	92.50±6.67	11.30	85.34±10.01^{++}	12.21	84.04±10.72^{++}	23.51
Capsicum annuum L.	30	90.66±6.91	13.30	87.76±7.27	8.13	87.13±6.98	21.43

$^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{+++}p \leq 0.001$, SD-standard deviation [%]

T a b l e 2

Seed longevity of plant species predicted after real long-term storage in National Gene bank of Bulgaria

Crops/Species	Number of access.	Seed moisture content [%]	Total storage period [year]	K_i	1/σ	σ [year]	$P_{10\%}$ [year]	$P_{50\%}$ [year]
Cereals								
Avena sativa L.	457	6.22±0.27	23.12	1.761	–0.002	500.00	338.5	880.00
Hordeum vulgare L.	1195	6.85±0.21	22.78	1.509	–0.003	333.33	179.0	502.67
Secale cereale L.	35	7.09±0.37	23.48	1.544	–0.027	37.03	20.55	55.85
Sorghum vulgare L.	99	6.59±0.26	24.13	1.241	–0.015	66.66	28.33	82.67
X Triticosecale Wittm.	52	6.91±0.50	24.00	1.327	–0.020	50.00	22.85	66.30
Zea mays L.	364	7.07±0.32	23.49	1.552	–0.011	90.90	50.81	141.00
Triticum aestivum L.	1547	6.91±0.26	23.03	1.695	–0.002	500.00	318.5	847.00
Triticum durum Desf.	389	7.09±0.42	27.29	1.675	–0.009	111.11	69.44	186.00
Cereal grasses								
Dactylis glomerata L.	55	6.12±0.34	22.78	1.027	–0.009	111.11	39.89	114.00
Bromus inermis L.	37	5.84±0.16	21.13	1.295	–0.004	250.00	111.2	323.50
Festuca arundinacea Schreb.	34	5.78±0.22	22.97	1.763	–0.023	43.47	29.47	76.61
Legumes								
Vicia faba L.	57	6.91±0.50	21.94	1.732	–0.012	83.33	54.92	144.25
Vicia sativa L.	131	6.81±0.28	24.16	1.275	–0.015	66.67	29.13	84.93
Lathyrus sativus L.	65	6.99±0.37	23.75	1.502	–0.019	52.63	28.11	79.00
Cicer arietinum L.	37	5.94±0.51	23.91	1.525	–0.009	111.11	60.56	169.33
Arachis hypogaea L.	54	3.37±0.28	22.35	1.347	–0.049	20.41	9.49	28.51
Lens esculenta L.	56	6.84±0.10	23.62	1.575	–0.016	62.50	35.94	98.38
Phaseolus vulgaris L.	56	7.25±0.25	21.69	1.293	–0.020	50.00	22.20	64.60
Glycine max Merr.	306	5.95±0.52	23.87	1.261	–0.022	45.45	19.64	57.27
Industrial crops								
Brassica napus L.	44	4.56±0.25	21.72	1.567	–0.028	35.71	20.25	55.93
Nicotiana tabacum L.	117	3.30±0.06	21.82	1.877	–0.026	38.46	28.92	72.15
Helianthus annuus L.	53	3.94±0.22	23.07	1.429	–0.003	333.33	166.60	476.00
Linum usitatissimum L.	67	4.59±0.29	23.22	1.780	–0.026	38.46	26.50	68.42
Vegetables								
Brassica oleracea L.	48	4.59±0.17	22.64	1.219	–0.014	71.43	29.86	87.00
Capsicum annuum L.	30	4.53±0.09	21.43	1.310	–0.009	111.11	50.11	145.44
Lycopersicon esculentum Miller	63	4.81±0.36	23.51	1.379	–0.019	52.63	25.21	72.53
Lactuca sativa L.	177	4.22±0.37	22.37	1.025	–0.021	47.62	17.10	48.76
Cucumis sativus L.	68	4.69±0.20	22.91	2.390	–0.018	55.56	64.44	132.72

K_i – *Probit* value of initial seed viability; 1/σ – measure of seed deterioration in storage; σ – standard deviation of seed death in storage; $P_{10\%}$ – time in years for seed viability reduction with 10%; $P_{50\%}$ – seed half-life or measure of time to 50% seed viability in storage

L., and 250 years for *Bromus inermis* L. Generally, 68.75% of the evaluated species are characterized with a short life span after the real long-term storage in National Genebank of Bulgaria (σ < 100 years) (Table 2). Longevity can also be described by determining the time taken for viability to fall to 50% ($P_{50\%}$). Walters *et al*. (2005b) reported that large families as *Asteraceae*, *Fabaceae*, *Poaceae*, and *Solanaceae* contained species with wide-ranging $P_{50\%}$ values, that is the time required for germination to decrease to half the initial value. Similarly, Probert *et al*. (2009) reported wide variation in $P_{50\%}$ between families, as well as within some families, but relatively little variation in longevity among genera within those families. Differences in seed longevity between cultivars and wild types of crops have been reported for diverse taxa (Walters *et al*. 2005b; Ellis & Hong 2007). In the presented study, there was wide variation between crops in the time taken for viability to fall to 50%. The calculated value was the lowest for peanuts (28.51 years), following by lettuce (48.76 years), rye (55.85 years), rapeseeds (55.93 years) and soybean (57.27 years). The crops can be classified into groups having high longevity ($300 < \sigma > 1000$) – oats, bread wheat, barley and sunflower have the highest $P_{50\%}$ values, respectively 880, 847, 502.67 and 476 years. The species with similar σ values are differentiated by $P_{50\%}$ values.

Seed aging during storage is an inevitable phenomenon, but the degree and speed of decline in seed quality depends strongly, beside storage conditions, on plant species stored and initial seed quality (Elias & Copeland 1994; Balešević-Tubić *et al.* 2005), as well as on the seed genetic traits (Malenčić *et al.* 2003). Milošević *et al.* (1996) also suggested that seed longevity is genetically determined, and that significant differences exist among cultivars of same crops in their ability to maintain quality during storage (Balešević-Tubić *et al.* 2010). Seed structure and climate of origin are also known genetic factors related to seed longevity (Probert *et al*. 2009).

As mentioned before, a decrease in seed viability of 10% could induce genetic shifts in heterogeneous seed accessions (Stoyanova 1991; 1992; 1996). The safe storage time ($P_{10\%}$) or the time for seed viability reduction of 10% was calculated using the viability equation proposed by Ellis and Roberts (1980). According to the results presented in Table 2, plant species described with shortest longevity (*Arachis hypogaea* L., *Lactuca sativa* L., *Glycine max* Merr., *Brassica napus* L.*, Secale cereale* L. and *X Triticosecale* Wittm.) should not be monitored later than 10–20 years from the beginning of storage. The safe storage time for *Sorghum vulgare* L., *Festuca arundinacea* Schreb., *Vicia sativa* L., *Lathyrus sativus* L., *Nicotiana tabacum* L., *Linum usitatissimum* L., *Brassica oleracea* L. and *Lens esculenta* L. is prolonged to 25–35 years, while for *Dactylis glomerata* L., *Zea mays* L., *Triticum durum* Desf., *Vicia faba* L., *Cicer arietinum* L., *Capsicum annuum* L. and *Cucumis sativus* L. is 40–60 years or more. When safe storage is longer than 100 years, the risk of losses in time is limited (Stoyanova 2001). The predicted storage times for *Bromus inermis* L., *Helianthus annuus* L. *and Hordeum vulgare* L. were above 100 years, while for plant species described with longest longevity (*Avena sativa* L. and *Triticum aestivum* L.) was above 300 years.

However, it should be pointed out that the *Probit* analysis for modelling was made on a storage period (–18°C) of around 20 years. This is a rather short time for such conditions, and therefore, future investigations need to be made in this area. On the other hand, Nagel *et al*. (2010) mentioned that viability equation can forecast a longevity tendency, but the behaviour of a specific genotype depends on more factors than moisture content, storage temperature and initial viability. Therefore, as a guarantee for prevention of changes, the predictions should be carried out on plant groups according to the conditions for compatibility in each species. Moreover, seed viability monitoring should be done individually, and should not be monitored selectively because there was a large variation of viability among accessions.

CONCLUSIONS

The results obtained from the monitoring tests indicate that the storage conditions in the National Seed Gene bank of Bulgaria are suitable for the preservation of the investigated species. Only two species (*Arachis hypogaea* L. and *Glycine max* Merr.) showed significantly decreased germination

percentages when compared with the germination percentages before storage, but the damages leading to loss of accessions were not observed. However, viability differs greatly between species and within a species, as does the response to storage. Seed longevity calculated as σ values varied from 20.41 to 500 years. Generally, 68.75% of the evaluated species are characterized with short life span after real long-term storage in National Gene bank of Bulgaria (σ < 100 years). The longest storability had bread wheat, oats, barley and sunflower (σ > 300 years). The predicted time of safe storage ($P_{10\%}$) for peanuts, lettuce, rye, triticale, rapeseeds and soybean was 10–20 years. The safe storage time for sorghum, tall fescue, common vetch, grass pea, tobacco, flax, rapeseed and lentil was prolonged to 25–35 years, while for orchard grass, maize, durum wheat, faba bean, chickpea, pepper and cucumber was 40–60 years or more. The predicted storage times for smooth brome grass, sunflower and barley were above 100 years, while for plant species described with longest longevity (oats and bread wheat) was above 300 years. The presented results are a useful tool for the monitoring of gene bank storage and the prediction of regeneration needs in the National Gene bank of Bulgaria.

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ENHANCING OF WINTER WHEAT PRODUCTIVITY BY THE INTRODUCTION OF FIELD PEA INTO CROP ROTATION

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The aim of our study was to find out the influence of different preceding crops and weather in particular years on the production ability of winter wheat in crop rotation with 80% share of cereals. The long-term field trial with 40, 60 and 80% share of cereals and two levels of fertilisation (H_1 mineral fertilisation + organic manure Veget®; H_2 mineral fertilisation only) was carried out in the very warm and dry area of continental climate on Luvi-Haplic Chernozem. In crop rotation with 80% share of cereals, winter wheat was sown after two preceding crops: pea and winter barley. In the years 2013–2015, the weight of 1,000 grains, bulk density, share of grains over 2.8 + 2.5 mm sieve, grain yield and straw yield were investigated. In crop rotation with 80% share of cereals, the significantly higher grain yield of winter wheat was recorded after preceding crop of field pea (6.18 t/ha) by comparison with winter barley as preceding crop (5.19 t/ha). The statistically significantly higher straw yield of winter wheat was observed after field pea (8.38 t/ha) in comparison to winter barley (7.29 t/ha). The weight of 1,000 grains, bulk density, share of grains over 2.8 + 2.5 mm sieve were statistically significantly higher after field pea in comparison to winter barley. The winter wheat grain yield can be increased by the preceding crop pea and the combination of mineral and organic fertilisation in substantial degree.

