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CONTENT

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***Gordan S. KARAMAN*[1]**

REDESCRIPTION OF PARTIALLY KNOWN *GAMMARUS PAVLOVICI* S. KARAMAN, 1929 (FAMILY GAMMARIDAE) AND ITS VARIABILITY (CONTRIBUTION TO THE KNOWLEDGE OF THE AMPHIPODA 311)

SUMMARY

The epigean freshwater species *Gammarus pavlovici* S. Karaman, 1929 (Amphipoda: Gammaridae) is redescribed from type-locality, Rašće springs near Skoplje (North Macedonia). This species has been only partially described and for long time considered as synonym of *Gammarus balcanicus* Schäferna, 1922 by numerous authors (G. Karaman, 1977; Barnard & Barnard, 1983, etc.).

As *Gammarus pavlovici* was one of the earliest described taxa affiliated to *G. balcanicus*, the recognition of morphological and taxonomical characters of this species is notably to show the morpho-taxonomical relation of this species regarding *Gammarus balcanicus* from type-locality (Kolašin, Montenegro). Variability of some morphological characters within the type-locality population of *G. pavlovici* is presented and some recent problems within the molecular/genetic and classic morphological approach to the recognition of single taxa are discussed.

Keywords: Amphipoda, Gammaridae, *Gammarus pavlovici*, *balcanicus*, taxonomy, redescription, North Macedonia

INTRODUCTION

The freshwater species *Gammarus pavlovici* (fam. Gammaridae) has been discovered and described by S. Karaman (1929b) from the large spring Rašće near Skoplje (North Macedonia). Later this species was attributed to the genus *Rivulogammarus* S. Karaman, 1931 as distinct species (S. Karaman, 1931; Schellenberg, 1937a, etc.), than as subspecies of *Gammarus* (*Rivulogammarus*) *balcanicus* Schäferna, 1922 (G. Karaman, 1966), and finally as synonym of *Gammarus balcanicus* (G. Karaman, 1977; G. Karaman & Pinkster, 1987, etc.).

The recent molecular and genetic investigations of *Gammarus balcanicus* Complex indicated that *Gammarus balcanicus* Schäferna, 1922 is limited on the *locus typicus* region in Crna Gora (Montenegro) only (Mamos et al., 2014), and that many other described taxa, affiliated to *Gammarus balcanicus* as synonyms, should be a putative distinct taxa. These opinions were later confirmed by results of various authors worked on delimitation of taxa within genus *Gammarus* in various parts of Europe (Copilaş-Ciocianu, D. & Petrusek, 2017).

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Notes: The author declare that they have no conflicts of interest. Authorship Form signed online.

Gammarus pavlovici, as one of the earliest taxa affiliated to *G. balcanicus*, is redescribed and figured from type-locality, to show the morphological characters of this species regarding these of *G. balcanicus* from type-locality (Kolašin).

MATERIAL AND METHODS

The collected samples of *Gammarus* were preserved in 70% ethanol. The specimens were examined and dissected in the mixture of glycerin and water, using a Wild M 20 stereomicroscope. Dissected specimens were transferred onto slides with Faure liquid used for final preservation. The advantage of Faure liquid is that it is possible to dissolve the liquid on slides using water, and remove the dissected pieces for further studies. The body- length of examined specimens was measured by tracing individual's lengths from tip of the rostrum to end of the telson. Drawings were made using a camera lucida attachment and manually inked.

Some morphological terminology and setae formulae follow G. Karaman`s terminology (Karaman, G., 1969) regarding article 3 of mandibular palpus [A= A-setae on outer face; B= B-setae on inner face; C= additional C-setae on outer face; D= lateral marginal D-setae; E= distal long E-setae]. Terms "setae" and "spines" are used based on its shape, not origin.

All studies in this work are based on the classic morphological, ecological and zoogeographical studies.

TAXONOMICAL PART

Order AMPHIPODA Latreille, 1816
Family GAMMARIDAE Leach, 1814

GAMMARUS PAVLOVICI S. Karaman, 1929

Figs. 1-9

Gammarus pavlovici pavlovici S. Karaman, 1929b: 95, fig. 9a, d; Karaman, G., 1974: 12;

Rivulogammarus pavlovici pavlovici S. Karaman, 1931: 51, fig. 9;

Gammarus (Rivulogammarus) pavlovici Schellenberg, 1937a*:* 270; 1937b: 509;

Gammarus (*Rivulogammarus*) *balcanicus pavlovici* Karaman, G., 1966: 117, figs. 21, 23-26;

Gammarus balcanicus (part.) Karaman, G., 1977: 47; Barnard & Barnard, 1983: 464; Karaman, G. & Pinkster, 1987: 213, fig. 2S.

MATERIAL EXAMINED: North Macedonia:

-33= Rašće spring near Skoplje, North Macedonia, May 1929, 9 exp. (leg. S. Karaman) [paralectotypes];

-138= ibid., 1930, 14 exp. (leg. S. Karaman);

-430= ibid., 1933, many exp. (leg. S. Karaman);

Sp. 496= ibid., 1934, 6 exp. (leg. S. Karaman);

S-5798= May 1960, 4 exp. (leg. G. Karaman);

S-2717= ibid., 8.5.1972, many exp. mixed with *Gammarus roeselii* f. *triacanthus* Schäferna, 1922 and *Gammarus dulensis* S. Karaman, 1929a (leg. G. Karaman);

S-5759= ibid., 17.8.1962, many exp. mixed with *Gammarus roeselii* f. *triacanthus* Schäf. 1922 and *Gammarus dulensis* S. Kar., 1929a (leg. G. Karaman).

DESCRIPTION.**Male 11.0 mm**. (S-2717): Body moderately slender, metasomal segments 1-3 with 2-4 short dorsoposterior marginal setae each (fig. 2F). Epimeral plate 1 quadrate, with poorly convex posterior margin bearing 4-5 setae, 5 setae appear at ventroanterior margin.

Epimeral plate 2 slightly pointed, with inclined posterior margin bearing 4-5 setae and with 5 facial and subventral spines. Epimeral plate 3 sharply pointed, with concave posterior margin bearing 2-4 marginal setae and with 3 subventral spines (fig. 2F).

Urosomal segments 1-3 low, poorly elevated, not compressed laterally. Urosomal segment 1 with one dorsomedian and 2 dorsolateral groups of 2 spines each, accompanied by single short setae (fig. 3D). Urosomal segment 2 on each dorsolateral side with group of 3 spines mixed with single short setae, and with one median group of 2 spines accompanied by single short setae. Urosomal segment 3 on each dorsolateral side with group of 3 spines mixed with single short setae, dorsomedian group consisting of 3 short setae only. Urosomal segment 1 at ventroposterior margin with distal spine near basis of uropod 1 peduncle and with one median group of 3 setae (fig. 3D).

Head with short rostrum and short more or less subrounded (not angular) lateral cephalic lobes; eyes elliptic to poorly reniform, as long as diameter of antenna 1 peduncular article 1 (fig. 1A).

Antenna 1 reaching nearly half of body, peduncular articles 1-3 progressively shorter (ratio: 52:33:22), scarcely setose (fig. 1B); main flagellum consisting of 27 articles scarcely setose. Accessory flagellum nearly as long as last peduncular article and consisting of 4 articles (fig. 1B).

Antenna 2 relatively slender: peduncular article 3 with distal setae at ventral margin; peduncular articles 4 and 5 of equal length, scarcely setose, each with several bunches of facial and distal setae not exceeding diameter of articles themselves (fig. 1C); flagellum relatively slender, longer than last peduncular article and consisting of 12 slender articles bearing short setae up to as long as diameter of articles; many of articles with one calceola. Antennal gland cone short (fig. 1C).

Mouthparts basic. Labrum short, broader than long (ratio: 54:38), with subrounded distal margin (fig. 1D). Labium broader than long, with broad entire convex outer lobes, inner lobes absent (fig. 2A).

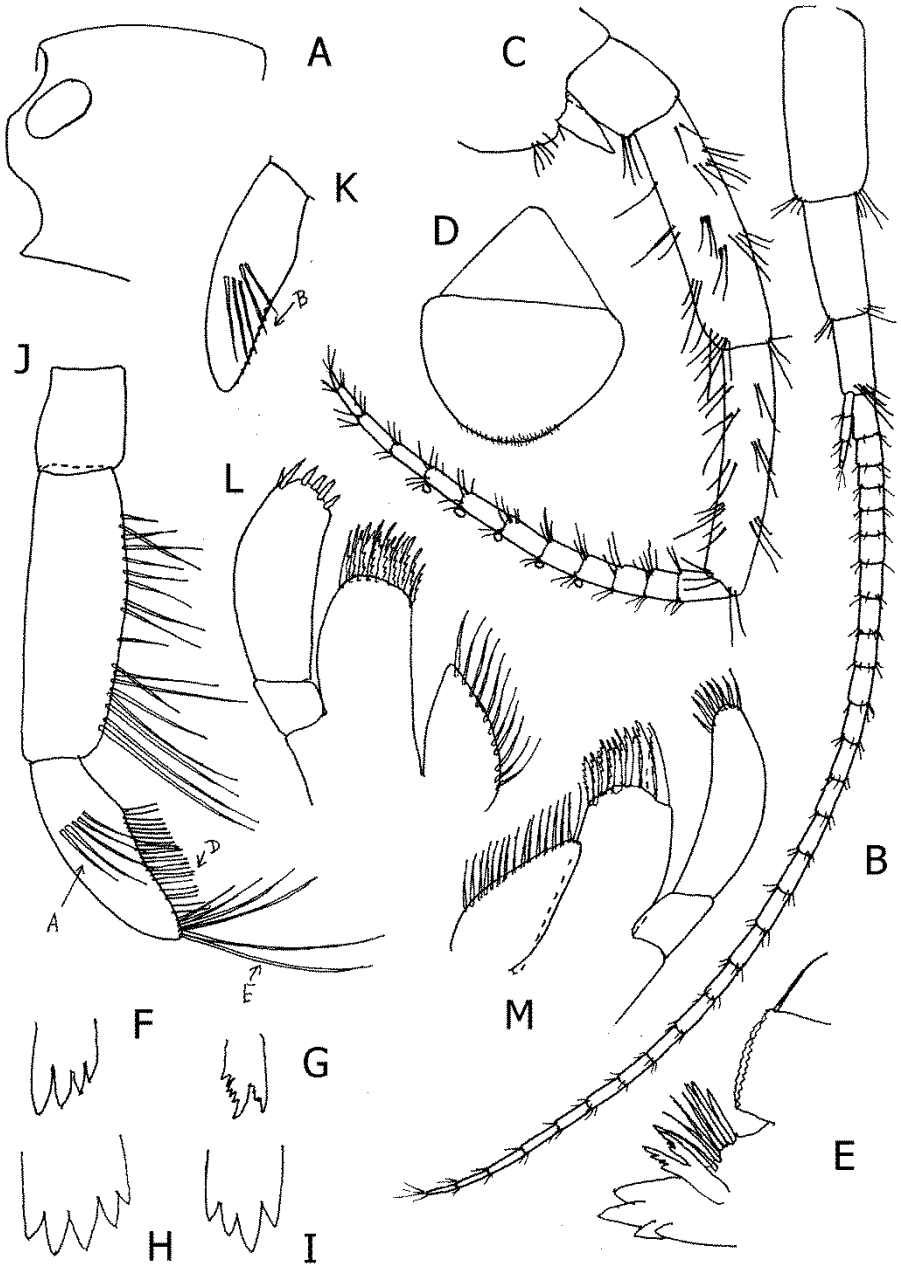

Fig. 1. *Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, male 11.0 mm: A= head; B= antenna 1; C= antenna 2; D= labrum; E= right mandible; F= right incisor; G= right lacinia mobilis; H= left incisor; I= left lacinia mobilis; J= mandibular palpus, outer face (D= D-setae; A= A-setae; E= E-setae); K= mandibular palpus, inner face (B= B-setae); L= right maxilla 1; M= left maxilla 1.

