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Synergism of Neem and Karanja Oils against Colorado Potato Beetle Larvae in Field Conditions

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Abstract: The increasing scarcity of active substances approved for use in plant protection is reflected in the growing effort to find suitable plant protection alternatives. Products based on plant oils could provide a promising environmentally friendly solution. In previous research in laboratory conditions, the synergistic effect of neem and karanja oils on *Leptinotarsa decemlineata* (CPB) larvae was observed. The aim of this current study was to verify whether the synergistic effect would also be observed in field conditions. The active substances used included azadirachtin A (NeemAzal® T/S); in both a reduced dose of 10.6 g/ha and a normal dose of 26.5 g/ha (Neem1, Neem2), *Pongamia pinnata* oil (Rock Effect New–REN); in a reduced dose of 1987.6 g/ha, and a mixture of both reduced doses (MIX). The protective effect was expressed by a visual estimation of the damaged leaf area on the potato plant. The MIX variant was always among the least damaged variants throughout the experiments, while the control was always the most damaged variant. A synergistic effect was observed at site I in 2021 when the MIX variant was more than 10 times less damaged than the control; in other cases, it was around 3 times less damaged. Treatment with MIX provided a protective effect comparable to NeemAzal® T/S in the full dose. This mixture can therefore be used to expand the portfolio of suitable preparations against CPB larvae in potato production.

Keywords: biopesticide; Colorado potato beetle; field efficacy; karanja oil; neem oil; synergism

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1. Introduction

Potatoes are the number-one non-grain food commodity and the fourth most important food worldwide [1]. They are an important source of nutrition for the growing global population, which is estimated to reach 9.9 billion individuals by the middle of the 21st century [2], i.e., by 0.9 billion more compared to the estimate of 2010 [3]. Potatoes are used in other industries, including feeds, starch, and seed potatoes. Although their acreage worldwide remains about the same (approximately 17.5 mil. ha between 1990–2018), in the same period, their production increased from approx. 267 mil. tons to approx. 370 mil. tons in 2018 [4]. The acreage used for potato production has constantly been decreasing in Europe, as well as in the Czech Republic.

According to FAO [5], up to 20–40% of global food production is lost to pests every year. The need for effective plant protection is clear; at the same time, many active substances are being prohibited, primarily due to the unsuitable ecotoxicological properties of the products. The European Union is planning to prohibit or restrict the use of chemical pesticides by up to 50% by 2030 [6], which will result in a global impact. The constantly shrinking spectrum of active substances not only makes the work of farmers more difficult but also increases the risk of selecting resistant pest populations. Decreasing the selection pressure delays the onset of resistance [7] and can be achieved by alternating the active substances used. The main potato pest, the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), which causes massive mechanical damage to plants, is very flexible and rapidly adapts to the active substances of plant protection products [8]. In addition, it is common to find different resistance levels among populations from different localities [9]. The arrival of neonicotinoid insecticides in 1995 brought a period of relief in areas where the beetles had developed resistance to other chemicals [10]. In 2008, the Colorado potato beetle was known to be resistant to 52 different insecticides [11], including spinosad and neonicotinoids [12]. At the same time, the Colorado potato beetle is an important potato pest that causes significant yield losses without a protective intervention [13–15]. In relation to yield reduction, potato plants are particularly sensitive to defoliation at the full-bloom stage [16].

Botanical pesticides show great potential in contemporary plant protection against pests, as they are based on the plants' own chemical defenses against pests, which have co-evolved with insects. As they originate from plants, they pose no burden to the environment regarding residues. They are mixtures of various bioactive substances [17] with different modes of action inevitably delaying resistance selection in pathogens and pests [18–20]. Moreover, their efficacy is usually comparable to that of synthetic products [21,22]. A product based on oil from the seeds of the neem tree, *Azadirachta indica* A. Juss, along with the active substance azadirachtin, is commonly used in plant protection. Azadirachtin is an insect deterrent that possesses anti-ovipositional, antifeedant and growth-disrupting properties and can also reduce the fitness and fecundity of insects [23]. However, the higher cost of this treatment comes as a disadvantage [20]. Other widely known biopesticides include karanj (*Pongamia pinnata* (L.) Pierre), mahua (*Madhuca indica* Gmel.) and chinaberry (*Melia azedirach* L.) [24].