Key words: share of cereals, legumes, preceding crop, grain yield, bulk density, weight of thousand grains

Economic conditions are forcing farmers to grow crops that yield high revenue, leading to cereal-dominated crop rotations with increasing risk due to unfavourable preceding crops or preceding crop combinations (Sieling & Christen 2015). Crop rotations in Slovakia are constantly adapted to economic conditions and political intentions. In agricultural co-operative farms, the so-called free crop rotation is applied. It means that farms grow one or two main crops. Cropping systems based on winter wheat are particularly widespread in non-irrigated land, wherein other higher yielding spring crops may fail, due to water shortage in summer. In these conditions, the frequency of wheat within rotations becomes very high and continuous cropping may often be the only profitable option. Rotations based on high frequency of wheat are at risk of reduced yield levels (Bonciarelli *et al.* 2016). Indeed, it has been shown that, in the short term, wheat following wheat gives a lower yield than wheat following a different crop (Sieling *et al.* 2005). Legume production has declined in most of Europe, from 4.7% of arable land in 1961 to 1.6% in 2013 (FAOstat 2015). There are many reasons why farmers do not grow legumes, including specialisation in cereal crop production, low and unstable yields (Cernay *et al*. 2015; Reckling *et al.* 2016), low and unpredictable policy support (Bues *et al*. 2013), and inability to recognise or evaluate the long-term benefits of legumes within cropping systems (Preissel *et al.* 2015). Grain legumes are known to increase the soil mineral nitrogen (N) content, reduce the infection pressure of soil borne pathogens and hence enhance subsequent cereals yields (Hauggaard-Nielsen *et al.* 2012). While

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the effect of legumes on yield of the following crop is easily measured, changes in root growth and pressures from pests and pathogens are harder to quantify. Legumes generally have lower gross margins than cereals or oilseeds, but their rotational effects increase the gross margins of subsequent crops, so assessment of legumes needs to be performed at the cropping system scale (Preissel *et al*. 2015). Sarunaite *et al.* (2013) evaluated the possibility to associate cereals with legumes as an alternative double-cropping system for supplying biological N to cereals. Pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.) and lupin (*Lupinus angustifolius* L.) grain legumes are the most adapted species to temperate growing conditions (Hauggaard-Nielsen *et al.* 2012). Field pea belongs among agricultural plants with great agronomical importance. The primary importance of legumes is their ability to fix atmospheric nitrogen (Danilovič & Šoltýsová 2010). Bielek (1998) reported that they fixed about 10 kg/ha/year of nitrogen in condition of Slovakia. The potential of field pea is markedly higher, pea may fix about 48–167 kg/ha N (Nielsen *et al.* 2009). The grain yield of spring wheat can be enhanced following field pea (Carr *et al.* 2008).

The aim of our research was to investigate the influence of preceding crop, fertilisation and weather in particular years on the grain yield and yield components of winter wheat in the crop rotation with 80% share of cereals.

MATERIAL AND METHODS

The research was realised in 2013–2015 in a long-term field trial. The trial was established in 1974 at Experimental Station Borovce, which belongs to Research Institute of Plant Production in Piešťany. In 2013–2015, the variety of winter wheat Bertold was used.

Long-term field trial is situated on Luvi-Haplic Chernozem, pH 5.5–7.2, with a humus content of 1.8–2.0%. The area has continental pattern of climate with long-term average sum of annual rainfall of 593 mm and 358 mm rainfall during vegetation period. Long-term average of annual temperature is

T a b l e 1

Weather conditions in the experimental years 2013–2015 on the stand Borovce

Month	n (1951–1980)		2013		2014		2015	
	x_{td} [C°]	∑ [mm]	x_{td} [C°]	∑ [mm]	x_{td} [C°]	∑ [mm]	x_{td} [C°]	$\sum_z$ [mm]
January	–1.8	32	–2.55	69.8	–0.21	34.4	–1.29	64.5
February	0.2	33	–0.77	90.3	1.22	33.2	–1.51	28.9
March	4.2	32	0.84	75.3	6.14	20.7	2.71	53.1
April	9.4	43	9.15	17.4	9.65	65.7	8.17	21.9
May	14.1	54	13.52	67.4	13.16	110.3	13.27	58.9
June	17.7	80	17.51	70.1	18.0	34.5	18.34	21.0
July	18.9	76	20.71	3.0	19.85	120.1	22.28	24.8
August	18.4	68	20.12	112.9	17.12	50.9	21.94	111.0
September	14.5	38	11.59	75.6	14.06	122.9	14.13	47.6
October	9.6	42	8.71	29.1	9.19	53.3	7.12	63.5
November	4.6	51	3.33	59.7	4.78	24.9	3.45	40.5
December	0.3	46	–0.57	9.9	–0.23	49.4	–0.06	22.0
x_{td} [°C]	9.2	–	8.47	–	9.40	–	9.05	–
$\sum_z$ [mm]	–	595	–	680.4	–	720.3	–	557.7

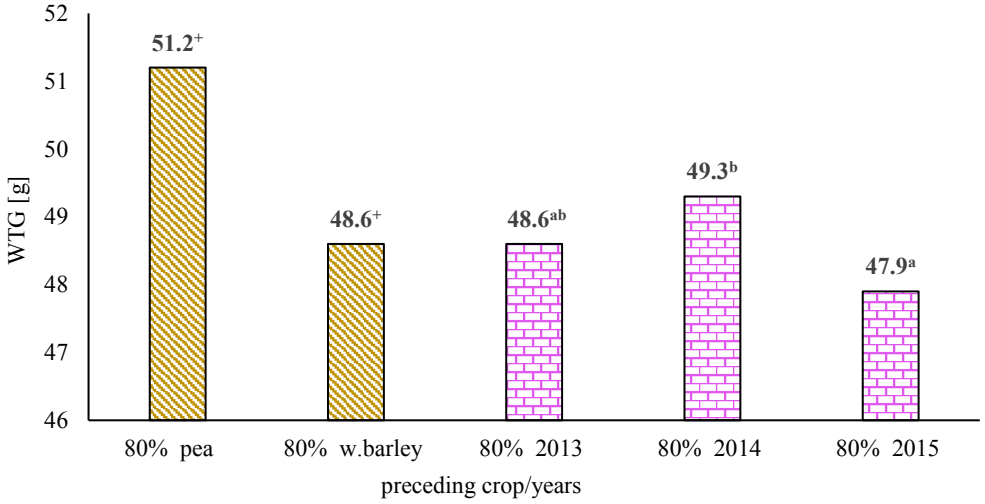
n – long-term (30-year) normal; x_{td} – average air temperature; ∑ – sum of precipitation

9.2°C, and in vegetation period, it is 15.5°C (Table 1). In the field experiment, there were crop rotations with 40%, 60% and 80% proportion of cereals. Crops in different crop rotations are presented in Table 2. In crop rotation with 80% share of cereals, winter wheat was grown after two preceding crops: field pea and winter barley. The fertilisation level H_1: fertilisation by phosphorus and potassium was carried out by balance method according Bizík *et al*. (1998). Nitrogen fertilisation was used according to the content of nitrogen in soil (soil samples were taken from 0.0 to 0.3 m of soil profile) + organic manure Veget® in dose of 5 tons per hectare. The composition of organic fertiliser Veget® [ČOV, a. s., (joint stock company Cleaner of Waste Water), Slovenská Ľupča, Slovakia] is as follows: dry matter content at a minimum of 85% (includes combustible matter content 75%; total N content 2.5–3.0%; total P_2O_5

T a b l e 2

The crop rotations with 40%, 60% and 80% share of the cereals

Crop rotations		
40% share of cereal	60% share of cereal	80% share of cereal
1. pea	1. pea	1. winter wheat
2. winter wheat	2. winter wheat	2. spring barley
3. silage maize	3. winter barley	3. pea
4. spring barley	4. silage maize	4. winter wheat
5. grain maize	5. spring barley	5. winter barley



Average weight of one thousand grains after different preceding crop – pea; w. barley – winter barley; +significant difference at the level α = 0.05. Average weight of one thousand grains in years after both preceding crops – different literae – significant difference at the level α = 0.05; equal literae – insignificant difference at the level α = 0.05.

Figure 1. The weight of one thousand grains [g] of winter wheat after field pea and winter barley in years 2013–2015

content 0.5–2.0%, and K_2O content 1.5%), ratio C:N 13:1, and pH (in water) 8.5. Veget® is produced from waste materials after industrial antibiotics production. The fertilisation level H_2: fertilisation of phosphorus, potassium and nitrogen was applied at the same level as H_1 except organic manure Veget®.

The data was computed by multiple-way analysis of variance (ANOVA) and the *LSD* multiple range test was used at the 0.05% level. STATISTICA 6. 1 (StatSoft Inc., Tulsa, USA) software was used.

RESULTS AND DISCUSSION

In 2013–2015, the average monthly temperatures and monthly sums of monthly rainfall (Table 1) differ from the long-term average (1951–1980). In 2013, the average monthly temperature was lower by 3.36°C than in the long-term average. In 2013, the monthly sum of rainfall was higher in February and in March than the long-term average. In 2014, the monthly sum of rainfall in May was higher by 56.3 mm than the long-term average. On the contra-

Table 3

The influence of fertilisation and preceding crop on the weight of one thousand grains of winter wheat (analyse of variance)

Factor	WTG [g]				
	df	MS	F	*P*	$LSD_{0.05}$
Fertilisation (A)	1	3.15	0.89	–	–
PC (B)	1	82.43	23.21	+	1.11
A × B	1	7.44	2.10	–	–
Years (C)	2	32.60	9.18	+	1.64
A × C	2	2.87	0.81	–	–
B × C	2	10.19	2.87	–	–
Total	47	7.10	–	–	–
Error	33	3.55	–	–	–

PC – preceding crop; *df* – degrees of freedom; MS – average squares, F – F-test; *P* – effect of a factor significant at the level 0.05; $LSD_{0.05}$ – least significant difference at the level α = 0.05

Table 4

The influence of fertilisation and preceding crop on weight bulk and share of grains over 2.8 + 2.5 mm sieve of winter wheat (analyse of variance)

Factor	Bulk density [g/l]					Share of grains over sieve 2.8 + 2.5 mm [%]				
	df	MS	F	*P*	$LSD_{0.05}$	*df*	MS	F	*P*	$LSD_{0.05}$
Fertilisation (A) (A [A]	1	70.1	0.26	–	–	1	7.76	0.69	–	–
PC (B)	1	3536.33	13.35	+	9.56	1	181.35	16.04	+	1.98
A × B	1	1.33	0.01	–	–	1	49.01	4.34	+	3.72
Year (C)	2	6460.4	24.39	+	14.13	2	205.00	18.14	+	2.92
A × C	2	492.7	1.86	–	–	2	14.98	1.33	–	–
B × C	2	2807.0	10.60	+	24.63	2	16.75	1.48	–	–
Total	47	710.47	–	–	–	47	25.11	–	–	–
Error	33	264.8	–	–	–	33	11.30	–	–	–

PC – preceding crop; *df* – degrees of freedom; MS – average squares, F – F-test; *P* – effect of a factor significant at the level 0.05; $LSD_{0.05}$ – least significant difference at the level α = 0.05

ry, the monthly sum of rainfall in June was lower by 45.5 mm than the long-term average. In 2015, the growth of winter wheat was negatively influenced by abnormal rainfall. The monthly sum of rainfall was higher by 32.5, 21.1 and 21.1 mm in January, March and April, respectively. On the contrary, the monthly sum of rainfall in June was lower by 59.0 mm than the long-term average. Weather conditions of particular years were the most important factor affecting the grain yield of winter wheat.