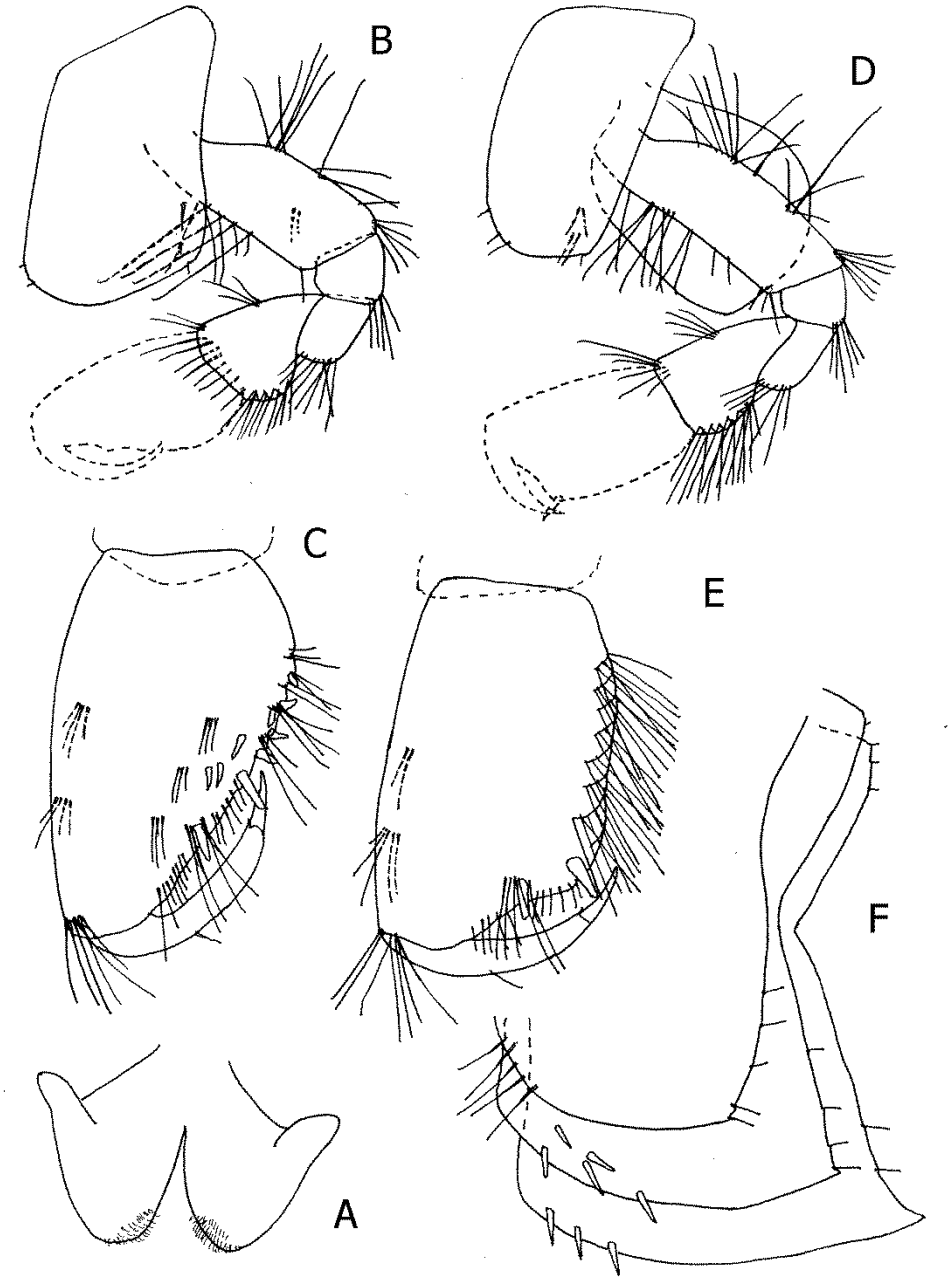

Fig. 2.*Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, male 11 mm: A= labium; B-C= gnathopod 1, outer face; D-E= gnathopod 2, outer face; F= epimeral plates 1-3.

Mandibles well developed, asymmetric to each other. **Left mandible**: molar triturative, with lateral short strong seta, incisor strong, with 5 teeth (fig. 1H), lacinia mobilis with 4 teeth (fig. 1 I), accompanied by nearly 8 strong rakers. **Right mandible**: molar triturative, with lateral long strong seta, incisor with 4 teeth (fig. 1F), lacinia mobilis bifurcate, serrate (fig. 1G), accompanied by nearly 7 strong rakers (fig. 1E). Mandibular palpus of left and right mandible symmetric to each other, consisting of 3 articles: first article short, naked, second article with nearly 18 setae (fig. 1J). Third article subfalciform, shorter than second article (ratio: 60:79), with over 20 D-setae and 6 E setae; on outer face appear one median group of 5 A-setae (fig. 1J), on inner face are attached 5 B-setae in 2 median groups (fig. 1K).

Left and right maxilla 1 asymmetrical to each other. **Left maxilla 1**: inner plate triangular, with row of mesial marginal setae (fig. 1M); outer plate with numerous serrate distal spines; palpus 2-articulated, second article slightly curved, narrow, bearing nearly 8 distal short spine-like setae. **Right mandible**: inner and outer plates like these in left mandible; palpus article 2 dilated, slightly curved, bearing 5-6 distal short strong spines (fig. 1L).

Maxilla 2 longer than broad, with inner plate slightly smaller than outer one bearing distolateral and faciolateral row of setae (fig. 3A); outer plate with distal setae only.

Maxilliped: inner plate longer than broad, with row of distal short spines and lateral row of setae (fig. 4A); outer plate not reaching distal tip of palpus article 2, with distal setae and lateral row of short spines. Palpus 4-articulated, article 2 along outer margin with one median and one distal bunch of setae; article 3 at outer margin with 2 median and one distal bunch of setae; article 4 (dactylus) with short nail and 3-4 short setae at inner margin near nail (fig. 4A), at outer margin with one median seta.

Coxa 1 longer than broad (ratio: 63:51), slightly dilated distally, subrounded ventral margin scarcely setose (fig. 2B).

Coxa 2 remarkably longer than broad (ratio: 68:43), ventral (distal) part subrounded and poorly more narrowed than proximal one, bearing nearly 4 short marginal setae; 3-4 short setae are attached at ventroposterior corner of the plate (fig. 2D).

Coxa 3 remarkably longer than broad (ratio: 77:42), with almost parallel lateral margins and subrounded ventral (distal) margin bearing 4 short setae only (fig. 3B).

Coxa 4 dilated, poorly longer than broad (ratio: 85:82), with strong ventroposterior lobe and poorly convex ventral margin bearing 1-2 short setae only (fig. 3D).

Coxae 5-7 much shorter than coxae 1-4. Coxa 5 much broader than long (ratio: 64:38), with short anterior lobe and 2 posterior marginal setae (fig. 4B). Coxa 6 smaller than coxa 5, broader than long (ratio: 49:28), anterior lobe small, posterior margin scarcely setose (fig. 4C). Coxa 7 entire, much broader than long (ratio: 49:25), ventral margin convex, with 3 posterior setae (fig. 4D).

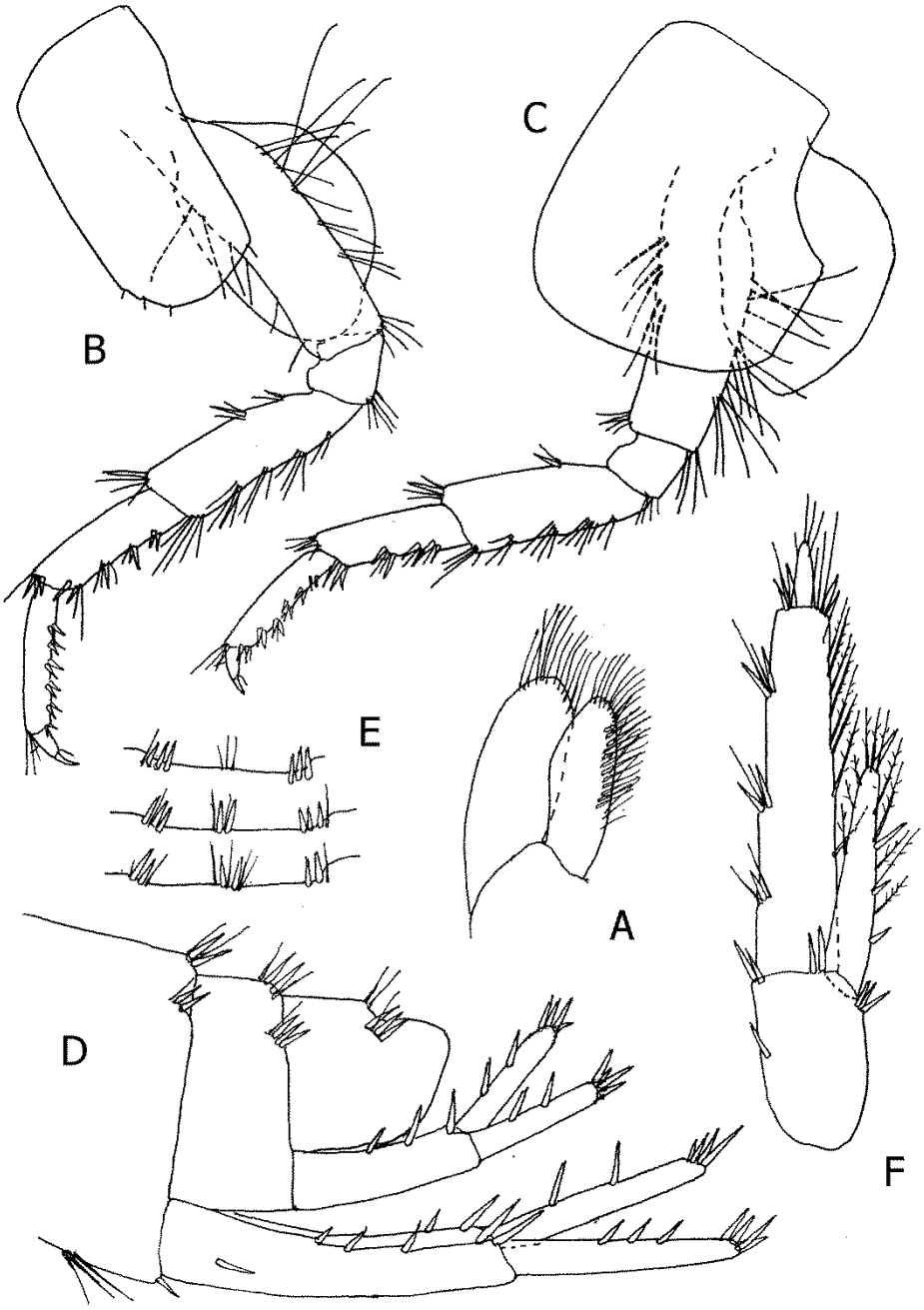

Fig. 3. *Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, male 11 mm: A= maxilla 2; B= pereopod 3; C= pereopod 4; D= urosome with uropods 1-2; E= urosome, dorsal projection; F= uropod 3.