Karanja oil often exhibits synergistic effects with many natural and synthetic products [24]. The synergism of karanja and neem plant extracts has already been observed in previous research. The methanolic extract of neem and karanja oil inhibited fungal growth [21], aphids and mites [25]. Pavela [18] described the connection between neem and karanja oils as beneficial for the preventive treatment of plants against diseases and pests in practice. In a previous study [26], on which this current research builds, a synergistic effect of neem and karanja oil mixtures against Colorado potato beetle larvae in the laboratory had been observed. On the other hand, exposure to weather conditions, in particular to UV radiation [27], can limit the efficacy of botanical products, so it is important to test how the products will behave in an open field. The aim of this study was to evaluate the protective effect of various potato treatments against Colorado potato beetle larvae and to verify the synergistic effect of karanja and neem oil (Rock Effect New and NeemAzal® T/S) based products in field conditions.

2. Materials and Methods

2.1. Experimental Sites

Experiments were carried out at the Potato Research Institute (PRI), Havlíčkův Brod, Czech Republic, in the summer seasons (June–July) 2020 and 2021 (site I, field coordinates: 49°1′14.231″ N, 16°36′57.049″ E in 2020 and 49°1′12.43″ N, 16°36′54.34″ E in 2021). Experiments were performed in accordance with the principles of Good Experimental Practices (GEP), and they were designed following EPPO guidelines [28] as completely randomized blocks (four blocks with five treatments). GEP codes are provided in Appendix A at the end of this paper. The total area of each treated plot was 3 × 8.4 m (25 m^2), of which 12.6 m^2 was for sampling. A similar but small-scale experiment (plots 1 × 2 m) was also established at the Crop Research Institute (CRI), Prague, Czech Republic, in June–July 2021 (site II, field coordinates: 50°5′14.930″ N, 14°17′54.678″ E).

At site I, the land is flat, with an altitude of 179–184 m. This area is among the warmest localities in the Czech Republic, with a mean annual temperature of 9–10 °C and a mean annual precipitation of 500–550 mm according to the long-term average 1991–2020 [29]. Potatoes (*Solanum tuberosum* L.), variety Rosara, were planted on 15 April 2020/23 April 2021 to a depth of 8 cm, in-row spacing of 30 cm, and inter-row spacing of 75 cm. The soil type at site I is sandy loam with a neutral pH and was not cultivated during the experiment.

The field was previously planted with winter wheat. Fertilization was conducted prior to the plantation on 7 April 2020/8 April 2021 with N (120 kg/ha), P_2O_5 (90 kg/ha), and K_2O (150 kg/ha). The last manure application (40 kg/ha) was done in October 2019/November 2020.

At site II, the land is also flat, with a higher altitude of 348–355 m. The mean annual temperature is 9–10 °C, and the mean annual precipitation is 500–550 mm according to the long-term average 1991–2020 [29]. Potatoes (*Solanum tuberosum* L.), variety Ditta, was planted on 22 April 2021 to a depth of 10 cm, in-row spacing of 30 cm, and inter-row spacing of 75 cm. The soil type at site II is clay loam and was not cultivated during the experiment. The field was previously planted with spring barley. The last manure application (40 kg/ha) was done in November 2020.

The experimental design followed completely randomized blocks (three blocks with six treatments). Temperature and precipitation measurements were taken at both sites using our own meteorological stations, with the data provided in Table 1.

Table 1. The sum of weekly precipitation and the average air temperature measured for four weeks after the application of the products (site I, 2020/site I, 2021/site II, 2021). Pesticide application was done on 24 June 2020, 21 June 2021 and 18 June 2021.