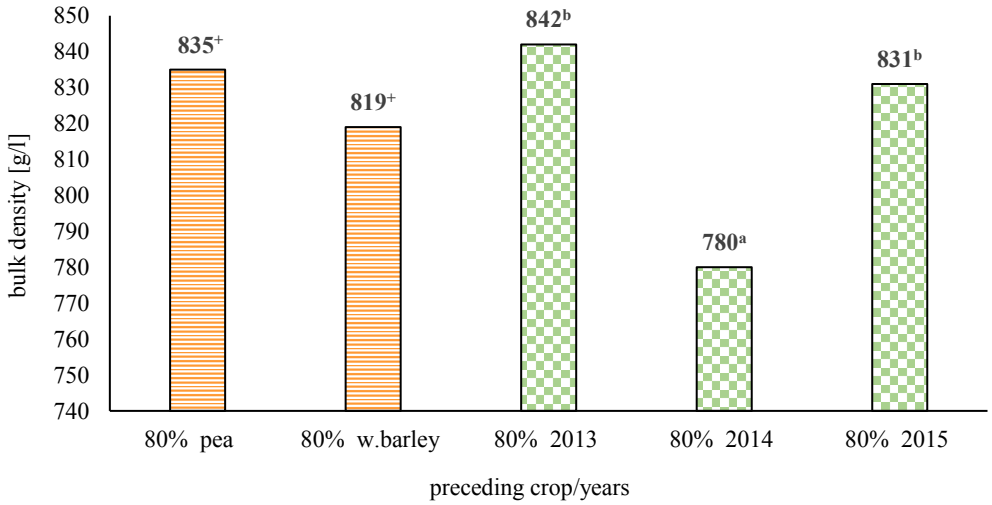
The weight of 1,000 grains of winter wheat was statistically significantly influenced by the preced-

T a b l e 5

The influence of fertilisation and share of cereals on grain yield and straw yield of winter wheat (analyse of variance)

Factor	Grain yield [t/ha]					Straw yield [t/ha]				
	df	MS	F	*P*	$LSD_{0.05}$	*df*	MS	F	*P*	$LSD_{0.05}$
Fertilisation [A]	1	3.46	9.75	+	0.35	1	6.02	2.74	–	–
PC(B)	1	11.91	33.54	+	0.35	1	14.21	6.48	+	0.87
A × B	1	0.01	0.02	–	–	1	0.01	0.00	–	–
Year (C)	2	17.61	49.59	+	0.52	2	26.27	11.97	+	1.29
A × C	2	5.24	14.77	+	0.90	2	1.18	0.54	–	–
B × C	2	2.35	6.61	+	0.90	2	6.86	3.13	–	–
Total	47	0.36	–	–	–	47	0.22	–	–	–
Error	33	1.83	–	–	–	33	3.81	–	–	–

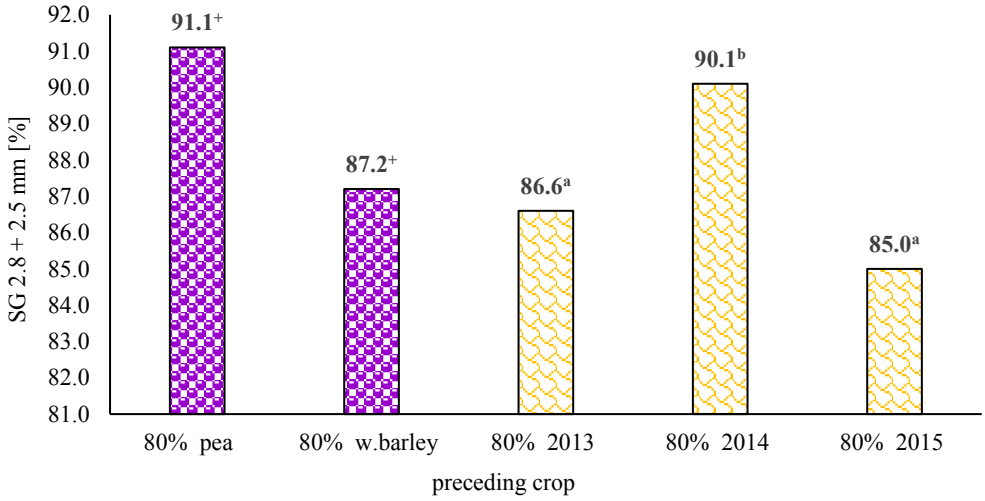
PC – preceding crop; *df* – degrees of freedom; MS – average squares, F – F-test; *P* – effect of a factor significant at the level 0.05; $LSD_{0.05}$ – least significant difference at the level α = 0.05



Average bulk density after different preceding crop – pea, w.barley – winter barley; +significant difference at the level α = 0.05. Average bulk density in years after both preceding crops – different literae – significant difference at the level α = 0.05; equal literae – insignificant difference at the level α = 0.05.

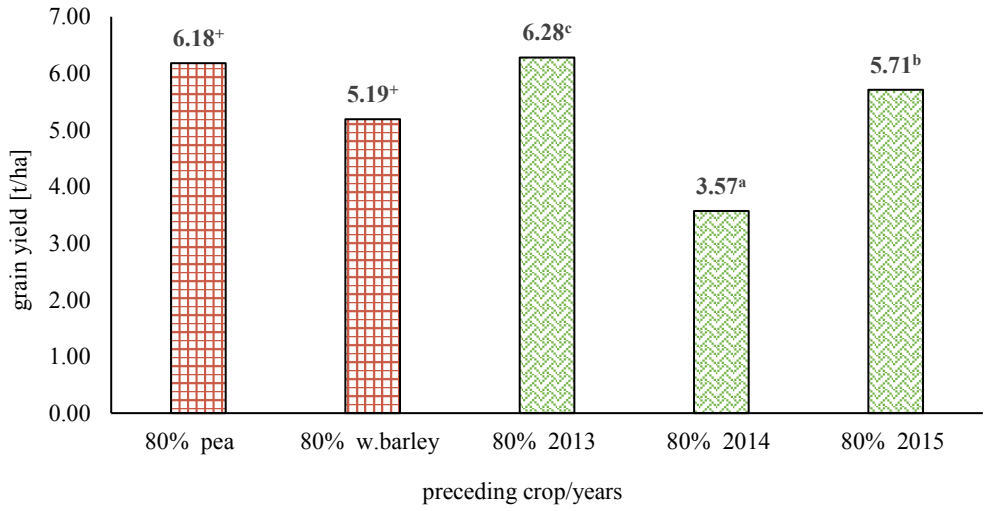
Figure 2. The bulk density [g/l] of winter wheat after field pea and winter barley in years 2013–2015

ing crop and weather in particular years (Table 3). Similarly, Jamriška *et al*. (2006) found out that nitrogen fertilisation was not effective on grain yield, but decreased values of the weight of 1,000 grains and the share of great kernels. Sieling *et al*. (2005) suggested that the yield losses due to an unfavourable preceding crop combination was mainly due to a reduced number of ears/m^2 and a decreased 1,000 grain weight. In 2014, the weight of 1,000 grains of winter wheat (Figure 1) was statistically significantly higher (49.3 g) than in 2015 (47.9 g). The weight of 1,000 grains after field pea was statisti-



Average share of grains 2.8 + 2.5 mm sieve after different preceding crop – pea; w.barley – winter barley; +significant difference at the level α = 0.05. Average share of grains 2.8 + 2.5 mm sieve in years after both preceding crops – different literae – significant difference at the level α = 0.05; equal literae – insignificant difference at the level α = 0.05.

Figure 3. The share of grains over 2.8 + 2.5 mm sieve [%] of winter wheat after field pea and winter barley in years 2013–2015



Average grain yield after different preceding crop – pea; w.barley – winter barley; +significant difference at the level α = 0.05. Average grain yield in years after both preceding crops – different literae – significant difference at the level α = 0.05; equal literae – insignificant difference at the level α = 0.05.

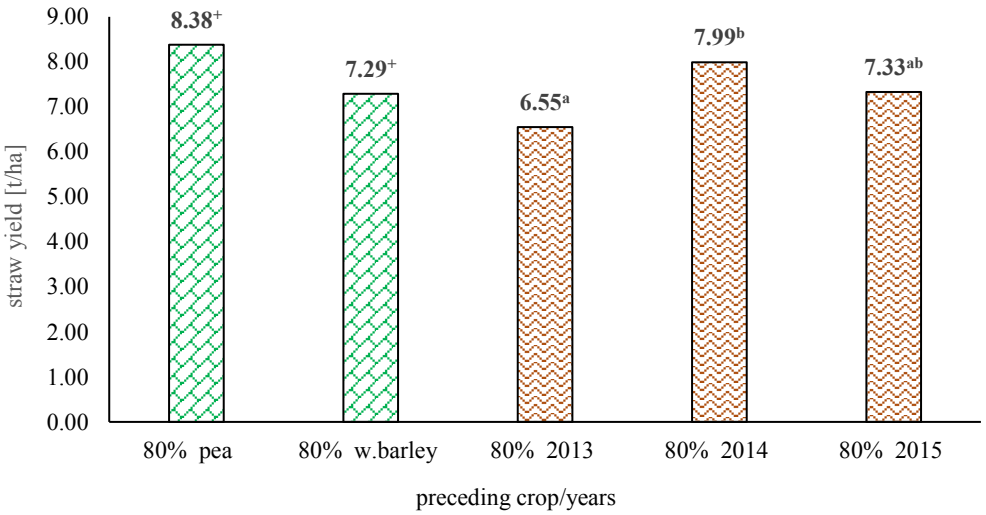
Figure 4. The grain yield [t/ha] of winter wheat after field pea and winter barley in years 2013–2015

cally significantly higher (51.2 g) than after preceding crop of winter barley (48.6 g). Sieling and Christen (2015) investigated the effects unfavourable preceding crops on the grain yield of winter oilseed rape, winter wheat and winter barley. Wheat as the preceding crop mainly decreased the 1,000 grain weight, and to a lesser extent, the ear density of the subsequent wheat crop.

The bulk density of winter wheat was statistically significantly influenced by preceding crop, weather in particular years and by interaction between the preceding crop and particular years (Table 4). The bulk density (Figure 2) after field pea was statistically significantly higher (835 g/l) than after preceding crop of winter barley (819 g/l). In 2014, the bulk density of winter wheat was statistically significantly lower (780 g/l) than in 2013 (842 g/l) and in 2015 (831 g/l).

The share of grains over 2.8 + 2.5 mm sieve of winter wheat was statistically significantly influenced by the preceding crop, weather in particular years and the interaction between fertilisation and weather in particular years (Table 4). The share of grains over 2.8 + 2.5 mm sieve (Figure 3) after field pea was statistically significantly higher (91.1%) than after preceding crop of winter barley (87.2%). In 2014, the share of grains over 2.8 + 2.5 mm sieve of winter wheat was statistically significantly higher (90.1%) than in 2013 (86.6%) and in 2015 (85.0%).

The grain yield of winter wheat was statistically significantly influenced by fertilisation, preceding crop, weather in particular years, the interaction between fertilisation and weather in particular years and the interaction between the preceding crop and weather in particular years (Table 5). The grain yield in the treatment with mineral fertilisation and organic manure Veget incorporation pea was statistically significantly higher (5.95 t/ha) than in the treatment with mineral fertilisation only (5.41 t/ha). The trend of increasing grain yield was also influenced by better crop management practices and more effective N use by the modern cultivars (Užík & Žofajová 2009). In 2014, the grain yield of winter wheat was statistically significantly lower (3.57 t/ha) than in 2013 (6.28 t/ha) and in 2015 (5.71 t/ha). In accordance with the results Užík *et al*. (2008), reasons of low grain yield in 2014 were low precipitations in June during the grain filling period. Similarly, Hudec *et al*. (2006) found out that drought stress (water deficiency) in the phase between the milk and wax grain ripeness decreased the grain



Average straw yield after different preceding crop – pea; w.barley – winter barley; +significant difference at the level α = 0.05. Average straw yield in years after both preceding crops – different literae – significant difference at the level α = 0.05; equal literae – insignificant difference at the level α = 0.05.