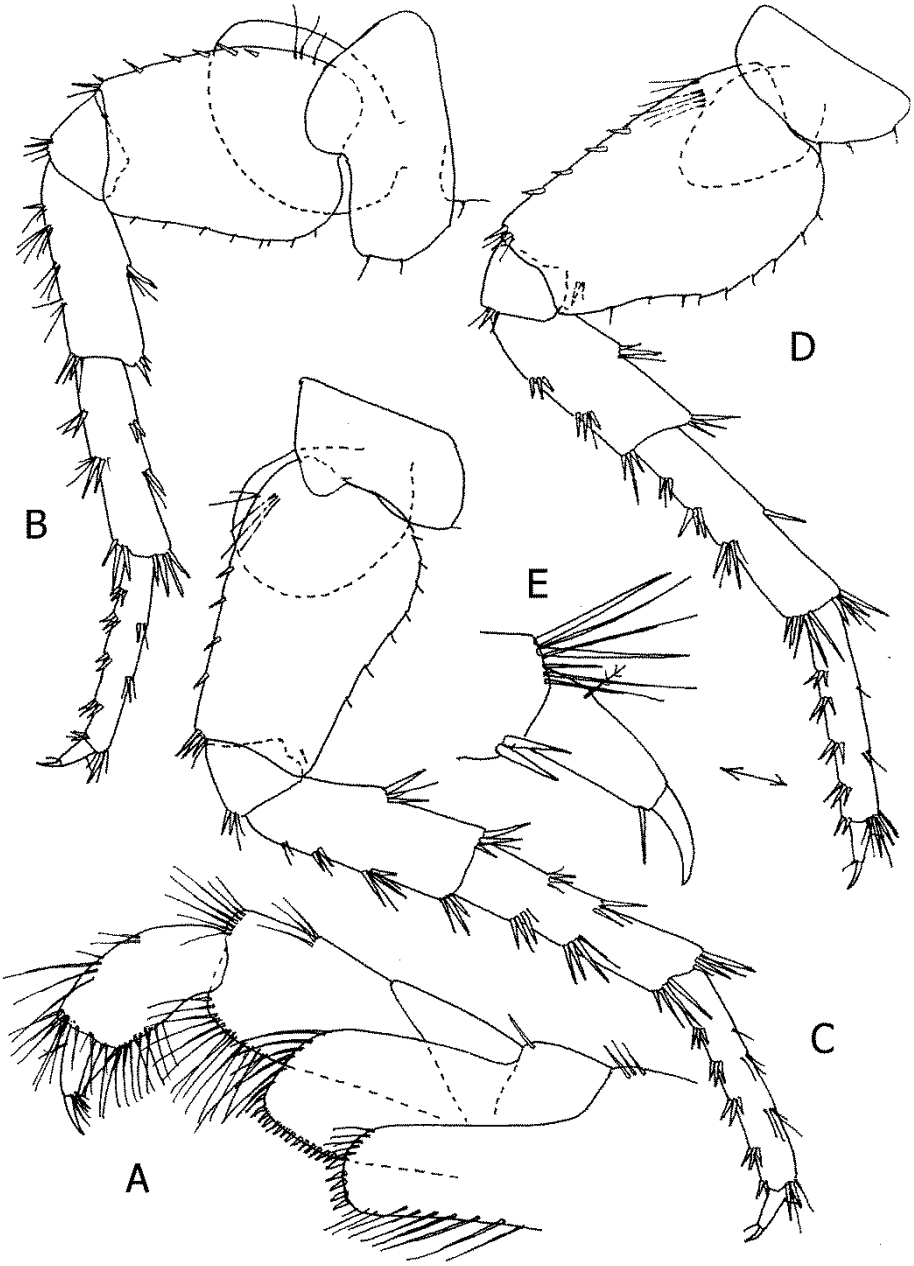

Fig. 4. *Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, male 11 mm: A= maxilliped; B= pereopod 5; C= pereopod 6; D-E= pereopod 7.

Gnathopod 1: article 2 along anterior and posterior margin with numerous long setae, setae in distal part are shorter than these in proximal part of article (fig. 2B); article 3 at posterior margin with one distal bunch of setae. Article 5 triangular, shorter than propodus (ratio: 35:50), along posterior margin with 3-4 transverse rows of short setae, along anterior margin with one median and one distal bunch of setae. Propodus longer than broad (ratio: 105: 62), pyriform, along posterior margin with 4 transverse rows of straight setae and several marginal spines; palm long, strongly inclined, with one corner and several facial spines; in the middle of palm is attached one strong spine accompanied by one bunch of setae much longer than spine itself. Dactylus along outer margin with one median seta, inner margin naked; nail short (fig. 2C).

Gnathopod 2: article 2 along anterior and posterior margin with long straight setae, especially in proximal part (fig. 2D); article 3 at posterior margin with one distal bunch of setae; article 5 triangular, shorter than propodus (ratio: 40:48), along posterior margin with 5-6 transverse rows of straight setae, along anterior margin with one median and one distal bunch of setae. Propodus quadrate, longer than broad (ratio: 95:60), along posterior margin with nearly 8 transverse rows of straight setae (fig. 2E), palm concave, inclined, with strong corner spine and one median palmar spine accompanied by bunch of long setae; dactylus along outer margin with one median seta, inner margin naked, nail short.

Pereopods 3 and 4 moderately strong. Pereopod 3: article 2 along both margins with long setae in proximal part and shorter setae in distal part. Articles 4-6 of unequal length (ratio: 55:38:39). Article 4 along posterior margin with 5 bunches of short straight setae not exceeding diameter of article itself, along anterior margin with 3 bunches of single setae mixed with spines (fig. 3B). Article 5 along anterior margin with distal bunch of short spines and single short setae, along posterior margin with 4 groups of short spines mixed with short straight setae; article 6 along posterior margin with 6 pairs of short spines, along anterior margin with distal bunch of setae. Dactylus short and strong, at inner margin with one seta near basis of the nail.

Pereopod 4 like pereopod 3 but with rather shorter setae along posterior margin. Articles 4-6 of unequal length (ratio: 50:35:36), article 4 along posterior margin with 5 groups of short setae, along anterior margin with one median and one distal spine mixed with single short setae (fig. 3C). Article 5 along posterior margin with 4 groups of short spines mixed with single short setae, along anterior margin with distal bunch of spine and short setae; article 6 along posterior margin with 6 pairs of short spines; dactylus short and strong.

Pereopods 5-7 moderately strong. Pereopod 5 slightly shorter than pereopods 6 and 7, article 2 longer than broad (ratio: 72:52), along anterior margin with row of short spines and proximal group of setae, along posterior margin with nearly 8 short setae, ventroposterior dilatation obtuse (fig. 4B). Articles 4-6 of poorly unequal length (ratio: 53:54:53), article 4 along anterior margin with 5 bunches of short setae, along posterior margin with 2 bunches of 1-3 short spines and setae; article 5 along both margins with 3 bunches of short

spines and single short setae. Article 6 along anterior margin with 5 groups of 2-3 short spines, along posterior margin with 3 groups of short spines; dactylus short and strong, with one strong seta at inner margin near basis of the nail, nail shorter than pedestal.

Pereopod 6: article 2 longer than broad (ratio: 80:48), along anterior margin with row of short single spines and proximal bunch of setae, along posterior margin with 7-8 short setae, ventroposterior dilatation obtuse, on inner face with proximal anterior group of submarginal setae (fig. 4C). Articles 4-6 of unequal length (ratio: 61:66:64); articles 4 and 5 along both margins with groups of short spines accompanied by single short setae; article 6 along anterior margin with 5 groups of short spines, along posterior margin with 3 lateral groups of short setae and distal bunch of short spines and setae. Dactylus short and strong, like that in pereopod 5.

Pereopod 7: article 2 longer than broad (ratio: 83:52), along anterior margin with row of nearly 5 spines, distal bunch of spine and short setae, and proximal group of setae, along posterior margin with nearly 9 short setae; on inner face appear distal subventral short spine and seta (fig. 4D), ventroposterior dilatation short, obtuse. Articles 4-6 of unequal length (ratio: 53:63:60); article 4 at anterior margin with 3 bunches of short spines, posterior margin with 2 groups of spines; article 5 along anterior margin with 4 bunches of short spines, along posterior margin with 2 groups of spines. Article 6 along anterior margin with 6 groups of short spines, along posterior margin with 3 median groups of short setae and distal bunch of short spines and setae. Dactylus like that of pereopods 5 and 6, at inner margin with strong seta near basis of the nail, along outer margin with one median plumose seta (fig. 4E); nail shorter than pedestal (ratio: 17:44).

Pleopods 1-3 with 2 retinacula. Peduncle of all pleopods with several bunches of short setae (fig. 5B, C, D).

Uropod 1: peduncle with dorsoexternal row of spines, dorsointernal margin with one median and 1-2 distal spines (fig. 3D); one spine is attached at outer face of peduncle; outer ramus is poorly longer than inner one, both rami with 2-3 lateral and 4 distal short spines.

Uropod 2: rami nearly equal or inner ramus is poorly longer than outer one, both rami with 2 lateral and 4-5 distal spines (fig. 3D).

Uropod 3: peduncle with one lateral and several distal spines; inner ramus reaching half of outer ramus, provided with plumose setae along both margins and with 3 lateral and one distal spine. Outer ramus 2-articulated, first article along outer margin with 4 bunches of spines mixed with single smooth setae, along inner (mesial) margin with numerous plumose setae (fig. 3F); second article short, not exceeding diameter of first article and provided with 2-3 lateral and 3-4 distal simple setae.

Telson slightly longer than broad (ratio: 80:75), each lobe with 2 distal spines accompanied by 2 simple setae (the longest setae exceeding length of spines), a pair of short simple setae attached on dorsal surface of each lobe, as well as a pair of very short plumose setae sitting in upper half of lobes (fig. 5A).

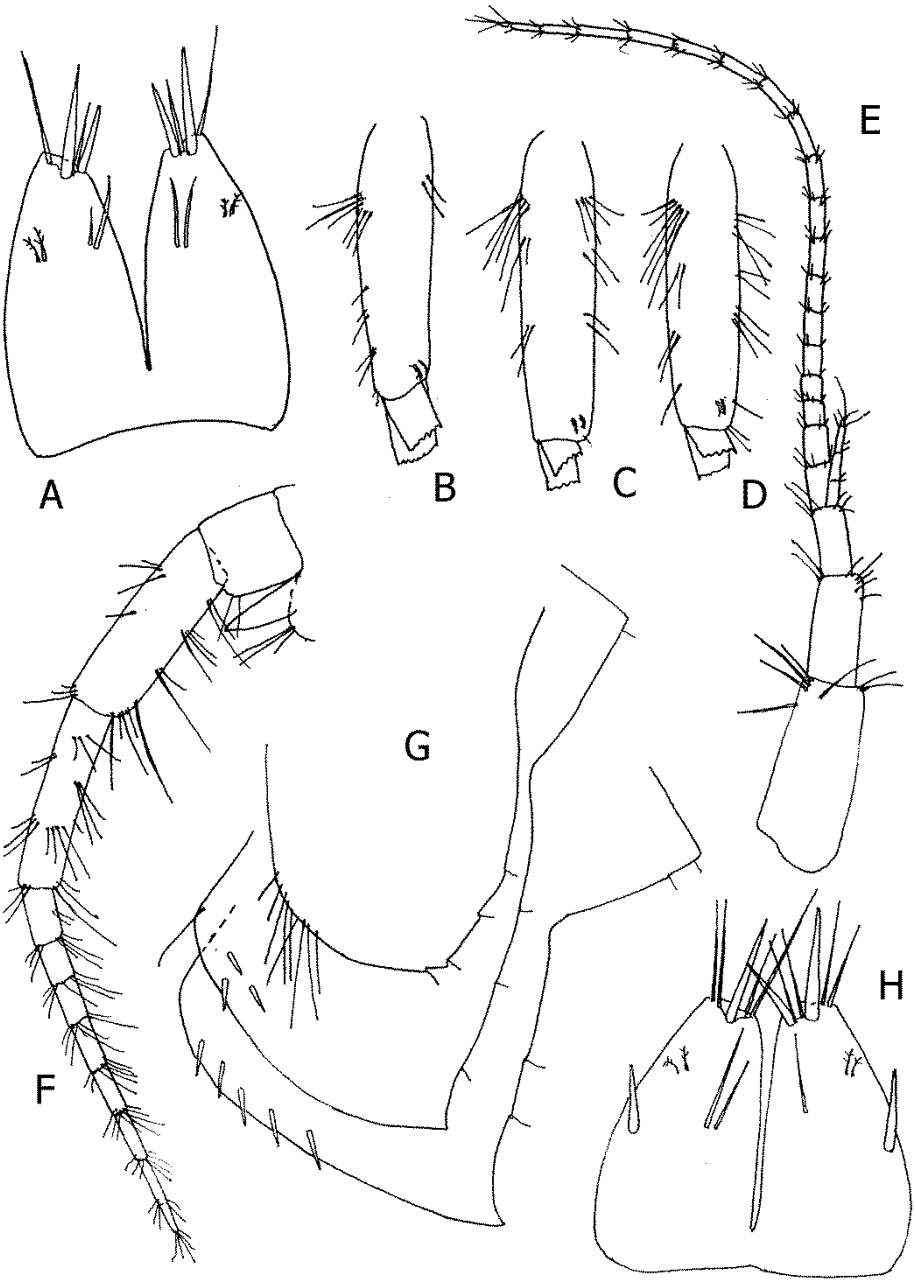

Fig. 5. *Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, male 11 mm: A= telson; B= peduncle of pleopod 1; C= peduncle of pleopod 2; D= peduncle of pleopod 3;
Female ovig. 8.2 mm: E= antenna 1; F= antenna 2; G= epimeral plates 1-3; H= telson.