Day	∑ Precipitation [mm]	Mean Temperature [°C]
0–7	54/39.4/45	19.9/23/22.5
8–14	4.5/9.3/18.3	19.7/20/19
15–21	17.9/10.9/17.9	18.2/22.5/19.7
22–28	29.7/6.4/35.5	18.7/23.3/19.8

2.2. Insecticide Applications

The plant protection products NeemAzal® T/S (Trifolio-M GmbH, Lahnau, Germany) and Rock Effect New (Agro CS a.s., Česká Skalice, Czech Republic) were used in the experiment; details of which are presented in Table 2. The recommended field dose of NeemAzal (Neem2) was used as a standard, and the test dose was reduced (Neem1). The variant MIX, used to determine any synergistic effect, was composed of NeemAzal and Rock Effect New (REN) in the volume ratio 1:4. Additionally, SpinTor (Dow AgroSciences Ltd., Norfolk, UK) was used at site II as a reference variant. The control variant was treated with pure water. The water doses for site I and site II were 400 L/ha and 700 L/ha, respectively, with spraying being conducted on 24 June 2020/21 June 2021 (site I) and on 18 June 2021 (site II) using a Vermorel 2000 Electric backpack sprayer.

Table 2. Overview and dosing of the used chemicals.

Insecticide	Active Ingredient	Label	Dose
NeemAzal® T/S	Azadirachtin A	Neem1	10.6 g/ha a.i.
NeemAzal® T/S	Azadirachtin A	Neem2	26.5 g/ha a.i.
Rock Effect New	*Pongamia pinnata* oil	REN	1987.6 g/ha a.i.
Neem1 + REN	Azadirachtin A + *Pongamia pinnata* oil	MIX	10.6 + 1987.6 g/ha a.i.
SpinTor	Spinosad	Spin	36 g/ha a.i.
Water	x	control	x

x = not applicable.

2.3. Field Trial

Ten plants (site I) and five plants (site II) from each plot were picked randomly for sampling. The plants were marked with plastic signal tape. Marked plants did not touch neighboring plants in order to prevent potential larval migration between them. Observations for visual estimation of percent defoliation of the potato plant by CPB larvae and the number of CPB larvae per plant were recorded on sampling days 0, 2, 7 and 14 (site I, 2020); days 0, 1, 8 and 17 (site I, 2021); and days 0, 2 and 6 (site II, 2021).

2.4. Laboratory Trial

Leaf sections, 3.5 cm in diameter, were placed in separate plastic boxes (5 cm diameter) with filter paper at the bottom. CPB larvae were of the same age and a single larva was placed in each of the boxes onto the leaf section. Four larvae were used for each treatment. The same preparations used for the field trials were used in the lab. A volume of approx. 0.2 mL of the prepared solutions were sprayed using a hand sprayer onto the leaves with larvae. The feeding test lasted 18 h at 24 ± 1 °C, 40 ± 5% RH, L:D = 15:9 h and was repeated twice. The missing leaf tissue area at the end of the experiment was measured [mm^2] and compared.

2.5. Statistical Analyses

Defoliation estimates [%] were based on a visual examination of potato plants. Final defoliation was calculated as the change between the start and the end of the experiment. An initial number of larvae was used as a covariate, repetitions as a random effect, and treatment as a fixed effect in a logarithmic glmmTMB model–using R software version 4.1.2 [30]. The efficacy of different treatments in protecting the leaf area from feeding was calculated based on posthoc pairwise comparisons using the Emmeans package [31]. Larval count data were analyzed using a negative binomial model with fixed effects (variants and sampling days) and random effects (repetitions and plant ids). Feeding test data were analyzed using a simple lm model with two factors (variants and repetitions of the experiment).

3. Results

3.1. Larvae Counts

In 2021, the number of larvae at the beginning of the experiment was balanced, and no statistically significant differences were found between the variants ($p > 0.1$). Prior to spraying, the number of larvae per plant at site I ranged from 31–42 individuals (Table 3) and 21–30 individuals at site II (Table 4). The number of larvae at site I in 2020 (Table 5) was uneven, and in the variants MIX (13 individuals) and REN (14 individuals), there were statistically fewer larvae ($p < 0.0177$) compared to the control (24 individuals). During the experiment, the number of larvae decreased in all variants.

Table 3. Larvae count at site I in 2021 on sampling days 0, 1, and 8.

Day	Variant	Response	Lower CI	Upper CI
0	control	31	25.5	36.9
	mix	39	32.5	46.5
	neem1	40	33.5	47.9
	neem2	38	31.6	45.2
	ren	42	35.5	50.5
1	control	31	25.8	37.4
	mix	33	27.4	39.5
	neem1	37	31.2	44.6
	neem2	36	30.4	43.6
	ren	38	31.6	45.2
8	control	16	12.8	19.6
	mix *	**7**	**5.8**	**9.6**
	neem1	12	9.3	14.6
	neem2 *	**9**	**7.2**	**11.7**
	ren *	**10**	**7.5**	**12.2**

* Variants marked with * differed from the control statistically ($p < 0.05$).