Figure 5. The straw yield [t/ha] of winter wheat after field pea and winter barley in years 2013–2015

yield significantly (an average by 34%), mainly as a consequence of the reduction of grain number per ear. The grain yield (Figure 4) after field pea was statistically significantly higher (6.18 t/ha) than after preceding crop of winter barley (5.19 t/ha). Stevenson (1996) found out that the inclusion of a pulse crop in rotation often leads to greater seed yields in succeeding cereal crop. Wheat seed yield was 43% greater (rotation benefit) when preceded by pea rather than wheat. Our results revealed that the increase of winter wheat grain yield after field pea in 2013–2015 was 19% in comparison with the preceding crop winter barley. In 2007–2009, the average winter wheat grain yield was 6.02 t/ha and the average winter wheat grain yield after winter barley was 4.75 t/ha. The increase in winter wheat yield after field pea cultivation was 26.74% (Babulicová *et al*. 2011). In 2010–2012, the winter wheat grain yield after field pea was at 1.22 t/ha (18%) higher than the winter wheat grain yield after winter barley (Babulicová 2014). Carr *et al*. (2008) suggested that wheat grain yield was 524 to 739 kg/ha greater in the winter wheat–field pea rotation compared with winter wheat–winter wheat rotation from 2000 to 2003, depending on the year. Similarly, Sieling and Christen (2015) suggested that unfavourable preceding crops significantly decreased the yield of winter wheat by 10% on average, however, with a large year-to-year variation. In double-cropping system, in which cereals and legumes were associated, the winter wheat yield was reduced by 50% or more in comparison with control plots (Sarunaite *et al.* 2013). Sieling *et al*. (2005) indicated that wheat following oilseed rape achieved 8 t/ha whereas wheat following wheat yielded 1 t/ha (13%) less compared with wheat after oilseed rape, due to a reduction of all yield components. Anderson (2008) found that winter wheat grain yield following oat-pea was by 28% higher than following spring wheat. The favourable impact of oat-pea on winter wheat yield may be related to suppression of root diseases (Wildermuth & McNamara 1991). Other research has shown that oat (Lockie *et al.* 1995), pea (Stevenson & Van Kessel 1996) and soybean (Vyn *et al.* 1991) can reduce disease severity in wheat. The importance of crop rotation on the grain yield and quality of wheat was confirmed by Borgi *et al*. (1995). In the wheat–maize rotation, maximum yield and quality was achieved with the highest rate of fertilisers even in the absence of manure. In the rotation that included alfalfa, maximum yield was obtained with the lowest rate of fertilisers but, to optimize quality, it appeared necessary to apply the highest rate of nitrogen (200 kg/ha). Hossain *et al*. (2016) found that the inclusion of legumes in the wheat–rice cropping sequence, particularly the use of mung bean, resulted in highest productivity. The wheat–mung bean–rice cropping system under integrated plant nutrition system with organic manure (especially 3–6 t/ha poultry manure) rendered 57% higher equivalent yield than wheat–fallow–rice rotation. Under European conditions, grain legume pre-crop effects are variable and increasing cereal yields by 0.5–1.6 t/ha (Preissel *et al*. 2015). According to the meta-analysis by Preissel *et al*. (2015), the pre-crop effect of grain legumes is highest under low N fertilisation to subsequent crops and comparable to non-leguminous oilseed crops.

The straw yield of winter wheat was statistically significantly influenced by preceding crop and weather in particular years (Table 5). The straw yield (Figure 5) after pea was statistically significantly higher (8.38 t/ha) than after the preceding crop of winter barley (7.29 t/ha). In 2013, the straw yield of winter wheat was statistically significantly lower (6.55 t/ha) than in 2014 (7.99 t/ha).

CONCLUSIONS

The weather had the strongest influence not only on the yield components but also on the grain yield and straw yield. The difference in grain yield depending upon weather in particular years was 2.71 t/ha. By including pea as the preceding crop in crop rotation with a high share of cereals, the statistically significantly increase of weight of 1,000 grains, bulk density, share of grains over 2.8 + 2.5 mm sieve, grain yield and straw yield were reached. These results suggest that preceding crop can positively influence the production potential of winter wheat despite unfavourable climatic conditions in 2013–2015. The increase of winter wheat grain yield at 0.99 t/ha was due to including pea as preceding crop of winter wheat. The results expressly confirm that despite the stress factors (e.g. irregular rainfall di-

vision in a vegetation period, increase of average monthly temperatures of air), the winter wheat grain yield can be increased to a substantial degree by the preceding crop pea and the combination of mineral and organic fertilisation.

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INCREASING ANTIOXIDANT CONTENT OF BROCCOLI SPROUTS USING ESSENTIAL OILS DURING COLD STORAGE

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Broccoli sprouts are natural functional foods for cancer prevention because of their high content of glucosinolate and antioxidant. Sprouts and mature broccoli are of potential importance in devising chemoprotective strategies in humans.The aim of the investigation was to study the effect of essential oils on broccoli seed germination, increase their antioxidant content and determine the glucosinolate concentration and other phytochemical parameters in 3-day -old sprouts during cold storage at 4°C and 95% RH for 15 days. The results showed that all treatments of essential oils increased germination index, seed germination percentage, seedling length, seedling vigour index, yield and the antioxidant content of broccoli sprout and reduced the microbial load compared to the control. Fortunately, the coliform bacteria was not detected in all treatments. Different essential oils of fennel, caraway, basil, thyme and sage were tested. The thyme oil was the best treatment, which increased the accumulation of the phenolic compounds and glucosinolate compared to the control at different storage periods. In the sprouts treated with thyme oil treatment and the control, at the end of cold storage, 1.98% and 28.06% of total phenolic content, 1.90% and 20.28% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, 1.39% and 58.33% of flavonoids, 1.93% and 36.25% of vitamin C, 2.95% and 22.02% of anthocyanin and 2.18% and 49.12% of glucosinolate were lost, respectively. A slight reduction differences in all detected compound concentrations occurred between the initial content and the end of storage period because of the application of thyme oil compared to the control. Therefore, the total glucosinolate level in the sprout (27.02 µg/g F.W.) was higher than that in the florets (7.37 µg/g F.W.). Glucoraphanin was the most abundant aliphatic glucosinolate present in the sprout and reached the highest value (16.24 µg/g F.W.) followed by glucoerucin (5.9 µg/g F.W.) and glucoiberm(1.2 µg/g F.W.).

Key words: broccoli, sprout, antioxidant, polyphonic compounds, flavonoids, essential oils

Broccoli sprouts are considered as a functional food. Essential nutrient content provides diverse secondary metabolites and phytochemicals (Villarreal -García *et al*. 2016). The phenolic compounds, especially flavonoids and anthocyanin, show a great ability to capture free radical that lead to oxidative

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stress; therefore, these compounds are attributed a beneficial effect in the prevention of cardiovascular diseases, circulatory problems, neurological disorders and cancer (Baenas *et al*. 2014). Broccoli has been identified as a vegetable with potential anticancer activity because of high levels of glucosinolates that are divided into three major categories: aliphatic, indole and aromatic glucosinolates (Yan & Chen 2007). The high content of the aliphatic glucosinolates in broccoli sprouts is mainly attributed to glucoraphanin. The degradation products of aliphatic glucosinolates are considered to have the higher phase 2 detoxification enzyme inducer ability than the other two groups, which is effective in blocking chemical carcinogenesis; therefore, they are thought to be the main contributor to the protection against carcinogenesis. The glucoraphanin can be hydrolysed tosulphoraphane (Figure 1). Sulphoraphane is a naturally occurring isothiocyanate in cruciferous vegetables and has a high capacity to induce phase 2 enzymes (López-Cervantes *et al*. 2013). This compound plays an important role in controlling, preventing or blocking any of the multiple stages of the carcinogenic process (Parnaud *et al*. 2004). Moreover, broccoli sprouts have 20–50 times higher concentration of glucoraphaninand 30–50 times higher concentration of sulphoraphane when compared to mature plants. Level of myrosinaseenzyme, which protects against carcinogens, in broccoli sprouts is 10–100 times higher than that in mature broccoli heads (Fahey *et al*. 1997). Use of sprouts in the daily nutrition maintains the nutritional value, protects from diseases and eliminates spoilage and pathogenic bacteria.

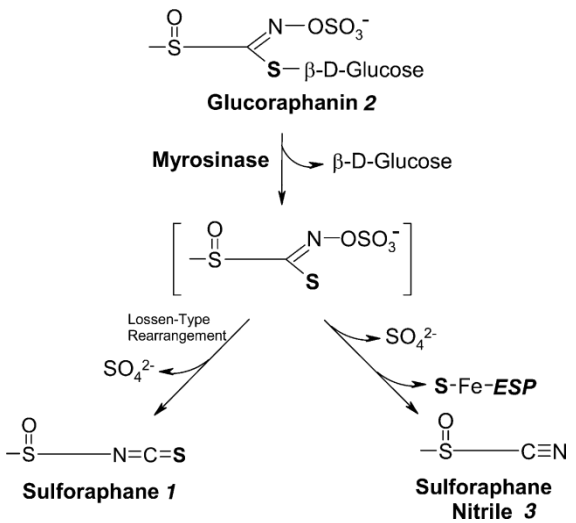

Figure 1. Formation mechanism of sulphoraphane and sulphoraphane nitrile in broccoli sprouts (Matusheski *et al*. 2004)

The use of treatments with essential oil rich in antioxidant to stimulate broccoli seed germination should be considered. Application of 4% thyme and basil oils reduced the pathogenic fungi from seed to seedling and had a positive effect on the seed germination of infected seeds (Nguefack *et al*. 2005). Aromatic plants, especially essential oils, are well known for their antioxidant and antimicrobial properties that prevent food degradation and alteration (Justesen & Knuthsen 2001), as they are rich in phenolic substances, usually referred to as polyphenols, which are ubiquitous components of plants and herbs.

Campas-Baypoli *et al*. (2010) indicate that 30 g of broccoli sprouts may have the amount of antioxidant present in 600 g of mature broccoli. Furthermore, broccoli sprouts are sold in sealed plastic containers and destined to be stored in domestic refrigerators for consumption. Although the concentration of bioactive compounds may be high at the time of harvest, there is no accurate data that document the stability of these phytochemicals during the storage (West *et al*. 2004). Several researches have shown that the temperature and storage time are the main factors responsible for the changes in the concentration of diverse compounds, such as glucosinolates (Fiskaa *et al*. 2009). Therefore, it is necessary to know the stability of glucosinolates in the food matrix under refrigeration storage.

The aim of this investigation was to use the essential oils during cold storage to produce healthy broccoli sprout with high nutritive values, quality and antioxidants content with low microbial load. After choosing the best oil treatment, the glucosinolate concentration, total phenolic content, flavonoids, vitamin C and DPPH radical scavenging capacity content in sprouts were determined during cold storage.

MATERIAL AND METHODS

1. Plant material and germination condition

The study was conducted in the Laboratory of Mansoura Horticultural Research Station, Horti-

culture Research Institute, Egypt, during the period from 2012 to 2014 seasons. Broccoli seeds (*Brassica oleracea* L. var. *italica* and the variety name is F1 Hybrid Sakura) from Tokita Seeds CO., LTD (Saitama, Japan) was used. Germination of broccoli seeds was carried out according to Pérez-Balibrea *et al*. (2011). The seeds (1,000 seeds, nearly 5 g) for each treatment were soaked in 0.5% v/v sodium hypochlorite solution for 15 min, then immersed in 50 ml of distilled water for 5½ hours with shaking for every 30 min and washed with distilled and sterilised water. On September 15, the seeds were sown broadcast over absorbent medical cotton in sprouting plastic containers (220 mm × 110 mm). The cotton was treated with emulsion of different natural essential oils at 0.05% concentration and emulsified using 1.5 ml/l of Tween 80, and the containers were closed immediately. Germination conditions were maintained at 25±2°C with a cycle of 16 h light and 8 h darkness, a relative humidity (RH) between 80% and 90% and light intensity of 7.4 lmol/m^2/s. After 3 days, sprouts were cut from their root mats, and 20 g of sprouts from each was weighed and placed in a container. The container was stored and placed at 4°C and 95% RH in the dark simulating a domestic refrigerator for 15 days for the best treatment and control.

2. Characterisation of essential oils

The major compounds of essential oil of caraway, *Carum carvi,* were carvone (57%) and limonene (30%). The other compounds were oleic acid (C 18:1), which accounted for 52.28%, and linoleic acid (C 18:2), which accounted for 30.84%. *Carum carvi* essential oil was characterised by the predominance of oxygenated monoterpenes (79.79%). In essential oil of fennel, *Foeniculum vulgare*, the most abundant component was trans-anethole (83.80%), then limonene (9.34%), fenchone (4.84%) and methyl chavicol (1.36%). The other compounds were monoterpenes (15.04%) and phenylpropanoids (84.79%). According to the results of the chemical analysis of thyme essential oil, *Thymus vulgaris*, 25 compounds were identified, which represented 94.53% of the oil content. The highest percentage of compounds includes three classes: monoterpene hydrocarbons, aromatic hydrocarbons and oxidised monoterpenes. More than half of the total compounds of thyme essential oil include six dominant compounds. Amongst them, the most dominant is the oxidised monoterpene thymol 40.12% and the monoterpene hydrocarbon p-cymene (21.15%). Other dominant components of this essential oil are the following: carvacrol (14.34%) and linalool (4.54%). The major constituents detected in our samples of basil, *Ocimum basilicum*, include eugenol (39.51%), linalool (27.24%), 1,8 cineole (17.88%) and ß-bisabolene (15.37%). The dominant constituent in essential oils of sage, *Salvia officinalis*, is thujone (41.33%). 1,8 cineole is also present in high amount (39.5%).