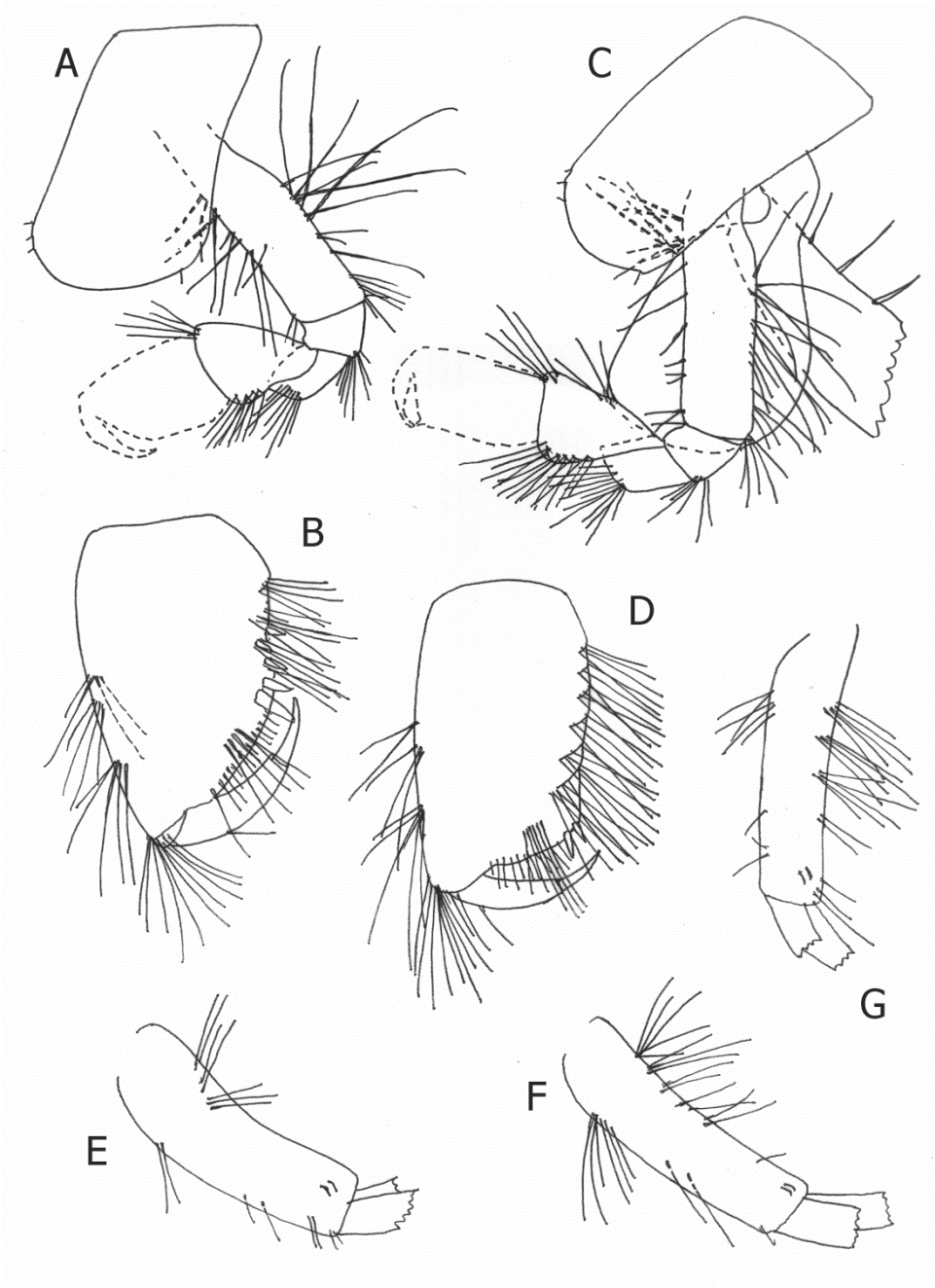

Fig. 6. *Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, female ovig. 8.2 mm: A-B= gnathopod 1, outer face; C-D= gnathopod 2, outer face; E= peduncle of pleopod 1; F= peduncle of pleopod 2; G= peduncle of pleopod 3.

Coxal gills ovoid, not exceeding the ventral tip of corresponding article 2 of legs; the smaller coxal gills appear on pereopod 7 (figs. 2D; 3B, C; 4B, D, E).

FEMALE with 14 eggs in marsupium, 8.2 mm: Body stout, metasomal segments with 2 dorsoposterior marginal short setae. Urosomal segments like these in males, low; first and second urosomal segment with one median and 2 dorsolateral groups of 2-3 short spines and short setae (fig. 9B). Urosomal segment 3 with 2 dorsolateral groups of 3 spines mixed with single short setae, median group is consisting of 2 short setae only. Urosomal segment 1 at ventroposterior corner near basis of uropod 1 peduncle with one spine and one lateral bunch of long setae (fig. 7C).

Epimeral plate 1 subangular, convex posterior margin bearing 4 short setae, at ventroanterior margin appear a bunch of setae (fig. 5G). Epimeral plate 2 pointed, with 2-3 posterior marginal setae, 3 facial spines appear in ventrodistal part. Epimeral plate 3 sharply pointed, with 2-3 posterior marginal setae and with row of 5 subventral spines.

Head like that in male, eyes elliptic to poorly reniform, not exceeding diameter of antenna 1 peduncular article 1.

Antenna 1 mostly like that in male but slightly shorter. Peduncular articles progressively shorter (ratio: 50:30:19), scarcely setose, but some setae can be longer than diameter of articles themselves (fig. 5E); main flagellum consisting of 19 slender articles scarcely setose. Accessory flagellum consisting of 4 articles (fig. 5E), longer than last peduncular article.

Antenna 2 relatively slender; peduncular articles 4 and 5 nearly of equal length or article 5 poorly longer, both articles along ventral margin with 4 bunches of setae (the longest setae exceeding diameter of articles themselves); flagellum relatively slender, consisting of 8 articles bearing setae as long as or longer than diameter of articles themselves, calceola absent (fig. 5F). Antennal gland cone poorly exceeding distal tip of peduncular article 3 (fig. 5F).

Mouthparts like these in male.

Coxa 1 longer than broad (ratio: 70:50), slightly dilated ventrally, bearing 5 short setae at convex ventral margin (fig. 6A). Coxa 2 longer than broad (ratio: 80:43), with 5 short setae at ventral convex margin (fig. 6C). Coxa 3 longer than broad (ratio: 82:50), at ventral convex margin with 5 short setae (fig. 7A). Coxa 4 slightly longer than broad (ratio: 83:65), with large ventroposterior lobe and scarce number of ventral marginal setae, several short setae are attached along posterior margin (fig. 7B).

Coxa 5 much shorter than 4, remarkably broader than long (ratio: 67:43) with small anterior lobe (fig. 8A). Coxa 6 is remarkably smaller than coxa 5, broader than long (ratio: 50:30) (fig. 8B). Coxa 7 only slightly smaller than coxa 6, entire, much broader than long (ratio: 50:25), with 2 setae at posterior margin (fig. 8C).

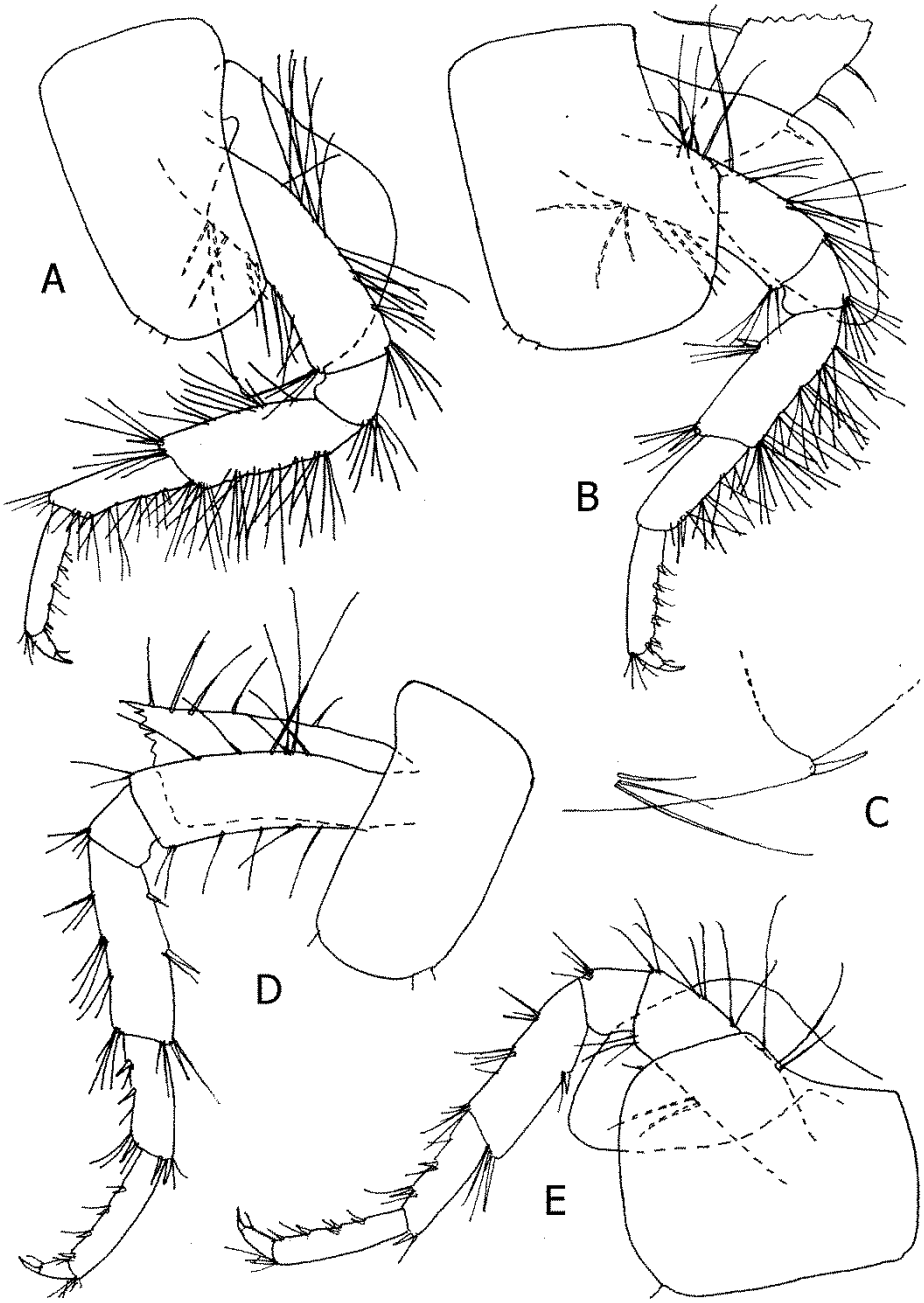

Fig. 7. *Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, female ovig. 8.2 mm: A= pereopod 3; B= pereopod 4; C= ventroposterior corner of urosomal segment 1.
Female ovig. 7.0 mm: D= pereopod 3; E= pereopod 4.