Table 4. Larvae counts at site II in 2021 on sampling days 0, 2 and 6.

Day	Variant	Response	Lower CI	Upper CI
0	control	30	22.7	38.5
	mix	30	23.0	38.9
	neem1	28	21.2	36.5
	neem2	30	22.6	38.5
	ren	21	15.4	27.7
	spin	30	23.3	39.4
2	control	26	19.9	34.3
	mix	**12**	**8.5**	**17.1**
	neem1	21	15.6	28.0
	neem2 *	**8**	**5.5**	**12.3**
	ren	21	15.9	28.4
	spin *	**2**	**0.8**	**3.4**
6	control	24	17.9	31.4
	mix *	**7**	**4.3**	**10.4**
	neem1	19	13.7	25.3
	neem2 *	**3**	**2.0**	**6.0**
	ren *	**9**	**6.2**	**13.7**
	spin *	**3**	**1.5**	**5.1**

* Variants marked with * differed from the control statistically, ($p < 0.001$).

Table 5. Larvae counts at site I in 2020 on sampling days 0, 2 and 7.

Day	Variant	Response	Lower CI	Upper CI
0	control	24	19.5	28.7
	mix *	**13**	**10.7**	**16.3**
	neem1	22	18.3	27.0
	neem2	18	14.6	21.7
	ren *	**14**	**11.4**	**17.3**
2	control	20	16.7	24.7
	mix *	**7**	**5.4**	**8.7**
	neem1 *	**12**	**9.9**	**15.1**
	neem2 *	**11**	**8.6**	**13.3**
	ren	13	10.9	16.5
7	control	8	6.4	10.0
	mix *	**3**	**1.9**	**3.5**
	neem1	6	5.0	8.1
	neem2 *	**4**	**3.2**	**5.5**
	ren	6	5.0	8.1

* Variants marked with * differed from the control statistically, ($p < 0.05$).

At site II, there was a significant decrease in the number of larvae between days 0 and 2 for the Spin, Neem2 and MIX variants ($p < 0.0003$). The Neem1 and control variants did not show a significant reduction in the number of larvae even after six days ($p = 0.6685$ and 0.9947, respectively). At the end of the experiment, the fewest larvae were present in the Spin and Neem2 variants (3 individuals); this did not differ significantly from the MIX variant (7 individuals). The REN variant (9 individuals) differed from the Spin and Neem2 variants. The Neem1 variant and the control (19 and 24 individuals) had significantly more larvae than all other variants.

At site I in 2021, no significant decrease in the larval count was found between day 0 and 1 in any variant, while the change in the count on day 8 was different for all variants. At the end of the experiment (day 8), the fewest larvae were present in the MIX variant (7 individuals), which differed significantly from the control (16 individuals). Neem2 and REN variants (9 and 10 individuals) also differed from the control. The Neem1 variant with

12 individuals did not differ significantly from the control. Other differences in the number of larvae were statistically insignificant.

At the site I in 2020, there was a significant decrease in the number of larvae between days 0 and 2 for variants MIX, Neem1 and Neem2 ($p < 0.0001$). On the seventh day, the number of larvae differed from the beginning in all variants. At the end of the experiment, the fewest larvae were present in the MIX variant (3 individuals), which differed significantly from all other variants except Neem2 (4 individuals). The differences between the Neem2, Neem1 (6 individuals) and REN (6 individuals) variants were insignificant. There were 8 individuals in the control and the difference between the REN and Neem1 variants were not significant.

3.2. Defoliation

Defoliation was counted as the difference in damaged leaf area between the start and the end of the field trial. Control was the most damaged variant throughout the experiment, while MIX was always amongst the two least damaged variants. Detailed results are summarized in Table 6.

Table 6. Summary of results for the different years and locations; response = defoliation change [%] between the start and end of the experiment [%].