3. Essential oils extraction methods

Seeds of fennel, caraway and herbs of basil, thyme and sage (200 g from each one) were used for oil extraction by hydro-distillation for 2–3 h according to Charles and Simon (1990). After extraction, essential oils were separated and their basic constituents were identified using gas liquid chromatography (GLC).

4. Experimental design for essential oils treatments

A completely randomised design with three replicates was used. The experiment included seven treatments of the above-mentioned essential oils in addition to hot water (36°C) treatment and tap water as a positive control.

Mature winter grown broccoli was sown on 18 November in both seasons and was harvested by hand on 28 January from the field of the Baramoon Experimental Farm. The variety used was the same as mentioned previously. The other agricultural practices were carried out as commonly followed in the district. Broccoli was harvested when the diameter of floret was about 0.35–0.40 cm. On 26 January, the germination of broccoli seeds has been done in incubator chamber at 20°C and 60% RH for 3 days. After germination, the samples of sprout were taken for chemical analysis to compare with samples of florets.

5. Recorded data

Vegetative characters of broccoli sprout

Germination [%] = Total number of normal seedlings / Total number of seeds (1)

Germination index (GI) was calculated according to the following formula:

$$GI = \Sigma T_i \times N_i/S \quad (2)$$

where: T_i is the number of days after planting, N_i is the number of seeds germinated on day I and S is the total number of planted seeds.

Seedling vigour index = Germination [%] ×Seedling length [cm] (3)

Germination index and seedling vigour index were calculated by the above equation of International Seed Testing Association (2010). At the end of germination (3 days), 10 seedlings from each treatment were taken randomly for the determination of sprout length [cm] and fresh weight of sprout [g].

Methanolic extracts for phytochemical determination

Extraction was performed under dark conditions using the solvent methanol/water at a ratio 8:2; specifically weighed 0.3 g of fresh broccoli sprouts were added to 5 ml of 80% v/v methanol, homogenised for 30 s, vortexed and sonicated for 5 min. The mixture was filtered using Whatman no. 41 paper (Du *et al*. 2009).

Total phenolic and total flavonoid contents measurements

Total phenolic content was determined using the Folin–Ciocalteu method. In an Eppendorf tube, 7.9 ml of distilled water, 100 µl of broccoli sprout extract and 500 µl of Folin–Ciocalteu reagent (1:1 with water) were added and mixed. Exactly after 1 min, 1,500 µl of sodium carbonate (20 g/100 ml) was added and the mixture was mixed and allowed to stand at room temperature in the dark for 2 h. The absorbance was read at 765 nm by spectrophotometer. Gallic acid was used for calibration curve. Results were expressed as mg GAE/100 g F.W. (Du *et al*. 2009).

In order to determine the total flavonoid content, 150 µl of broccoli sprout extract, 1,700 µl of 30% ethanol, 150 µl of 0.5 mol/l $NaNO_2$ and 150 µl of 0.3 mol/l $AlCl_3.6H_2O$ were added and mixed. After 5 min, 1 ml of 1 mol/l NaOH was added, and the mixture was measured spectrophotometrically at 506 nm. Results were expressed as mg/100 g F.W. of flavonoids content in sprouts (Du *et al*. 2009).

Vitamin C and Anthocyanin

Vitamin C was determined by titration of fruit juices with indophenol method and indicated as mg ascorbic acid per 100 ml (AOAC 2000).

Total anthocyanin content was measured using a spectrophotometric differential pH method following the procedure of Yuan *et al*. (2010). Frozen samples (100 mg) were crushed into powder and extracted separately with 2 ml of pH 1.0 buffer containing 50 mM KCl and 150 mM HCl as well as 2 ml of pH 4.5 buffer containing 400 mM sodium acetate and 240 mM HCl. The mixtures were centrifuged at 12,000 g for 15 min at 4°C. Supernatants were collected and diluted for direct measurement of absorbance at 510 nm. Total anthocyanin content was calculated using the following equation:

$$\text{Amount (mg/g F.W.)} = (A_{510\,nm} \text{ at pH } 1.0 - A_{510\,nm} \text{ at pH } 4.5) \times 484.8/24.825 \times \text{Dilution factor}$$

The molecular mass of cyanidin-3-glucoside-chloride is 484.8 and its molar absorptivity (e) at 510 nm is 24.825. Each sample was analysed in triplicate, and the results were expressed as the average of ± SD.

Total chlorophyll

Total chlorophyll content of broccoli was determined by using a spectrophotometry (Sabir & Agar 2011). One gram of blended broccoli portions was homogenised with 10 ml of chloroform–methanol mixture (2:1 v/v) for 1 min. Extracts was filtered with filter paper. The residue was resuspended in 10 ml of chloroform–methanol mixture and then filtered. All the filtrates were combined and solutions were supplemented with chloroform–methanol mixture to 25 ml final volume. Total chlorophyll was determined by measuring absorbance of the solution in UV spectrophotometer at 663 and 645 nm against chloroform–methanol blank. The total chlorophyll was estimated by the following formula:

$$\text{Total chlorophyll [(mg 100/g)]} = 8.02 \times (A663) + 20.02 \times (A645)$$

Measurement of DPPH radical scavenging capacity

2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity was determined by Brand-Williams *et al*. (1995). A working solution of 0.1 mM DPPH radical was prepared in 80% methanol,

which shows an absorbance of 1.237 at 515 nm. The DPPH radical scavenging capacity of the sample was expressed as mM Trolox equivalent (TE) per 100 g F.W.

Extraction and Desulphation of Glucosinolates

Desulphoglucosinolate contents were determined according to Kiddle *et al.* (2001). Each sample (20 µl) was analysed on a Merck-Hitachi HPLC system (Merck-Hitachi Ltd., Tokyo, Japan) consisting of a variable UV detector set at 227 nm and a Lichosphere RP-18 column (Merck, Darmstdat, Germany) (RP18, 25 cm × 0.4 cm, 5 µm particle size). The mobile phase was a mixture of water (A) and acetonitrile (B). Desulphoglucosinolates were eluted off the column in 28 min with a linear gradient starting with 1% B and reaching 20% B at 28 min and 90% B at 30 min. The flow rate was 1.5 ml/min. Extraction and desulphation were done according to Vallejo *et al.* (2002).

Microbial evaluation in the stored broccoli sprouts

The pour plate method was used for the enumeration of the microbial community in broccoli sprouts through the 15-day storage period, with 5-day interval. The total bacterial, yeast and fungal counts were determined using the media of Collins and Lyne (1985) and Marshall (1992), after 2, 3 and 5 days of incubation at 30°C. The enumeration of coliform bacteria was performed according to Lorenz *et al*. (1982). The counts of the different groups were expressed as colony forming unit (CFU) per gram of fresh sprouts.

6. Statistical Analysis

Data were analysed using analysis of variance technique, and the differences between individual pairs of treatment means were compared using Duncan's multiple range test at 5% according to Snedecor and Cochran (1989).

RESULTS

1. *Vegetative characters of broccoli sprout*

The treatments with essential oil rich in antioxidant stimulates broccoli seed germination. All different essential oils had significant effects on germination index [%], germination [%], seedling length [cm], seedling vigour index and yield [g] compared with the control during the two seasons (Table 1). The fennel, caraway and thyme oils increased the seed germination index of the seeds by 171.43%, 170.29% and 148.02% (an average of the two seasons), respectively, compared with the control (100%). The increases in seed germination percentage over the control (tap water) reached to 12.73%, 13.74% and 15.82% for the most effec-

Table 1

Vegetative characters of broccoli seeds treated with different essential oils before cold storage

Treatment		Germination index [%]		Seed germination [%]		Seedling length [cm]		Seedling vigour index [cm]		Yield [g] container / 242 cm^2	
		2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
1	Water (control)	13.36^{e}	12.96^{d}	86.67^{c}	86.00^{a}	4.67^{c}	4.00^{b}	4.03^{c}	3.44^{c}	36.40^{e}	34.20^{d}
2	Hot water	14.61^{de}	13.02^{d}	93.78^{b}	90.44^{bc}	5.00^{c}	4.80^{b}	4.71^{c}	4.33^{c}	40.88^{de}	37.21^{d}
3	Fennel oil	22.01^{a}	23.01^{a}	97.33^{ab}	97.33^{a}	7.33^{ab}	7.67^{a}	7.13^{ab}	7.47^{ab}	56.90^{b}	49.17^{c}
4	Caraway oil	21.94^{a}	22.88^{a}	97.33^{ab}	99.00^{a}	8.00^{ab}	8.33^{a}	7.79^{a}	8.25^{a}	54.97^{bc}	67.75^{a}
5	Basil oil	20.22^{ab}	21.82^{ab}	94.67^{b}	92.33^{b}	7.00^{b}	7.67^{a}	6.63^{b}	7.07^{b}	64.87^{a}	68.17^{a}
6	Thyme oil	18.81^{bc}	20.14^{b}	100.00^{a}	100.00^{a}	8.20^{a}	8.30^{a}	8.20^{a}	8.30^{a}	66.54^{a}	67.75^{a}
7	Sage oil	16.91^{cd}	17.76^{c}	100.00^{a}	100.00^{a}	7.83^{ab}	7.83^{a}	7.83^{a}	7.83^{ab}	47.83^{cd}	49.17^{c}

Means followed by the same letter (s) within each column do not significantly differ using Duncan's multiple range test at the level of 5%

tive treatments, respectively. Thyme, caraway and fennel oils had significant increases in the seedling vigour index and yield over the control to 50.25%, 73.82% and 90.22%, respectively (means of the two seasons).

2. *Phytochemical characters*

All treatments significantly surpassed over the control in broccoli sprout bio-constituents, that is, total phenolic acid, total flavonoid content, anthocyanin and ascorbic acid, whilst the control treatment gave the highest DPPH radical scavenging

T a b l e 2

Phytochemical screening by GLC for 3-day-old broccoli sprouts produced from seeds treated with essential oils before cold storage (0 time)

Treatment		Total phenolic acid [mg/100 g F.W.]		Total flavonoids [mg/100 g F.W.]		Anthocyanin [mg/100 g F.W.]		Ascorbic acid [mg/100 g F.W.]		DPPH [Mmol TE/g F.W.]	
		2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
1	Water (control)	83.33^{d}	84.11^{e}	91.99^{d}	95.18^{e}	7.13^{d}	7.70^{d}	70.58^{e}	81.23^{d}	23.66^{a}	24.66^{a}
2	Hot water	88.71^{c}	88.56^{c}	100.95^{c}	101.03^{d}	8.62^{c}	8.77^{c}	86.81^{c}	86.81^{c}	23.54^{b}	23.66^{a}
3	Fennel oil	88.46^{c}	88.90^{c}	107.66^{b}	107.72^{c}	8.86^{c}	8.87^{bc}	87.66^{c}	88.00^{c}	21.98^{d}	21.98^{c}
4	Caraway oil	87.90^{c}	88.13^{cd}	104.66^{b}	104.73^{c}	9.84^{bc}	9.84^{bc}	77.33^{d}	85.80^{c}	21.96^{de}	21.96^{c}
5	Basil oil	122.06^{b}	122.29^{b}	113.00^{a}	113.00^{b}	11.71^{a}	12.05^{a}	94.67^{b}	94.67^{b}	21.94^{de}	21.94^{c}
6	Thyme oil	131.66^{a}	131.60^{a}	115.66^{a}	116.24^{a}	12.09^{a}	12.14^{a}	102.33^{a}	103.33^{a}	21.86^{e}	20.03^{d}
7	Sage oil	87.90^{c}	84.74^{de}	104.33^{bc}	104.59^{c}	10.38^{b}	10.38^{b}	82.33^{cd}	86.69^{c}	22.79^{c}	22.79^{bc}