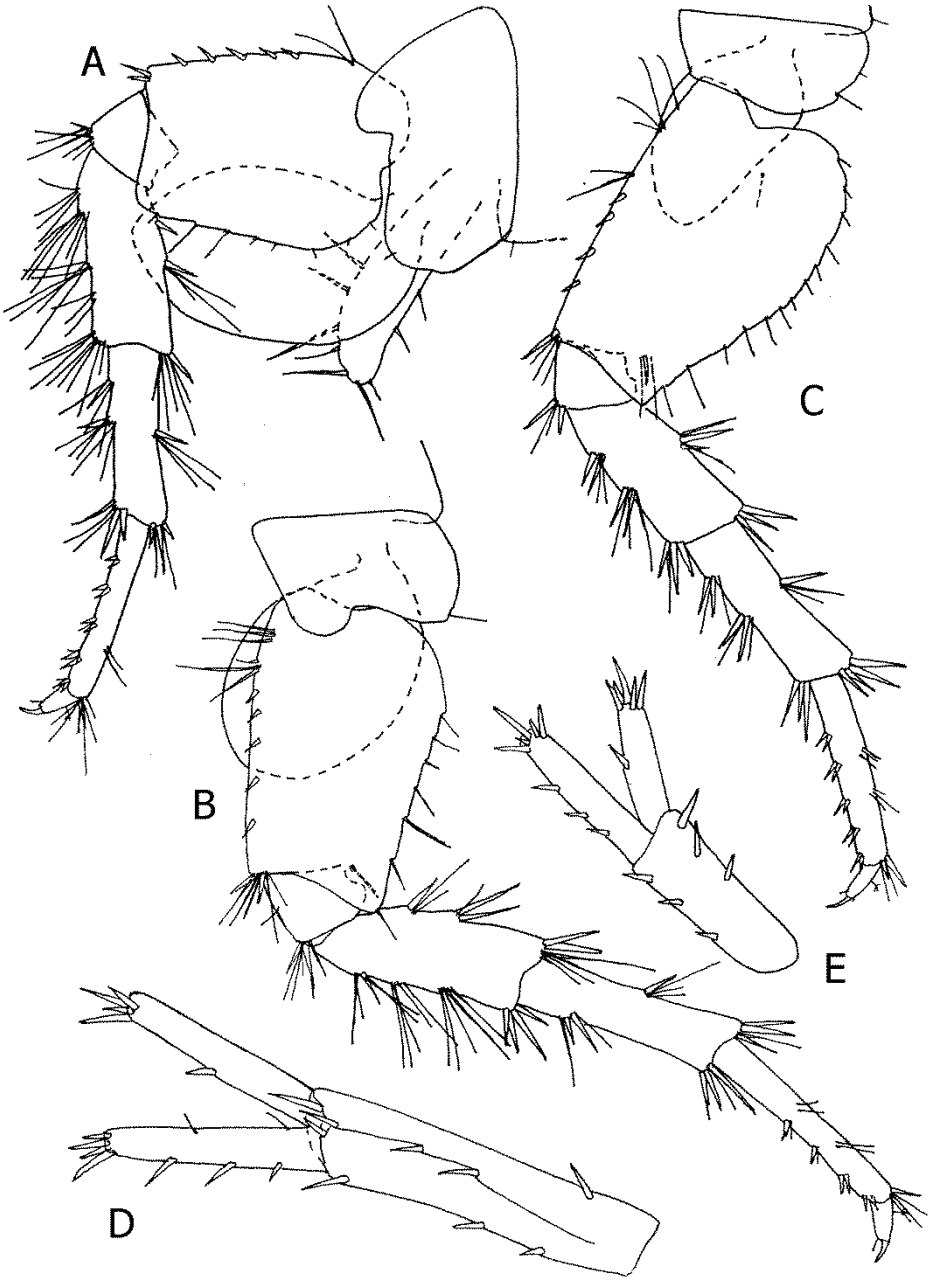

Fig. 8. *Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, female ovig. 8.2 mm: A= pereopod 5; B= pereopod 6; C= pereopod 7; D= uropod 1; E= uropod 2.

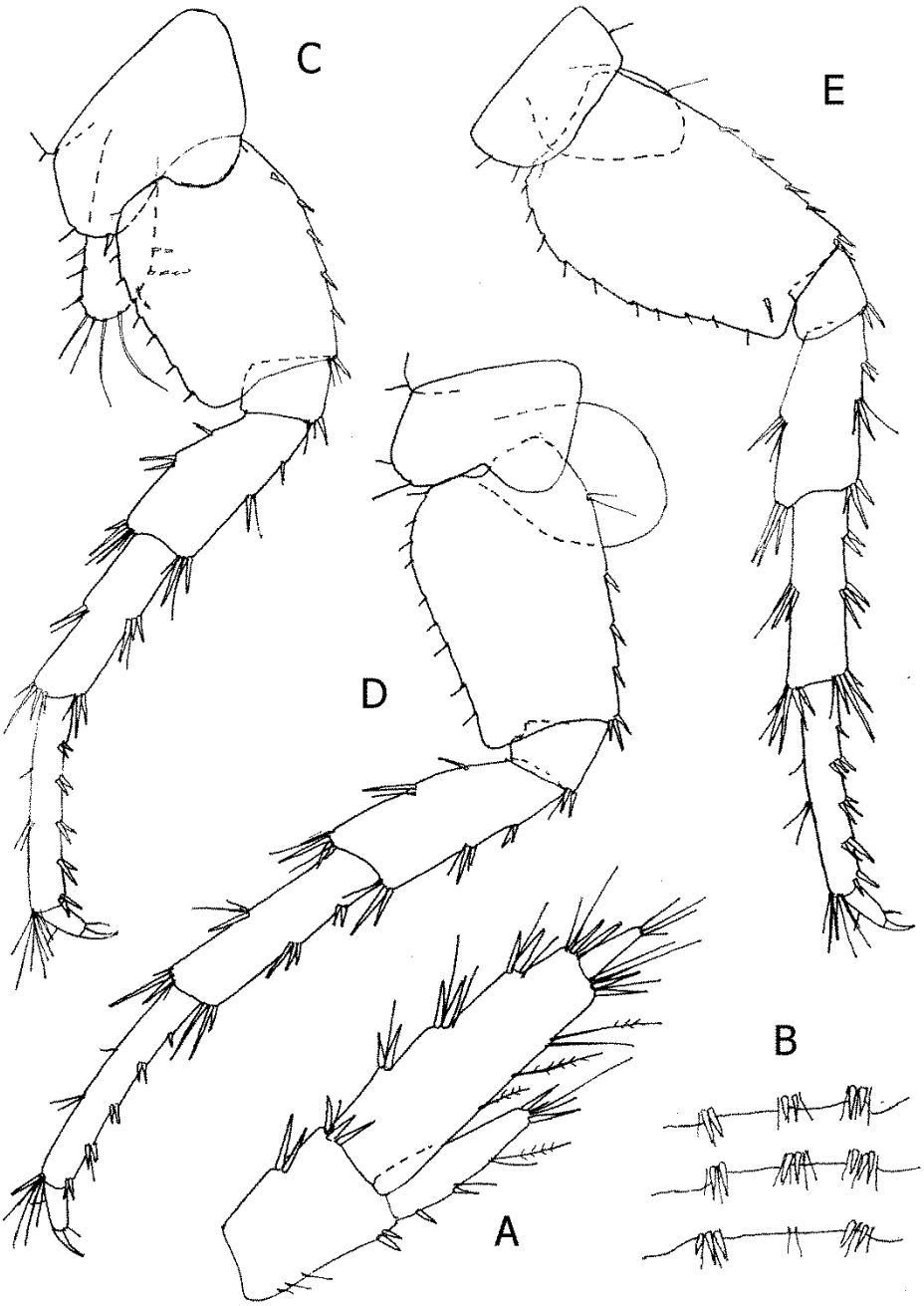

Fig. 9. *Gammarus pavlovici* S. Karaman, 1929, Rašće spring, Skoplje, female ovig. 8.2 mm: A= uropod 3; B= urosome , dorsal projection.
Female ovig. 7.0 mm: C= pereopod 5; D= pereopod 6; E= pereopod 7.

Gnathopods 1-2 smaller than these in male. Gnathopod 1: article 2 along anterior and posterior margin with numerous long setae in proximal and median part, setae in distal part of article are shorter; articles 3-4 like these in male. Article 5 triangular, almost as long as propodus, along posterior margin with several transverse rows of setae, along anterior margin with distal bunch of setae (fig. 6A). Propodus subpyriform, longer than broad (ratio: 83:54), along posterior margin with 3 transverse rows of setae and several marginal spines; palm inclined, slightly convex and defined by 2 strong corner spines; median palmar spine absent, but replaced by group of 4-5 long setae (fig. 6B). Dactylus along outer margin with one median seta, inner margin naked.

Gnathopod 2: article 2 along both margins with numerous long setae in proximal part, setae rather shorter in distal part. Article 5 triangular, narrow, nearly as long as propodus, along posterior margin with several transverse rows of setae, along anterior margin with 2 bunches of setae (fig. 6C). Propodus quadrate, longer than broad (ratio: 81:47), along posterior margin with 6 transverse rows of setae, along anterior margin with 3 bunches of long straight setae. Palm slightly inclined, with 2 corner spines, median palmar spine absent and replaced by group of 5-6 long setae; dactylus like that in gnathopod 1 but shorter, along outer margin with one median seta (fig. 6D).

Pereopods 3 and 4 more setose and with longer setae than these in male. Pereopod 3: article 2 along anterior and posterior margin with numerous long setae (fig. 7A). Articles 4-6 of unequal length (ratio: 48:32:34). Articles 3 and 4 along posterior margin with distal bunch of longer setae. Article 5 along posterior margin with numerous bunches of simple setae longer than diameter of articles themselves (fig. 7A), along anterior margin with 4 bunches of long setae. Article 5 along posterior margin with nearly 4 bunches of long straight setae remarkably longer than diameter of article itself, along anterior margin with distal bunch of setae only. Article 6 along posterior margin with 5 groups of short spines mixed with short single setae, along anterior margin with distal bunch of setae. Dactylus short and strong, like that in pereopod 7.

Pereopod 4: pilosity of articles 2-6 like that in pereopod 3, with numerous straight long setae (fig. 7B). Articles 4-6 are of unequal length (ratio: 43:31:32), dactylus short and strong, like that in pereopod 3.

Pereopods 5-7 more setose and with longer setae than these in male. Pereopod 5: article 2 longer than broad (ratio: 70:51), along anterior margin with row of short spines and proximal pair of short setae, along posterior margin with nearly 8 setae (fig. 8A); articles 4-6 of unequal length (ratio: 52:45:48); article 4 along anterior margin with 5 bunches of setae (the longest setae exceeding diameter of article itself), along posterior margin 3 single spines and several long setae are attached; article 5 along anterior margin with 3 bunches of setae mixed with single spines, along posterior margin with 2 bunches of setae mixed with spines; article 6 along anterior margin with 5 pairs of short spines, along posterior margin with 2 bunches of setae and single spines; dactylus short and strong.

Pereopod 6: article 2 longer than broad (ratio: 80:51), with subangular ventroposterior corner, along anterior margin with row of short spines and with 2 proximal groups of setae, along posterior margin with nearly 7 longer setae; on inner face is attached one subventral seta. Articles 4-6 of poorly unequal length (ratio: 57:55:58); article 4 along anterior margin with 3-4 bunches of longer setae and distal bunch of spines, along posterior margin with 3 bunches of spines mixed with single setae (fig. 8B); article 5 at anterior and posterior margin with 2 bunches of spines mixed with single setae longer than spines (fig. 8B); article 6 along anterior margin with 4 groups of short spines, along posterior margin with 3 groups of short setae and one distal spine. Dactylus short and strong.

Pereopod 7: article 2 longer than broad (ratio: 84:55), along anterior margin with row of short spines and 3 groups of proximal setae, along posterior margin with nearly 13 setae, on inner face appear one subventral spine and 2 setae. Articles 4-6 of poorly unequal length (ratio: 47:49:50), bearing less number of setae than these on pereopods 5 and 6 (fig. 8C). Article 4 along anterior margin with 3 bunches of spines mixed with setae (setae not exceeding diameter of article), along posterior margin with 2 bunches of spines mixed with single setae. Article 5 along anterior margin with 3 bunches of spines mixed with single setae, along posterior margin with 2 bunches of spines and single setae; article 6 along anterior margin with 5 groups of short spines; dactylus short and strong.

Pleopods 1-3 with 2 retinacula. Peduncle of all pleopods covered with several bunches of long setae (fig. 6E, F, G).