Locality	Year	Variant	Response	Lower CI	Upper CI
Site I	2020	control	10.2	8.1	12.9
		mix *	**3.1**	**2.3**	**4.1**
		neem1 *	**4.2**	**3.3**	**5.3**
		neem2	8.6	6.9	10.8
		ren *	**5.8**	**4.5**	**6.18**
	2021	control	56.7	45.9	70.0
		mix *	**4.1**	**3.3**	**5.0**
		neem1 *	**16.5**	**13.4**	**20.24**
		neem2 *	**2.7**	**2.2**	**3.3**
		ren *	**36.3**	**29.5**	**44.7**
Site II	2021	control	23.5	11.7	47.1
		mix *	**6.9**	**3.4**	**13.7**
		neem1 *	**7.7**	**3.8**	**15.4**
		neem2 *	**7.9**	**3.9**	**15.8**
		ren	22.0	10.9	44.1
		spin *	**1.5**	**0.8**	**3.0**

* Variants marked with * differed from the control statistically, ($p < 0.05$).

Significant differences at site I in 2020 were found between control and variants MIX, Neem1 and REN. MIX also differed from variant Neem2 ($p < 0.0001$) and REN ($p = 0.0097$), and finally, Neem1 differed from Neem2 ($p = 0.0002$). MIX and Neem1 were the least damaged variants.

At site I in 2021, all the variants were significantly different from each other. Neem2 and MIX were the least damaged variants, followed by Neem1 and REN. Control was the most damaged variant. Significant synergism of neem and karanja oils (variant MIX), according to the Colby equation [32], was confirmed at site I in 2021.

At site II the control was the most damaged variant and differed significantly from all other variants except REN. Variants MIX, Neem1 and Neem2, did not differ from each other. Variant spin was by far the least damaged variant.

3.3. Feeding Test

The most damaged variant in both cases was the control, followed by the variants REN, Neem1, Neem2, and MIX, with the least damaged variant being Spin (Table 7). Defoliation between variants was significantly different (LM, $F_{5,41} = 7.6$, $p < 0.0001$).

Table 7. Summary of defoliation [mm^2] done by one larva to potato leaf in feeding test in the laboratory during 18 h.

Variant	Response	Lower CI	Upper CI	Repetition
control	64.8	48.4	81.1	1
mix *	**25.5**	**9.1**	**41.9**	
neem1	36.8	20.4	53.1	
neem2	32.1	15.7	48.5	
ren	54.9	38.5	71.3	
spin *	**7**	**−9.4**	**23.4**	
control	87	70.6	103.4	2
mix *	**47.8**	**31.4**	**64.1**	
neem1	59	42.6	75.4	
neem2	54.4	38	70.8	
ren	77.1	60.7	93.5	
spin *	**29.2**	**12.9**	**45.6**	

* Variants marked with * differed from the control statistically, ($p < 0.05$).

4. Discussion

Products based on azadirachtin and karanjin provide a significant antifeedant effect [23,24,33]. Although they also exhibit an insecticidal effect [24], they do not kill Colorado potato beetle larvae and imagoes immediately–mortality occurs later, primarily at the time of moulting [20]. Initially, larvae hit by the sprayed product remain on the plants and show no signs of poisoning except for a reduction in feeding. For example, no larvae died during the feeding test in the laboratory (18 h) except those of the Spin variant, but in the field, we found out that the mortality occurred on the 2nd day after spraying for variants MIX and Neem2. Mailloux and Bostanian [14] stated that treating potatoes based on the level of defoliation would be better than a decision based on pest abundance. Therefore, potato leaf defoliation was chosen as the main parameter of this experiment instead of the usual pest mortality. For the same reason, it is not suitable to express defoliation as a variable that is directly dependent on the number of larvae on plants, as it depends mainly on the effects of individual sprays.

In 2021, the incidence rate of Colorado potato beetle was high at both sites; more than 30 larvae were present on the plants prior to treatment. Oviposition continued during the experiment, and although the new untreated egg clutches were removed on a regular basis, the experiment was terminated after the 6th day at site II because some plants were fully consumed after this period, particularly in the control and REN variants. Bhatnagar and Sharma [34] found that the residual toxicity of both neem and karanja oils was almost completely lost 4–5 days after the spray, with karanja oil being even less persistent than neem. For the same reason, the results from site I after the 8th day of the experiment were not included in the model.