Means followed by the same letter (s) within each column do not significantly differ using Duncan's multiple range test at the level of 5%

T a b l e 3

Phytochemicals of sprouts treated with essential oils after 15 days of cold storage at 4°C

Treatment		Total phenolic acid [mg/100 g F.W.]		Total flavonoids [mg/100 g F.W.]		Anthocyanin [mg/100 g F.W.]		Ascorbic acid [mg/100 g F.W.]		DPPH [Mmol TE/g F.W.]	
		2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
1	Water (control)	60.33^{d}	59.81^{e}	39.19^{d}	40.28^{e}	5.34^{d}	5.87^{d}	43.75^{e}	46.28^{d}	28.06^{a}	27.85^{a}
2	Hot water	87.27^{c}	86.45^{c}	98.59^{c}	99.23^{d}	7.92^{c}	8.07^{c}	82.08^{c}	82.51^{c}	24.15^{b}	24.86^{a}
3	Fennel oil	86.54^{c}	87.19^{c}	105.76^{b}	105.17^{c}	8.16^{c}	8.17^{bc}	85.16^{c}	86.00^{c}	22.08^{d}	22.00^{c}
4	Caraway oil	85.23^{c}	86.11^{cd}	102.96^{b}	102.57^{c}	9.04^{bc}	9.24^{bc}	76.83^{d}	81.90^{c}	21.98^{de}	22.00^{c}
5	Basil oil	120.86^{b}	119.99^{b}	110.60^{a}	111.00^{b}	11.21^{a}	11.75^{a}	92.66^{b}	92.16^{b}	22.03^{de}	21.99^{c}
6	Thyme oil	130.33^{a}	130.50^{a}	113.16^{a}	114.02^{a}	11.92^{a}	12.84^{a}	101.93^{a}	102.81^{a}	21.96^{e}	20.33^{d}
7	Sage oil	86.05^{c}	84.48^{de}	100.33^{bc}	102.25^{c}	10.08^{b}	10.03^{b}	82.00^{cd}	86.06^{c}	22.94^{c}	22.29^{bc}

Means followed by the same letter (s) within each column do not significantly differ using Duncan's multiple range test at the level of 5%

capacity, in both the seasons (Table 2). The thyme oil treatment produced significant increases in total phenolic content, total flavonoid content, anthocyanin content and ascorbic acid. Thyme and basil oils decreased significantly the DPPH radical scavenging capacity. Accordingly, this treatment (thyme oil) has been chosen to study the storage behaviour, in addition to control treatment. After 15 days of cold storage, all treatments had significant effects on all phytochemical characters compared to the control, in the two seasons (Table 3). The control decreased than the initial time (0 time, Table 2) in all studied traits. Thyme oil treatment gave the best results. So, it was chosen to compare with the control during storage.

3. *Antioxidant activity during cold storage*

3.1. *Total phenolic content and DPPH radical scavenging capacity*

A gradual increase in the total phenolic content reached a maximum value at day 5 and 10 (132.67 and 135.04 mg GAE/100 g F.W.) compared to the initial value but this concentration decreased to 129.03 mg at day15 because of the application of thyme oil (Figure 2). Keeping in view that the control treatment decreased to 73.84 GAE/100 g F.W. at day 5.

On the 15 days-old sprout from storage, the DPPH radical scavenging capacity in the control was reduced to 28.57% when compared to thyme oil treatment (1.98%). The DPPH radical scavenging capacity in control increased significantly until day 10 (29.43 mg /100 g F.W.) and finally decreased (28.46 mg/100 g F.W.) at day 15, the loss increased to 20.28% compared with the initial value (Figure 3). However, some authors confirm that low storage temperature causes an accumulation of total polyphenols (Policegoudra & Aradya 2007).

3.2. *Total flavonoid contents*

Total flavonoid content (Figure 4) was found in a higher concentration in 3-day-old sprouts of thyme oil treatment, with values of 115.95 mg/100 g F.W.; after 5 and 10 days of storage, it slightly decreased to 0.021% and 0.086%, respectively, when compared with the initial value and, finally, reduced by 1.39%. The high loss of flavonoids reached to 10.59% and 47.89%, after 5 and 10 days, respectively, and at 15 days, the loss increased to 58.33% for the control treatment (average two seasons).

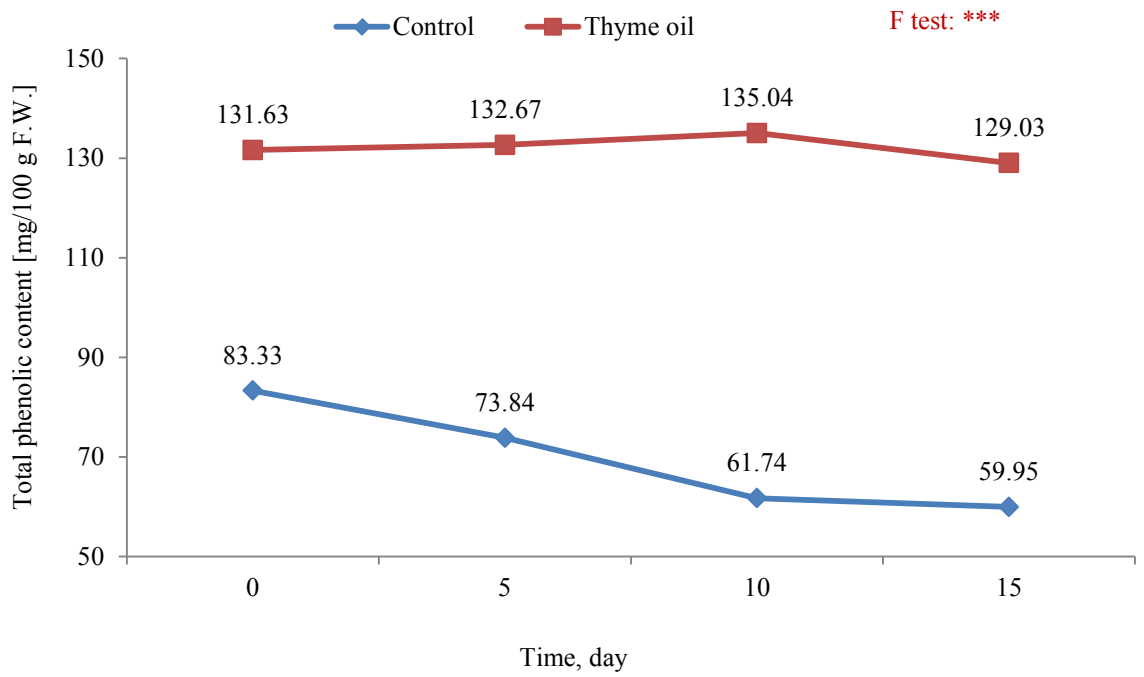

Figure 2. Total phenolic content as affected by the thyme oil compared to control treatment at different storage period

3.3. *Ascorbic acid*

Thyme oil application slightly decreased vitamin C content (0.37% loss) when compared with the initial value after 5 days of storage; the respective losses at the end of cold storage were 1.93%. At day 5 of storage, the vitamin C content in control treatment was decreased by 37.71%. Finally, the losses reached to 36.25% (average two seasons, Figure 5). This behaviour was clearly in contrast to that found for phenolic compounds and glucosinolates.

3.4. *Total chlorophyll*

Changes in chlorophyll amount of broccoli sprouts are illustrated in Figure 6. During the 15 days of storage, no significant change was observed in broccoli sprouts in samples treated with thyme oil compared to the control.

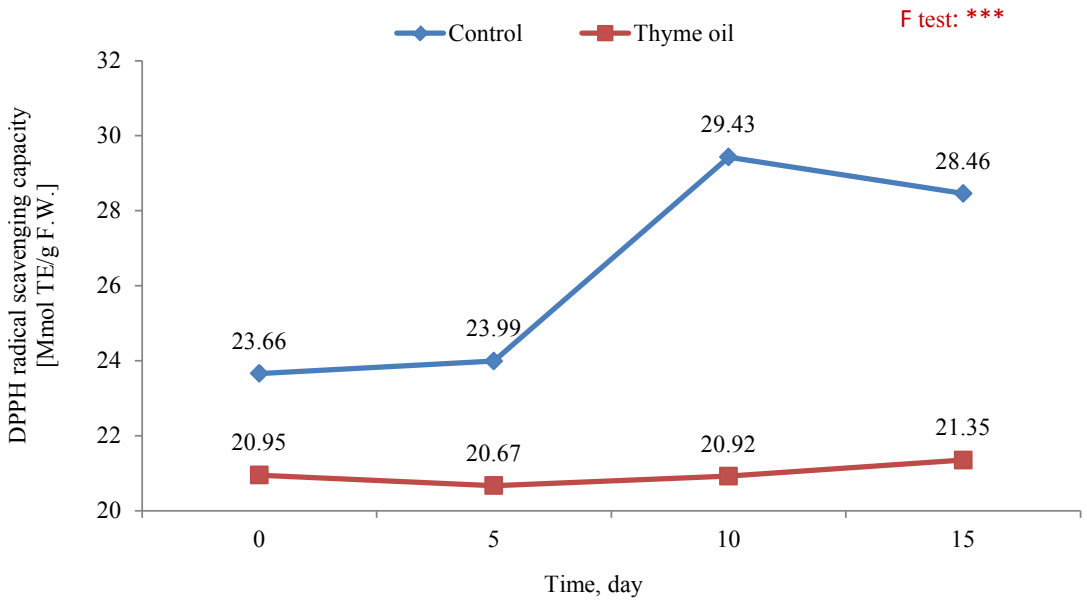

Figure 3. DPPH radical scavenging capacity as affected by the thyme oil treatment compared to the control treatment at different storage period

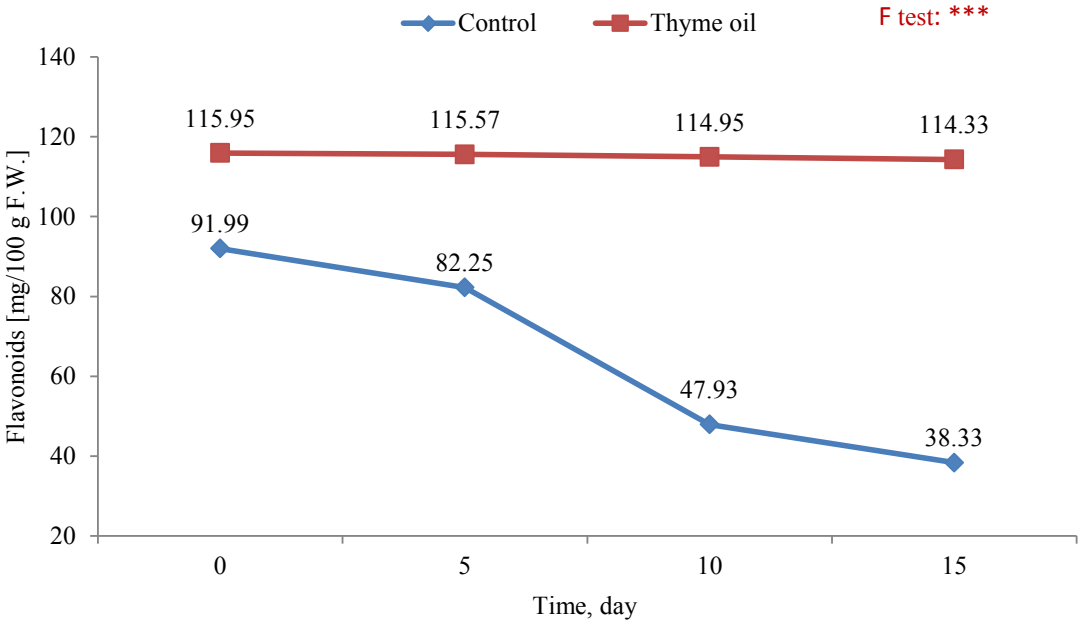

Figure 4. Total flavonoids content as affected by the thyme oil compared to the control treatment at different storage period

3.5. *Total anthocyanin*

Thyme oil treatment in 3-day-old sprout broccoli increased the level of anthocyanin compared with the control. A slight decrease in anthocyanin content was observed (2.95%) at the end of storage periods, compared with the initial value. A gradual decrease was noticed in control treatment and the changes reached about 22.019% at the end of storage period (average two seasons, Figure 7).