Uropod 1: peduncle with dorsoexternal and dorsointernal row of spines (fig. 8D); outer ramus poorly longer than inner one, both rami with lateral and with 4 distal short spines.

Uropod 2: inner ramus poorly longer than outer one, both rami with lateral and with distal 5 spines.(fig. 8E)

Uropod 3 slightly shorter than that in male; peduncle with single lateral and distal spines; inner ramus reaching nearly half of outer ramus, along outer margin with 2-3 lateral and distal short spines accompanied usually with single plumose setae (fig. 9B), inner (mesial) margin is naked. Outer ramus consisting of 2 articles: first article along outer margin with 4 bunches of spines mixed with simple setae, along inner (mesial) margin with row of plumose and single setae; second article short, not exceeding diameter of first article and bearing 4 distal simple setae.

Telson slightly broader than long (ratio: 82:74), each lobe with 1-2 spines accompanied by 4 setae (the longest setae exceeding length of spines); dorsal face of each lobe is covered by one proximal spine and 1-2 single setae, as well as by pair of short plumose setae in distal part of each lobe (fig. 5H).

Coxal gills large, ovoid, reaching or exceeding ventral margin of corresponding article 2 of pereopods (gnathopod 2 and pereopods 3-5) (figs. 6C; 7A, B; 8A), or gills are shorter (pereopods 6 and 7) (fig. 8B, C).

Oostegites moderately narrow, appear on gnathopod 2 and pereopods 3-5, with long marginal setae (figs. 6C; 7B; 8A).

VARIABILITY OF MORPHOLOGICAL CHARACTERS BY SPECIMENS FROM TYPE-LOCALITY

Body of females usually slightly more stout than that in male, with slightly longer extremities.The shape of eyes, usually semireniform, sometimes more elliptic, always reaching diameter of antenna 1 peduncular article 1. Lateral cephalic lobes subangular to obtusely subrounded. Metasomal segments 1-3 at dorsoposterior margin with 2-4 short setae only; urosomal segments 1-3 low, never compressed laterally, with one dorsomedian and 2 dorsolateral groups of elements (spines and setae), consisting of 2-3 spines accompanied by 1-4 short setae; only dorsomedian group on urosomal segment 3 always without spines, but with 2-4 short setae.

Antenna 1 reaching 2/5- 3/5 of body length, scarcely setose, rather longer, with higher number of articles in males than in females; accessory flagellum consisting of 3-4 articles, as long as or longer than last peduncular article.

Regarding the variability of morphological characters in females, to avoid possibility to compare abnormal or intersex specimens, we compare the females with eggs in marsupium. In the same sample two types of females were observed: females with strong pilosity of body, especially pereopods (figs. 7A, B; 8A, B, C), and females with relatively scarce pilosity of pereopods (figs. 7D, E; 9C, D. E).

In males in hands the pilosity of body and pereopods is more or less constant.

We suppose that this variability of pilosity is not just simple variability of single specimens, but probably exist the seasonal variability of the pilosity of body. Rather similar seasonal differences in the pilosity of body is observed in *Echinogammarus tibaldii* Pinkster & Stock, 1970 in Italy, where the species *E. bolo* G. Karaman & Tibaldi, 1973 and *E. roco* G. Karaman, 1973, despite the remarkable morphological differences, were later considered by some authors as seasonal different forms of *E. tibaldii* (**Pinkster, 1988**). There are no molecular/genetic investigations of this problem to support this conclusion.

This kind of possible seasonal variability has been mentioned already by G. Karaman **(1977**) in some populations of *Gammarus balcanicus* (sensu auct.) from Bosnia and Herzegovina (Buna River; Bosna River), North Macedonia (Bjelica River near Kičevo; Skopska Crna Gora Mts.), Croatia (Jadro River near Split; Krka River near Knin, etc.] where various types of morphological differences have been observed. The seasonal variability of *Gammarus* population is still poorly known, including molecular/genetic data of this phenomenon, and needs more detailed investigations.

Gammarus pavlovici (from type-locality) is very similar to *Gammarus balcanicus* Schäf. (from type-locality) by various general characters (scarce pilosity of body, scarce pilosity of article 2 of pereopods 5-7, presence of

calceola in males, slender antenna 2), but more detailed observations show differences [lacking dorsolateral groups of spines and setae on urosomal segment 1 in *balcanicus*, present in *pavlovici*; rather longer inner ramus of uropod 3 in *balcanicus*, shorter in *pavlovici*, etc.] (for details, see **Karaman, G., 1977**). For this reason, for delimitation of *G. balcanicus* and *G. pavlovici*, as well as for other taxa of *Gammarus balcanicus* superspecies is necessary use also many other characters (molecular/genetic, zoogeography, ecology, etc.]. As populations of *G. balcanicus* Complex are still in process of differentiation, the category subspecies must be not ignored, as well as presence of various intermediate populations.

LECTOTYPE: Male 8.0 mm (No. 33) and paralectotypes are deposited in KARAMAN`s Collction in Podgorica, Montenegro.

LOCUS TYPICUS: Rašće spring near Skoplje, North Macedonia.

ECOLOGY. In type-locality of *G. pavlovici*, spring Rašće near Skoplje (North Macedonia), three *Gammarus* species were observed in mixed populations: *Gammarus roeselii* f. *triacanthus* Schäferna, 1922 (sensu auct.), *Gammarus dulensis* S. Karaman, 1929b, *Gammarus pavlovici* S. Karaman, 1929b and *Niphargus macedonicus* S. Karaman, 1929a. All specimens are with distinct morphological characters of corresponding species, and no transitive specimens among *Gammarus* specimens have been observed.

DISCUSSION

Gammarus balcanicus Complex [or *Gammarus balcanicus* multispecies] show large plasticity and variability of numerous morphological characters between various populations as well as between specimens of one population as respond on different ecological and other conditions and events.

One species (multispecies) is consisting of numerous populations with rather different morphological characters each. If these populations with stable different morphological characters, settled well limited areal, these populations were considered, according classical taxonomy, as possible subspecies (as recognized category). If some of them acquire reproductive isolation, these populations were considered a distinct species.

Recently numerous scientists are providing various molecular/genetic studies of *Gammarus* taxa, with remarkably different approach to delimitation of various taxonomical categories.

Hebert et al. (2003) have opinion than the taxonomic expertise is collapsing, and they suggested: ”We are convinced that the sole prospect for a sustainable identification capability lies in the construction of systems that employ DNA sequences as taxon ‘barcodes’. We establish that the mitochondrial gene cytochrome c oxidase I (COI) can serve as the core of a global bioidentification system for animals.”

Sukumaran & Knowles, L. (2017) discussing about multispecies, and delimitation of taxa, considered “speciation as an extended process rather than an instantaneous event and carry out species delimitation inference on these data

under the multispecies coalescent.” They suggested “that the multispecies coalescent diagnoses genetic structure, not species, and that it does not statistically distinguish structure associated with population isolation vs. species boundaries. Because of the misidentification of population structure as putative species, our work raises questions about the practice of genome-based species discovery.”

About importance of recognition and delimitation of species and other taxonomical categories **Sukumaran & Knowles (2017**), pleading that: "Not all populations become species. Instead, speciation theory points to a continuum for the probability that a population lineage will evolve into a new species. Depending on the extent and duration of isolation and the form and strength of selection, speciation becomes more or less a protracted process, with new lineages only gradually and stochastically evolving from the initially isolated lineages into true species over time”. They well underlines that ” Misidentification of population structure as putative species is therefore emerging as a key issue that has received insufficient attention, especially with respect to methodologies for delimiting taxa based on genetic data alone”.

The introduction of cryptic species and the problem of recognition of cryptic species regarding rather morphologically different populations of already known taxa, remains very serious problem in taxonomy today, and many authors have different approach and suggestions. **Fišer et al. (2018)** by this way, cited **Ryberg (2015)** that “cryptic species are *de facto* taken into consideration by an increasing number of biodiversity studies that use sequence clusters rather than nominal species as units for taxonomic diversity”.

Taxonomical investigations are today in transitional period when the "classical taxonomy", based mainly on external morphology is considered by new genetic-molecular researchers as invalid or scarce valid, suggesting that only genetic-molecular approach [despite using limited methods of research at the moment] is crucial and valid. Combination of all kinds of research (which include morphology, molecular and genetic studies, ecology, histology, anatomy, etc.) will resolve the problems of delimitation of various taxa and understanding evolutive and taxonomical position of single populations, without underestimation of any of them. Similar opinion wrote also **Copilas-Ciolpan et al (2018)** mentioning that "The multidisciplinarity of integrative taxonomy is particularly useful for clarifying the systematics of speciose groups that are poorly differentiated morphologically, and this approach can also illuminate their evolutionary history and biogeography".

In this light, **Thomson et al. (2018**) mentioned: "Discovery of new organisms together with advances in methodology continue unabated, leading to a constant reevaluation of the boundaries between taxonomic entities. Species (and higher taxa) comprise related organisms that may be clustered together differently depending on which sets of criteria are emphasized”. They remember that "Through taxonomic research, our understanding of biodiversity and classifications of living organisms will continue to progress. Any system that

restricts such progress runs counter to basic scientific principles, which rely on peer review and subsequent acceptance or rejection by the community, rather than third-party regulation".

Further studies of these problems by numerous scientists will help us to understand the domains and limitations of various methods for the classification and phylogeny of various *Gammarus* populations, but always with "open mind" approach.

The increasing flow of described new species of Amphipoda in the next future, **Arfianti et al. (2018)** predicted, using a nonhomogeneous renewal process model, discovery 5600 to 6.600 new Amphipoda by the year 2100], what will show the richness of Amphipoda taxa, but increasing number of delimitation problems of various taxa also.

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EFFECT OF GENOTYPE ON MORPHOLOGICAL AND QUALITY FEATURES OF SUGAR BEET

SUMMARY

The importance of sugar beet is great because it gives root yield of about 50 t ha^{-1} and 30 - 40 t of fresh leaves and heads of beet that is used for feeding cattle. In the Republic of Serbia are favorable agro-ecological and soil conditions for the production of sugar beet. In the structure of total arable land of the Republic of Serbia, sugar beet accounts for about 2% where 94% of it is in Vojvodina, which has the most favorable climate and soil conditions, tradition and proximity of capacities for root production and processing. In addition to its adaptability to climatic conditions, sugar beet reacting quickly to changes in meteorological factors which greatly affect root yield and sugar content.

In the three-year trials it was examined morphological characteristics and the amount of a-amino N in five genotype of sugar beet. According to the survey results, the largest number of leaves at the genotypes at technological maturity had the genotype Otis (35.33) while the smallest had had genotype Severina (15:53). Genotype Otis, as a whole, had the highest leaf area ($\overline{X}$ =10193.13 and Xmax = 11483.79), and the lowest had genotype Severina ($\overline{X}$ = 4242.33; Xmin = 3982.44). The lowest average value of the amount of α -amino N indicators were found for Chiara and Laetitia ($\overline{X}$ = 2.23). The observed traits were largely depended on the genotypes and years. Genetic variation is necessary for successful breeding.

Keywords: sugar beet, genotype, morphological traits, α –amino N.

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is dicotyls plant, belonging to the family Amaranthaceae. Although in the above genus are annual and biennial species, grown sugar beet is a biennial plant species. Genus *Beta*, is a member of the family Chenopodiaceae, which in the cultivated group comprises sugar beets

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(*Beta vulgaris saccharifera*), fodder beets (*Beta vulgaris crassa*), leaf beets (*Beta vulgaris cicla*) and garden beets (*Beta vulgaris rubra*) (Ninfale and Angelino, 2013, Trifunovic *et al.* 2015).