The change in defoliation was minor in the variant Spin, confirming the rapid onset of the effect of the treatment. Reduced defoliation between days 2–6 was also observed in variants Neem2 and MIX. Low efficacy of REN alone in the chosen dose is quite common, given that karanja oil becomes effective only in higher concentrations [24,26]. The favorable temperatures in the year 2021 were also reflected in increased activity and feeding of the Colorado potato beetle. Larval development becomes faster at higher temperatures, with an optimum at 28 °C, while higher instar larvae can consume more food [35–37]. Products that did have an effect did not exhibit any considerable increase in defoliation between days 1–8, regardless of the number of larvae, because the larvae stopped feeding after being hit with the product.

The year 2020 was rather cold and rainy, and there were also fewer larvae on the plants. Thus the differences in defoliation between individual treatment variants were small, and no synergistic effect was observed that year. On the other hand, the trend in the efficacies of individual variants was apparent.

Based on plant defoliation results obtained during the experiment, a synergistic effect of neem and karanja oils in field conditions was demonstrated in 2021 at site I. It was further observed that satisfactory results in potato protection against the Colorado potato beetle could be achieved by combining low doses of both oils. Regarding the effects of individual treatments, the results of the laboratory feeding test were consistent with the field observations, although synergism was not achieved, probably due to the short feeding period.

Mailloux and Bostanian [14] reported that potatoes could sustain 12.5–25% defoliation (depending on the cultivar) without serious harm. Only the untreated control and the REN variant exceeded this value. Greater variability has been observed with Neem1, which may be a result of the reduced dose, which is not always completely reliable. With underdosing of pest control products, the risk of selection of resistant populations can also increase. REN alone in a reduced dose in a year with a favorable temperature development did not provide reliable protection against CPB larvae.

SpinTor (variant Spin), based on spinosyns, was included as a matter of interest at site II due to its high efficacy against Colorado potato beetle larvae, which has been observed in other experiments (unpublished). Depending on the application and under suitable conditions, SpinTor had the potential to kill up to 100% of larvae, which occurred very rapidly after the treatment was delivered. Feeding was stopped almost immediately, and resulting defoliation tended to be minimal. When spinosad is used, possible risks for non-target organisms need to be considered; this applies particularly to predatory mites, earwigs and hymenopteran parasitoids and pollinators [38]. Cases of resistance to spinosad have also been reported in the world, including in the US [12]. Resistance to spinosad has not been detected in the Czech Republic, and the same applies to azadirachtin, chlorantraniliprole, and cyantraniliprole diamides [39]. These active substances are thus often recommended for use in potato protection in practice. The synergistic mixture of neem and karanja oils from our experiments could become an addition to this portfolio.

Currently, the utilization rate of the neem tree and derived products is huge, which can result in the exploitation of the forests in the affected areas and the loss of biodiversity. However, the area available to grow neem trees is limited, and its usability in combination with karanja oil should increase interest in this biopesticide as well. As foretold by Deshmuk and Borle [40], the worse efficacy of karanja oil compared to neem will lead to a lack of interest in its mass utilization among growers. Additionally, the mixture of neem and karanja oils might reduce the economic costs of plant protection procedures. Other benefits include reduced environmental impact due to the botanical origin of the oils compared to synthetic products. Furthermore, it is a potential tool for managing resistance to pests by combining active substances–mainly limonoid triterpene azadirachtin and furanoflavonoid karanjin contained in neem and karanja oils, which provide a synergistic effect under optimal conditions.

5. Conclusions

In the field conditions, a statistically significant synergistic effect of neem and karanja oils (MIX variant) compared to the equivalent doses of separately used products (Neem1 and REN) was proved at site I in 2021. At site I in 2020 and at site II, the effect was not large enough to be statistically significant. Nevertheless, in all the experiments, variant MIX was always more efficient than Neem1 or REN, and defoliation was always the lowest on average in MIX–by 2.7% compared to Neem1 and by 8.4% compared to REN in the field trials. The effect of the MIX variant was virtually identical to that of Neem Azal T/S in the recommended field dose. All these findings are consistent with the results of the laboratory experiment.

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Appendix A

GEP codes: CZOR-VUB21-SOLTU-007VUB (No. 21/QK1920214/mand/Zab/VUB) and CZOR-VUB20-SOLTU-002VUB (No. 20/QK1920214/mand/Zab/VUB).

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