3.6. *Glucosinolates content*

The results of the samples analysed showed significant differences with respect to storage time. Thyme oil application increased glucosinolates content in 3-day-old sprouts compared to control treatments (Figure 8). Thyme oil had a high value of glucosinolates (27.02 µg/g F.W.), and it slightly decreased up to a concentration of 26.43 µg/g F.W. on day 15. The change percentage decreased to about 2.18% at the end of storage. The reduction in total glucosinolate content was observed in control treatment, in which the change percentage reached about 49.12% at the end of storage.

3.7. *Microbial population in the stored broccoli sprouts*

The microbial populations were presented in terms of bacterial (total and coliform), fungal and yeast counts (Table 4).

Marked variations were detected amongst the different essential oil treatments from one side and the different microbial groups in the other side. Regarding bacteria, it recorded the highest numbers amongst the groups, along the storage period; fortunately, the coliform bacterial count was not detected at all, indicating the suitability of such preparation for the nutritional aspects. Coliform bacteria are described and grouped based on their common origin or characteristics, as either total or faecal coliform.

T a b l e 4

Count (C.F.U.) of microbial groups on the sprouts of broccoli as a function of cold storage

Microbial group	Treatment	0 day	5 days	10 days	15 days
Bacteria*	Water (control)	110	8,000	19,700	200,500
	Hot water	100	10,000	18,850	21,800
	Fennel oil	0	330	6,530	15,670
	Caraway oil	0	670	3,670	4,670
	Basil oil	0	1,650	2,760	2,950
	Thyme oil	0	500	500	550
	Sage oil	20	980	1,750	2,009
Fungi	Water (control)	0	0	10	40
	Hot water	0	0	0	10
	Fennel oil	0	0	0	0
	Caraway oil	0	0	0	0
	Basil oil	0	0	5	10
	Thyme oil	0	0	0	0
	Sage oil	0	0	0	0
Yeast	Water (control)	0	20	40	50
	Hot water	0	0	0	10
	Fennel oil	0	0	0	10
	Caraway oil	0	0	0	10
	Basil oil	0	0	10	20
	Thyme oil	0	0	0	0
	Sage oil	0	0	20	30

*No coliform bacteria were detected; C.F.U. – colony forming unit

The total group includes faecal coliform bacteria that exist in the intestines of warm-blooded animals and humans and are found in bodily waste, in animal droppings and naturally in soil. Most of the faecal coliform in faecal material (faeces) is known to cause serious human illness. Oppositely, fungi group was the lowest detected groups in the tested treatments. Yeast count came in moderate numbers. However, the numbers of such groups are low enough to indicate the high quality of broccoli sprouts. Thyme oil followed by sage oil recorded the lowest bacterial load. On the other hand, the tested oils except basil oil completely inhibited any fungal growth on broccoli sprouts, along the storage period. Regarding the yeast group, thyme oil was the best. Generally, the microbial load of broccoli sprouts as results of the tested oils was reasonably accepted especially with the absence of any form of coliform bacteria and relatively low microbial load as a whole, representing that there is no any restriction for broccoli sprouts for human consumption.

4. *Mature versus sprout broccoli glucosinolate content*

The total glucosinolate level in sprout (27.02 µg/g F.W.) is higher than that in florets (7.37µg/g F.W.) (Figures 9 and 10). Glucoraphanin is the most abundant aliphatic glucosinolate present in sprout and reached the highest (16.24 µg/g F.W.) followed by glucoerucin (5.9 µg/g F.W.) and glucoiberm (1.2 µg/g F.W.). However, the florets contain the highest level of aromatic/indolylglucosinolates and neoglucobrassicin (2.11 µg/g F.W.) followed by glucobrassicin (1.67 µg/g F.W.). Our results are in agreement with those obtained by Fahey *et al*. 1997.

DISCUSSION

The essential oils are frequently known to induce stimulatory or inhibitory effects on the germination of seeds and other physiological processes depending on their basic component, concentration, allelochemicals interaction and selectivity due to the site of application and plant species. The lower doses of essential oil showed a stimulatory activity (Leth 2002).

The obtained results of current study revealed that the used essential oils improved the germination of broccoli seeds. However, thyme oil gave 100% of sprouts after germination (Table 1). The

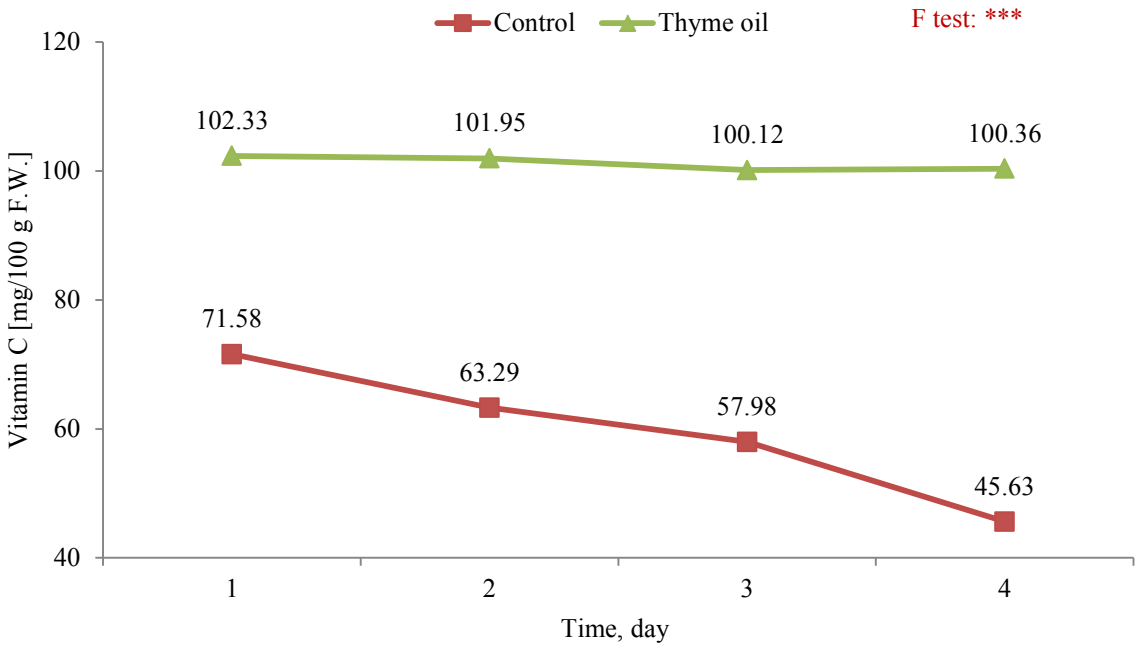

Figure 5.Vitamin C content as affected by the thyme oil compared to the control treatment at different storage period

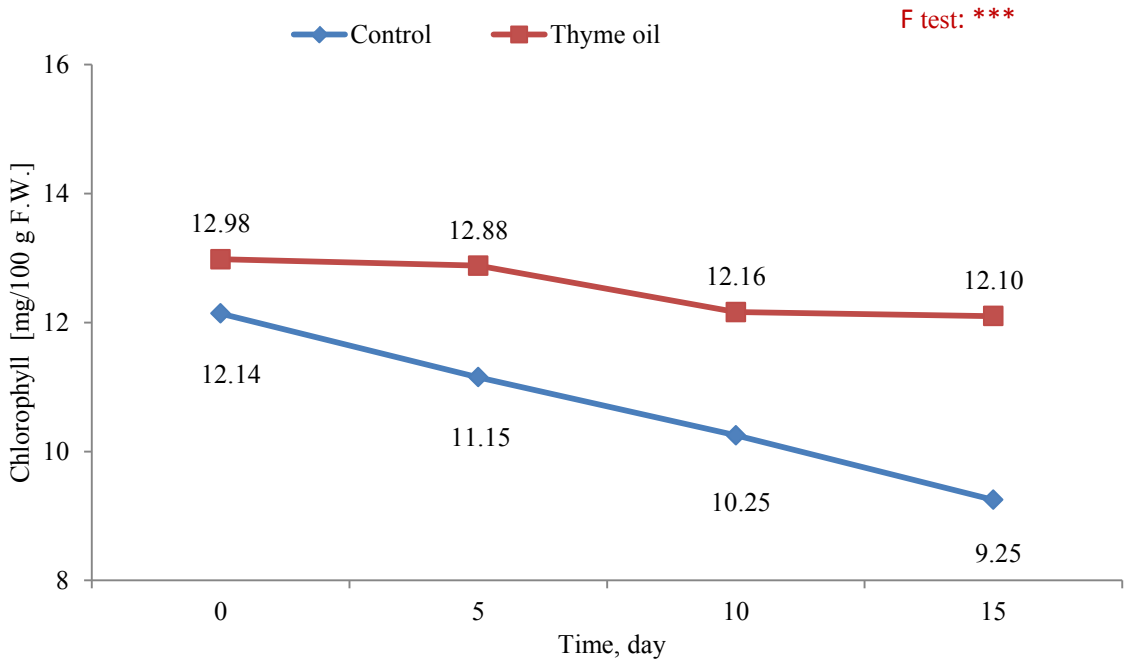

Figure 6. Total chlorophyll content as affected by the thyme oil compared to the control treatment at different storage period

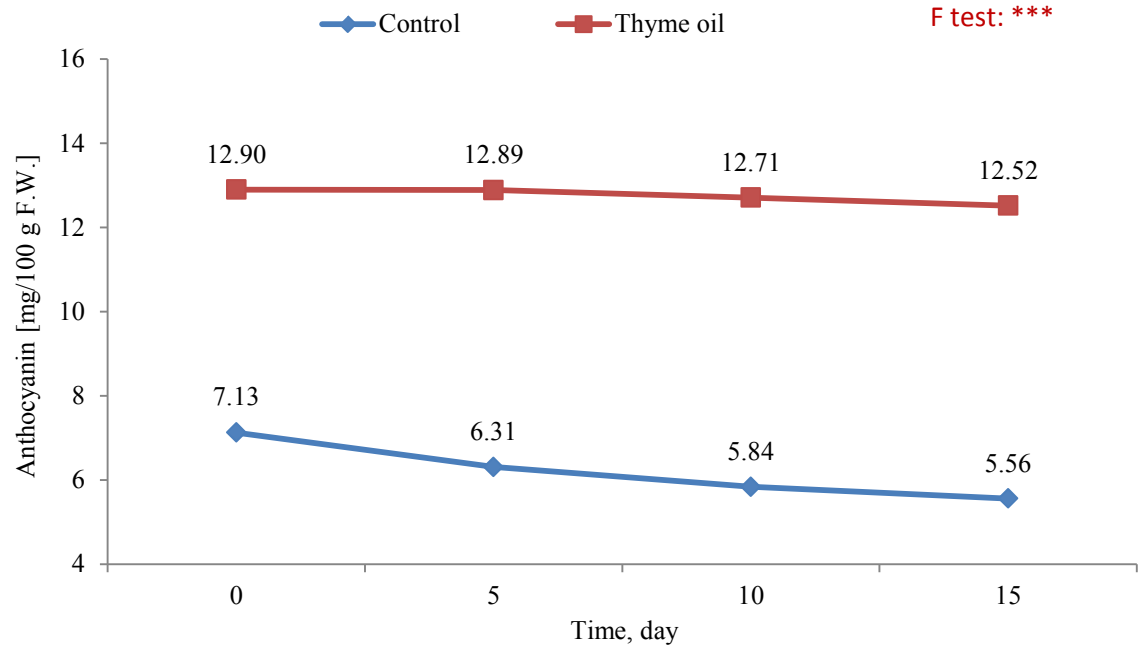

Figure 7. Total anthocyanin content as affected by the thyme oil compared to the control treatment at different storage period

impact of essential oils on seed germination of other plant species was reported as 24 out of 47 tested terpenoids enhanced the seed germination of *Lactuca sativa* (Vokou *et al*. 2003). Also, the positive impact of thyme oil on broccoli seeds could be due to its active ingredients. Kulisic *et al*. (2004) found that the phenolic compound containing thymol and carvacrol as major components exhibited strong antioxidant activity. The essential oils play an important role in the reduction of time need for broccoli germination, where it gives full germination on 3 days compared to normal conditions (7 days). So, we can produce two growing and production cycle in 7 days.