Sugar beet leaf is simple and consists of leaf area and stem. The first leaves are much smaller than those that appear later. The stalk is thickened and ribbed. The first pair of opposite leaves emerges for 10 - 12 days after germination. Every 2 - 3 days appear over a new leaf which emerges at an angle of 135 to the previous one. The rosette leaves are spirally arranged and rosette itself can make 50 - 60 leaves and more. Leaves from 10th to 25th have the highest leaf area. Life of leaf runs from 27 to 70 days or more depending on the order of appearance, variety and production conditions. During the growing season leaves gradually die off. The stem is straight, ribbed, branches and grows up to 2 m and from the armpit leaves formed lateral branches of the first order, from which form the branches of the second order, and so on. In the axils of leaves branches last row formed flowers (in the second year of vegetation). Pollination is by insects. The fruit is coalesced groundnut (Glamočlija, 1990, Filipović *et al.* 2007, 2009).

Compared with some major field crops in Serbia, sugar beet production in the past has been characterized by the cultivating of both domestic and foreign varieties (Nenadic *et al.* 2003). In the Republic of Serbia are favorable agro-ecological and soil conditions for the production of sugar beet. In the structure of total arable land, sugar beet accounts for about 2%. 94% of the area under sugar beet is in Vojvodina, for its favorable climate and soil conditions, tradition and proximity of root processing capacities.

The proposed percentage relationship of sugar beet types in total sowed surface creates the possibility that the sugar beet gradually collecting from the fields and processing according to the technological maturing. Bojović (2014) suggest that such a combination of types provides a good digestion from the beginning of treatment and, at the same time, successive sowing of winter crops after removing of sugar beet.

The aim of this study was to investigate the influence of variety on the productive and morphological characteristics of sugar beet in agro-ecological conditions of South Banat.

MATERIAL AND METHODS

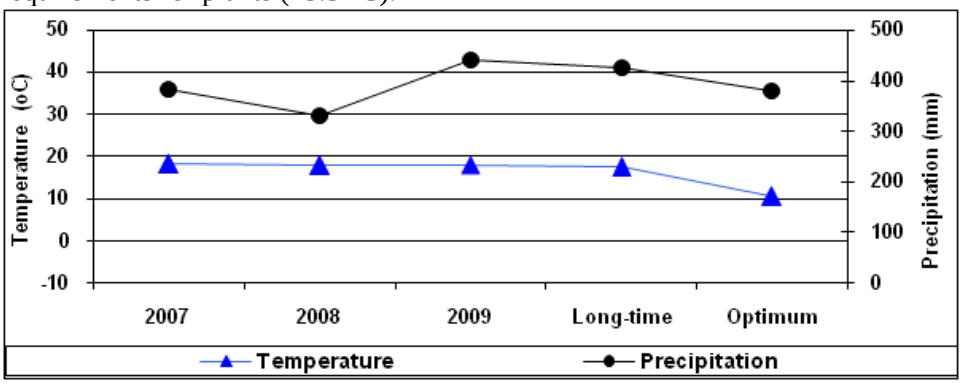
The study of the genotype influence on the morphological and technological characteristics of sugar beet was done in the period of 2007-2009. in agro-ecological conditions of South Banat at the experimental fields of the Institute Tamis PSS. Subjects of study were Otis, Chiara, Laetitia, Irina and Severina. Genotype Otis (producers Strube – Dickmann) is intended for removing the medium term. Genotypes Laetitia and Severina (producer KWS) recommended for removing medium terms. Genotype Chiara (producer KWS) is tolerant to drought conditions and is recommended for middle and later periods of extracting and NS genotype Irina (producer Institute of Field and Vegetable

Crops in Novi Sad) has well balanced root yield and sugar content and recommended for later removing from fields. The experiment was a randomized block system with four replications. Preceding crop, in all three year, was wheat. Land treatment was in two stages (August and October). Seedbed preparation and seed sowing were done at the end of March. Care and protection of crops were standard. Soil type on which are experiment derived is carbonate chernozem formed on loess terrace with high natural fertility. In texture it is loam (Bojović *et al.* 2014). Samples for determining the aboveground biomass were taken at the experimental plots. To determine the technological value of sugar beet root all the samples taken from the experimental plots were prepared for chemical analysis in the laboratory of sugar factories AD Unity in Kovacica. Laboratory analysis determined the content of "harmful" - α-amino nitrogen expressed in mmol / 1000S. The data were analyzed using the analytical statistical method STATISTICA 12 for Windows. The results are presented in tables and graphs.

Meteorological data.

In addition to the genotype and soil conditions, production technology and meteorological conditions have a great impact on the growth of plants (Djekic et al., 2017; Đekić et al., 2017; Živanović et al., 2017; Terzić et al., 2017; Ugrenović et al., 2018; Maksimović *et al.* 2018, Stevanović *et al*. 2018). One of the most important agro-technical measures in the sugar beet production is sowing. Proper sowing ensures optimal crop density and high yield. In our country, sowing of sugar beet usually is in the second half of March. Thermal conditions can then be unfavorable and unstable so it needs knowledge of seed and seedling traits in relation to possible adverse conditions in the soil at sowing time, seed germination and seedling emergence (Bojović, 2014).

Based on data Hydro-meteorological Station Pancevo monthly mean air temperatures are ranged from 18.00 °C (in 2008) to 18.20 °C in 2007 (Bojović *et al*. 2014). Thermal conditions during the study were 0.5 °C higher than the perennial average (17.6 °C) and higher than the conditional-optimum requirements for plants (15.3 °C).



Graph. 1. Temperature, °C, and precipitation, mm, Pančevo, Serbia

Total monthly rainfall had the great variation by years. Smallest total precipitation was in 2008 (331 mm), then in 2007 (384mm) and the highest in 2009 (440 mm). Total precipitation average during the growing season in the study period were lower by 41 mm compared to the multi-year average (426 mm) and at the level of conditionally-optimum requirements for plants (380 mm). Total rainfall during the growing season in 2007 were at the level of conditionally-optimal needs of plants, while in 2008 they were lower by 49 mm, and in 2009 more by 60 mm compared to the level of conditionally-optimal needs of plants (380 mm) vegetation period (Graph.1).

RESULTS AND DISCUSSION

Number of leaves. According to the results, the highest average number of leaves in the technological maturity of the genotypes, had the genotype Otis (35.33), and the smallest had genotype Severina (15.53), Table 1.

Table 1. Descriptive statistics for the number of leaves of sugar beet genotypes

Genotype	X_{min}	X_{max}	$\overline{X}$	$S_{\bar{x}}$	S	Cv (%)
Otis	30.97	36.13	35.33	0.49	1.55	4.38
Chiara	27.00	27.70	27.29	0.06	0.19	0.72
Laetitia	16.77	18.50	17.47	0.17	0.55	3.13
Irina	22.77	23.37	23.05	0.06	0.20	0.88
Severina	14.70	16.17	15.53	0.13	0.40	2.58

The variation of the average values of the number of leaves of sugar beet genotypes are expressed by the coefficient of variation and standard deviation. Analysis of the data was observed low variability where the greatest variation was in the genotype Otis (CV = 4.38%) and the lowest in genotype Chiara (CV = 0.72%).

Leaf area. Variety Otis, as a whole, had the highest leaf area ($\overline{X}$ = 10193.13 and Xmax = 11483.79), and the lowest genotype Severina ($\overline{X}$ = 4242.33, Xmin = 3982.44).

Table 2. Descriptive statistics of the studied genotypes of leaf area

Genotype	X_{min}	X_{max}	$\overline{X}$	$S_{\bar{x}}$	S	Cv (%)
Otis	8471.68	11483.79	10193.13	237.89	752.26	7.38
Chiara	8135.,79	9340.91	8579.29	127.94	404.59	4.72
Laetitia	5047.58	6376.05	5584.02	143.95	455.20	8.15
Irina	6056.21	6902.36	6410.64	75.50	238.75	3.72
Severina	3982.44	4426,29	4242.33	44.09	139.41	3.29

The coefficient of variation, as a measure of dispersion, with this indicator ranged 3.29% <Cv <8.15% which leads to the conclusion that it is a relatively low level of variation of this indicator within the genotype (Table 2).

The amount of α-amino nitrogen content. Smallest average value of α-amino N were at the genotypes Chiara and Laetitia ($\overline{X}$ = 2.23). The largest amount of this indicator ($\overline{X}$ = 5.88 and Xmax = 6.50) had a genotype Irina. For other genotypes values digestion almost halved (tab. 3).

The dispersion of the observed indicators of the highest in the genotype Chiara (Cv = 45.08%).

Table 3. Descriptive statistics for the amount of the α -amino N studied varieties

Variety	X_{min}	X_{max}	$\overline{X}$	$S_{\overline{x}}$	S	Cv (%)
Otis	2.71	3.58	3.19	0.10	0.31	9.70
Chiara	1.47	4.53	2.23	0.32	1.00	45.08
Laetitia	1.59	2.93	2.23	0.14	0.44	19.93
Irina	5.27	6.50	5.88	0.14	0.43	7.41
Severina	2.10	3.22	2.59	0.13	0.42	16.17

Correlation between the studied parameters of sugar beet.

Studied correlation indicators were expressed by Pearson's correlation coefficient. Among the indicators there is a weak dependence if |0.00|<r<|0.50|, medium dependence if |0.50|<r<|0.75|, dependence strong if |0.75|<r<|0.90| and very strong dependence if |0.90|<r<|0.99|.

Correlation between the studied indicators individually for each genotype, are given in Table 4.

Based on the strength connections between the studied indicators, we can conclude that there was a correlation connection of all indicators, but of different intensities (tab. 4). Very strong positive correlation was between the number of leaves and leaf area (r = 0.828) and between the leaf surface and α amino N (r = 0.628) in average for all examined genotypes.

The observed traits largely depend on the genotypes. Genetic variation is necessary for successful breeding. In a breeding program it is therefore of utmost interest to quantify the variation among breeding lines and wild relatives of the crop (Ghasemi *et al.* 2014).

From sugar beet, beside the main products - sucrose, by various technological processes, from harvest residues obtained after processing we are getting a large number of different products. The heads and leaves that remain in the field, have great nutritional value as they contain 13% dry matter, of which

6.8% BEM, 2.4% of total protein, 2.4% of mineral salts and 0.9% cellulose 0.5 % of oil (Glamočlija, 1986). The yield and technological quality of roots were significantly associated with climatic conditions, soil (composition, structure and processing) and the most important agro agrotechnical measures, tillage, sowing, the correct choice of varieties, plant nutrition and crop protection. Size of root is in inverse proportion to the sugar content (Dobrovnaya *et al*, 2009, Bojović, 2014).

The great importance of the sugar beet is show in data that the yield of 50 t ha^{-1} of root gives 7 tons of sugar, 2.5 tons of dry noodles, 2 tons of molasses and 2.5 t saturation sludge. Further processing of molasses it can be obtained 650 liters of alcohol or more than 1250 kg of yeast. There is also a 30 - 40 t of fresh leaves and heads of beet for feeding cattle (Bojović *et al.* 2014).

Table 4. Correlation matrix parameters examined

Indicators	Number of leaves	The leaf area	The amount of α amino N
Correlation matrix for all examined genotypes			
Number of leaves	1	0.828**	0.189^{ns}
The leaf area	-	1	0.628*
The amount of α-amino N	-	-	1
Correlation matrix for genotype Otis			
Number of leaves	1	0.801**	-0.362^{ns}
The leaf area	-	1	0.001^{ns}
The amount of α- amino N	-	-	1
Correlation matrix for genotype Chiara			
Number of leaves	1	0.770**	-0.724*
The leaf area	-	1	0.629*
The amount of α- amino N	-	-	1
Correlation matrix for genotype Laetitia			
Number of leaves	1	0.990**	0.418^{ns}
The leaf area	-	1	0.405^{ns}
The amount of α- amino N	-	-	1
Correlation matrix for genotype Irina			
Number of leaves	1	0.485^{ns}	0.197^{ns}
The leaf area	-	1	0.133^{ns}
The amount of α- amino N	-	-	1
Correlation matrix for genotype Severina			
Number of leaves	1	0.361**	0.057^{ns}
The leaf area	-	1	0.219^{ns}
The amount of α- amino N	-	-	1
ns–without a significant correlation; * and **- significant correlation - $p<0.05$ i $p<0.01$;			

The quality of sugar beet is complex characteristic, caused by genetic differences between varieties and diverse environmental conditions in the area of cultivation (Rosso and Candolo, 2001). In all cultivated plants there is interaction between genotype (variety) and external environment (Čačić *et al*., 1997, Pejić *et al*., 2010, Kolarić *et al*. 2015).