The thyme oil treatment had significant increases in total phenolic content, total flavonoid content, anthocyanin content and ascorbic acid (Table 2). The majority of the antioxidant activity attributes to phenolic compounds, flavonoids and ascorbic acid in essential oils (Heim *et al*. 2002). Moreover, the antioxidant effect was due to the presence of hydroxyl groups in their chemical structure. Milos *et al*. (2000) found that the oregano essential oil inhibited hydroperoxide formation and that the CHO fraction showed the highest antioxidative activity.

The thyme oil showed very poor radical scavenging capacity (Table 2). All other antioxidants showed high and almost the same DPPH radical scavenging capacity effect. It was described that radical scavenging abilities of some compounds can be influenced by their different kinetic behaviour (Kulisic *et al*. 2004). For slow-reacting compounds, the influence was attributed to the complex reacting mechanism. In our study, probably, the constituents from thyme essential oil involved one or more secondary reactions, which result in the slower reduction of DPPH solutions (Kulisic *et al*. 2004). After 15 days of storage (Table 3), application of thyme oil may help in the maintenance of the stored sprouts reserves, keeping the internal biochemical enzymatic activities in minimum level and in more stable case, thereby prolonged their shelf life. Also, this treatment was highly effective in the protection of sprouts against the known degradable effects of higher free radicals during storage conditions.

Application of 4% thyme and basil oils reduced the pathogenic fungi from seed to seedling (Table

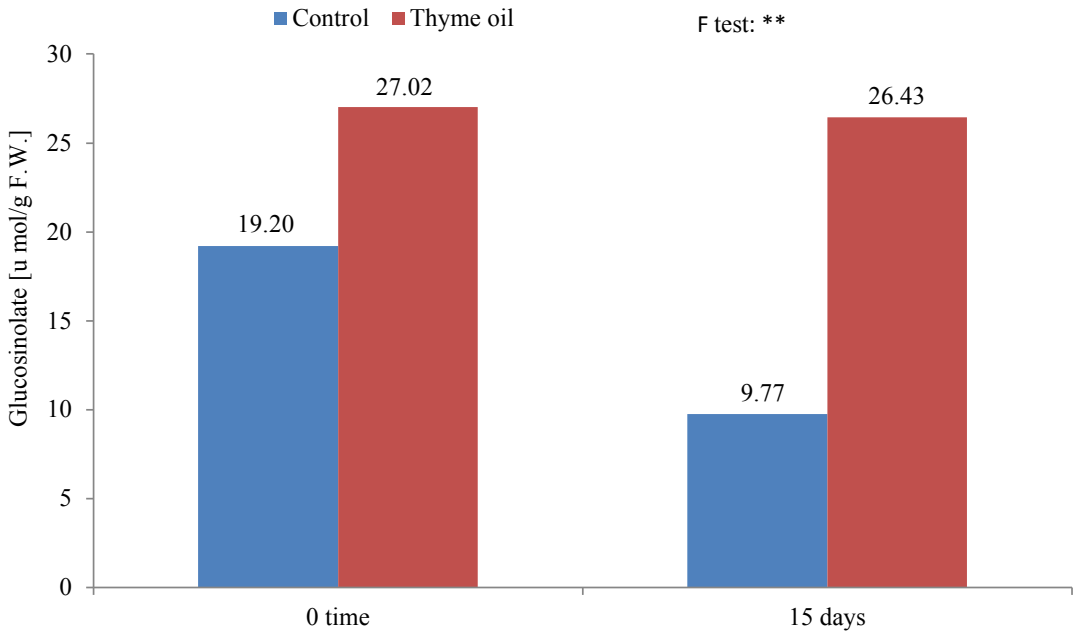

Figure 8. Total glucosinolate content as affected by the thyme oil compared to the control treatment at 0 time and 15 DAS

4) and had a positive effect on the seed germination of infected seeds, and basic constituents (monoterpenes and antioxidants) tended to slow down the activity of carbohydrates and protein breakdown associated enzymatic systems as well as enzyme involved in respiration and energy metabolism (Ngueface *et al*. 2005).

Application of thyme oil increases phenolic compound content especially in 10 days compared to that in the initial period of cold storage (Figure 2). The results obtained in this study are in agreement with the phenolic profile reported by Pająk *et al*. (2014). However, some authors confirmed that low storage temperature causes an accumulation of total polyphenols (Villarreal-García *et al*. 2016).

During cold storage (Figure 3), at day 15 the DPPH in control was reduced to 28.57% compared to that in sprouts treated with thyme oil (1.98%) Nath *et al*. (2011) observed a steady decrease in the DPPH radical scavenging capacity for 144 h of storage of broccoli inflorescences. The above behaviour may be due to the constant changes in plant metabolism during storage as a result of oxidative stress, which may include structural changes in synthesis or antioxidant compounds (Xiao *et al*. 2014).

In our study, thyme oil treatments possibly affected the environmental stresses of sprout broccoli; however, in contrast with control treatment, total flavonoid content decreased to 58.33% compared to that measured at initial period (Figure 4). An explanation for this could be the very high respiratory rate of broccoli (Izumi *et al*. 1996) that could increase the metabolism and, therefore, the degradation of the phenolic compounds.

During cold storage, broccoli's potential for maintaining the stability of vitamin C levels found in the fresh product was due to thyme oil application (Figure 5). Thus, according to previous report (Davey *et al*. 2000), broccoli retain its vitamin C levels because of the protection of other oxygen scavengers.

Total chlorophyll and total anthocyanin contents slightly decreased at 4°C because thyme oil application prevented the degradation of anthocyanin and chlorophyll (Figures 6 and 7), but this might be lost at the control, which may be associated with water loss (Haminiuk *et al*. 2012; Sabir 2012).

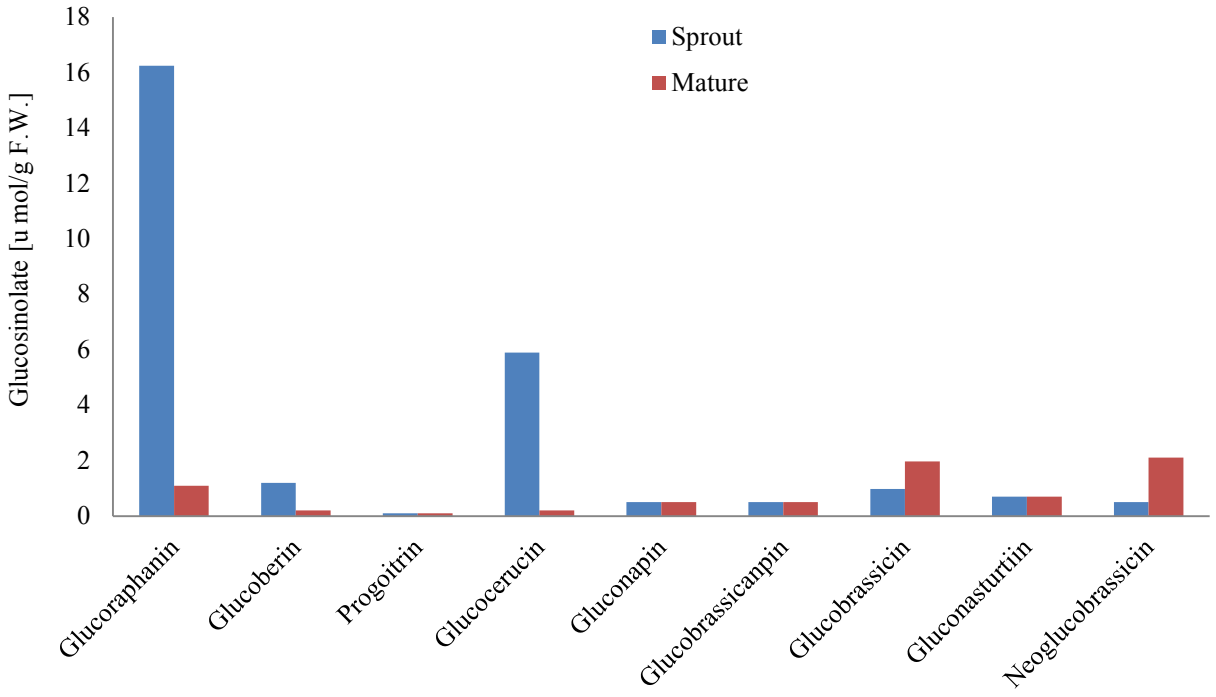

Figure 9. Total and individual levels of aliphatic, aromatic and indole glucosinolates in 3-day-old broccoli sprout and mature at harvest

Figure 10.(a) Mature plant and (b) sprout of broccoli

Thyme oil increased glucosinolates content in 3-day-old sprouts, compared to control treatments (Figure 8). The highest decrease in total glucosinolate content was observed in control treatment at the end of storage. This behaviour is consistent with experiments conducted by Howard *et al*. (1997), who reported a decrease of 50% on day 14 of storage at 4°C in broccoli sprouts. Cultural practices, handling and storage conditions, as well as the vegetable preparation, have a potential impact on the glucosinolates content, causing a change in the rate of formation of sulphoraphane (Jeffery & Araya 2009). However, the total glucosinolate content of the seedling was highest at both 4 and 7 days. Glucosinolates are plant defence compounds and, consistent with this function, are accumulated preferentially in the organs that contribute most to plant fitness at a particular moment in the growth cycle (Halkier & Gershenzon 2006).

The total glucosinolate content in sprout is higher than that in florets (Figures 9 and 10). Glucosinolates are divided into three major categories: aliphatic, indole and aromatic glucosinolates (Yan & Chen 2007). The high content of the aliphatic glucosinolates in broccoli sprouts is mainly attributed to glucoraphanin (16.24 µg/g F.W.). The glucoraphanin can be hydrolysed to form sulphoraphane. This compound plays an important role in controlling, preventing or blocking any of the multiple stages of the carcinogenic process (Parnaud *et al*. 2004). The florets contain the highest level of aromatic/indolylglucosinolates and neoglucobrassicin (2.11 µg/g F.W.) followed by glucobrassicin (1.67). Indole-3-carbinol (C_9H_9NO) is produced by the breakdown of the glucobrassicin. Indole-3-carbinol is a powerful strategy for achieving protection against carcinogenesis, mutagenesis and other forms of toxicity.

The impact of harvest and storage techniques on phytochemical has only recently begun to be explored. In general, phenolic compounds are considered to be relatively stable at cool temperature storage.

CONCLUSIONS

The results of this study show that the content of phytochemicals such as phenolic compounds, total flavonoids, anthocyanin, chlorophyll, ascorbic acid and glucosinolate in broccoli sprouts is stable during storage after 15 days at 4°C because of the application of essential oils. Thyme oil gave the best results on the content of phytochemicals and had a highest content of the glucosinolate and reduced the microbial load compared to the control. Fortunately, the coliform bacteria was not detected in all treatments. The results also indicate that the total glucosinolate content in sprout is higher than that in florets. The sprouts had significant values of glucoraphanin that can be hydrolysed to sulphoraphane. This compound plays an important role in controlling, preventing or blocking any of the multiple stages of the carcinogenic process. Therefore, the consumption of this food can play an important role in the prevention of related diseases with free radical generation, considering broccoli sprouts as a functional food.

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