Sugar beet, in addition to its adaptability to climatic conditions, reacts quickly to changes in meteorological factors which greatly affect root yield and sugar content. For a good yield it requires a mean temperature of 15.3 to 16.4 °C (Bojović et al. 2014). Klenter et al. (2006), in their experiments, found that the optimum for plant growth is at 18 °C. The high temperatures in July and August reduced the yield, but, at the end of season plant growth is not dependent on thermal conditions.

Surplus moisture in the air leads to a decrease in transpiration and productivity and, in the period of technological maturity, a negative impact on the accumulation of sugar. Great need sugar beet has in water, which provides over ¾ of its yield. For successful production, optimal amount of rainfall is 600 mm, although irrigation significantly increased the root yield (Bojović et al. 2014).

Drought is a major limiting factor in sugar beet yields in many areas. Varieties with increased tolerance to drought can partially mitigated the harmful consequences of drought. These varieties have certain genotypic and phenotypic characteristics. Genotypic characteristics is the root system with a larger absorption surface, more developed palisade tissue, thicker cuticle, fewer stomata, higher content of bound water, more osmotic active substances and the like. From phenotypic characteristics should be noted that these varieties have larger leaves shine, greater turgescence in petioles and leaflets, easily breakable leaves and susceptible to stretching. Particularly important is the rapid growth in the spring of these plants because if a drought occurs in the early stages of growth of root growth is slowing down, which can lead to yield losses of up to 46%, while in the later stages of the growth this loss is less significant. Proper protection of crops is very important for a good sugar beet yield. Proper protection is use of chemical preparates on seeds and young plants. In the area of South Banat (where experiments are performed) recorded the presence of 160 weed species (Nestorović, 2009). Suppression of herbicides is justified only if they achieve high yield (Ivanović et al. 1999). The most common diseases on sugar beet causing pathogens Cercospora and Rhizomania with which must be combated with chemical preparations. In the absence of chemical treatment of the foliage can be completely collapse, leading to a complete loss of yield. Attack of the parasites can reduce 1-2% of sugar content in the roots (Kuzevski *et al.* 2008, Bojović *et al.* 2014). Soil characteristics influence nutrient solubility, but also microbial activity and root growth (Knežević *et al.* 2009, 2014).

The proper selection of varieties for a particular production area contributes to greater and more stable production of cultivated plants. In order to cost-effective production and processing of sugar beet cultivation it is reasonable

to saw varieties in all three main directions of selection: Z (high content of sugar), N (normal) and E (high yield). The proposed manufacturing process for beet varieties share in the total sowed surface should be: 20% Z, 50% of N and 30% E type, but each manufacturer must be free to determine the basis of its own organizational and economic opportunities (Bojovic *et al.* 2014).

CONCLUSIONS

Based on the results obtained in this research can be done the following conclusions:

- The highest leaves number were formed at the genotype Otis, both individually and overall average, while the lowest leaves number have a genotype Severina. The variation in the number of formed leaves per nutrition variants was highest in genotype Laetitia and lowest at the genotype Otis.
- The largest leaf area, in total and individual average had genotype Otis and lowest values of this parameter had the genotype Severina. Variations in leaf area was the highest at genotype Leatitia and smallest in the genotype Severina.
- Indicator of root technological quality, amount of α-amino N, was the largest individually and in overall at the genotype Irina, while the lowest individual value had Chiara. The lowest average of amount of α-amino N had Chiara and Laetitia (2.33 mmol / 1000S). Difference between the largest and smallest average amount of α-amino N was highly significant (around 160%).
- Based on the strength of the connection between the studied indicators, we can conclude that there was a correlation connection of all indicators, but of different intensity. Very strong positive correlation was between the number of leaves and leaf area (r = 0.828) and between the leaf surface and α-amino N (r = 0.628) in average for all examined genotypes.

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Farhad ASADI, Afrooz ALIMOHAMADI [1]

ASSESSING THE PERFORMANCE OF POPULUS CASPICA AND POPULUS ALBA CUTTINGS UNDER DIFFERENT IRRIGATION INTERVALS

SUMMARY

Understanding the function of poplar species under different irrigation regimes is critical for water resources and ecosystem sustainable management. This study was conducted in order to understand the performance (survival and height growth) of two poplar species (*Populus alba* L. and *Populus caspica* Bornm.) cuttings in two lengths (15 cm and 25 cm) under three irrigation treatments (7-day, 14-day and 21-day intervals). One trial was established using split-split-plot design with three replications. Every two weeks height and number of remaining cuttings were measured. Results showed the survival average at the end of periods for 7-day, 14-day and 21-day irrigation treatments were 83.33 %, 22.08 % and 0 %, respectively, for *P. alba* and *P. caspica* were 25.89 % and 44.39 %, respectively and for cuttings in 15 and 25 cm were 29.61 % and 40.66 % respectively. The results of two-way analysis of variance of the survival among cuttings indicated that the differences survival among cuttings were all marked under the four treatments and analysis of variance of the height growth indicated that except under the size treatment, the differences height growth among cuttings were all marked under the other three treatments.

Keywords: *Populus caspica, Populus alba, irrigation interval, height growth, survival.*

INTRODUCTION

Poplar is an excellent candidate for short rotation coppice cultures, which rely on species that is characterized by fast growth and dynamic production of biomass (Payamnour *et al*, 2013). This species as an alternative source for wood production (Alimohamadi *et al*. 2012) have been planted in Iran for many years. Annual production of poplar plantations, according to available statistics, is more than triple the production of Caspian forests (the only commercial forests in Iran) while their area is less than 10 percent of Caspian forests area (Bozorgmehr *et al*. 2014). Poplar growth is highly dependent on the moisture content of the soil (Bagheri *et al*. 2012) and soil water can exert an important control on poplar growth (Dong *et al*. 2011, Hogg *et al*. 2013). Accordingly having a permanent resource of water is a primary need of poplar plantation. Since Iran is in the arid

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zone which receives less than a third of world average precipitation (Badripour, 2006), shortage of water resources is a major obstacle to successful poplar cultivation. In order to increase the productivity of poplar plantations in arid and semiarid lands, high efficiency irrigation strategies must be used. The efficiency of water use can be examined through assessment of poplar responses which differ from one species to another and from one age to other age. Various indices have been used to find the poplar responses to irrigation which consist of: a) growth traits such as diameter at breast height (Bagheri *et al*. 2012, Shock *et al*. 2005), basal area at breast height (O'Neill and Allen 2015, Voltas *et al*. 2006), total height (Bagheri *et al*. 2012, Shock *et al*. 2005), stem height (Bagheri *et al*. 2012), crown diameter (Bagheri *et al*. 2012), relative growth rate (Karačić and Weih 2006), volume (O'Neill and Allen 2015, Bagheri *et al*. 2012, BaoFang *et al*. 2007, Shock *et al*. 2005), biomass (Monclus *et al*. 2009, Karačić and Weih 2006), survival (O'Neill and Allen 2015, Saeidi and Azadfar 2009, Van den Driessche *et al*. 2003), radial growth (Giovannelli *et al*. 2002), root number (BaoFang *et al*. 2007); b) physiological characteristics such as foliar nutrition (Shock *et al*. 2005), mesophyll-to-stomatal conductance ratio (Rancourt *et al*. 2015), photosynthetic performance (Zhu *et al*. 2014; Saeidi and Azadfar, 2009, Fengjun *et al*. 2006), transpiration (Samuelson *et al*. 2007), leaf-specific hydraulic conductance (Samuelson *et al*. 2007), canopy stomatal conductance (Samuelson *et al*. 2007), stem water potential (AiHong *et al*. 2009); c) morphological traits such as leaf area (Monclus *et al*. 2009, BaoFang *et al*. 2007, Samuelson *et al*. 2007); d) phenology events such as bud-burst (Bagheri *et al*. 2012, Sera and Pons 2013), leaf expansion (Bagheri *et al*. 2012), leaf abscission (Bagheri *et al*. 2012), flowering (Bagheri *et al*. 2012; Sera and Pons 2013) and e) anatomical characters such as wood anatomy (Cocozza *et al*. 2011).

In order to develop efficient water use strategies in poplar plantations, different irrigation regimes can be conducted. Irrigation regime which is determined by the rate of irrigation, irrigation frequency (intervals), and time of water applications to crops. Some research has been undertaken on high efficiency irrigation regime in poplar plantations. Zhu *et al* (2014) investigated the effects of changed irrigation (water-saving irrigation and flood irrigation) on two poplar species (*P. euphratica* and *P. russkii*) growing in arid ecosystems. Results showed that reduced water availability during water-saving irrigation had a moderate but not significant impact on the photosynthesis of the two poplar species. Sera and Pons (2013) analyzed the dynamics of poplars between 2002 and 2008 which comprised periods of water surplus and water scarcity. No difference was observed between periods of water scarcity and water surplus. Bagheri *et al* (2012) showed significant difference between the 4, 8 and the 12 day irrigation intervals in respect to growth parameters of *P. euramericana*, *P. trichocarpa*, *P. alba*, *P. nigra* and *P. deltoids*. Cocozza *et al* (2011) investigated the correlation between the main ring traits of young poplar clones (*P* × *canadensis* and *P. deltoides*) and irrigation regimes (irrigated with 70 mm of water every week and no-irrigated). Results showed *P. deltoides* has the potential

to recover promptly after drought stress. AiHong *et al* (2010) in temperate desert zone analyzed the change of water potential of *Populus euphratica* Oliv. and *P. Russkii* Jabl under different irrigation volumes. Saeidi and Azadfar (2009) investigated the effect of drought and hydromorphy stresses on net photosynthesis rate and survival of *P. nigra* , *P. deltoides* and *P. euramericana*. Result showed *P. deltoides* and *P. nigra* had more resistance to drought stress than hydromorphic. Zomer *et al* (2007) irrigated their poplar plantations during April to June, when the atmospheric temperature is high with a low level of relative humidity. Fengjun *et al* (2006) applied four water treatments: well-watered condition, slight water stress, moderate water stress, and severe water stress. The results revealed that the clones with higher long-term water use efficiency always had strong photosynthetic capacity and optimum root/shoot ratio. Shock *et al* (2005) applied five irrigation treatments for hybrid poplar consisted of three water application rates using micro sprinklers and two water application rates using drip tape during five years. Result showed drip irrigation with two tapes per tree row resulted in higher tree growth than micro sprinkler irrigation.

In Iran, most of the researches about efficient irrigation management have been done on agricultural crops. Furthermore, there is no research available to determine growth response of *P. alba* and *P. caspica* as a local and critically endangered species in Caspian forest of Iran (Falah *et al* 2011) under different irrigation treatments. Given the importance of Poplars and for optimum use of water resources in arid season, in this study we explored the growth response and survival of *P. caspica* and *P. alba* cuttings in two lengths to different irrigation intervals.

MATERIAL AND METHODS

Site

Experiments were carried out in the Chamestan forest and rangeland research station located in Noor city (36° 25’ N & 51° 55’ E, 70 m a.s.l), Mazandaran province, Iran. Mean annual temperature and precipitation are 15.8 C° and 840 mm, respectively. Absolute minimum and maximum temperature are -8.5 C° and 36 C°, respectively. Average relative humidity is 78%.

Experiment design

Cuttings of *P. caspica* and *P. alba* in two lengths (15 and 25 cm) were established in a field trial, as a split-split-plot design with three replications. The species as factor A was assigned to whole plots, then the cutting length as factor B was assigned to split-plots within the applications of Factor A, and then split the experimental units used for factor B into sub-sub-plots to receive different irrigation intervals as factor C. For this purpose a total of 108 (three treatments of irrigation intervals, two treatments of cutting lengths, two kinds of species, three pots and three replications) plastic bags were put into a water container with 15 cm depth and 46 cm diameter (Figure 1). The culture medium was a soil with 33 percent clay, 47 percent loam and 21 percent sand. Soil pH and electrical

conductivity were 6.58 and 0.57 ds/m, respectively. Percentage of lime, organic carbon and total nitrogen were 1.19, 2.78 and 0.29, respectively and finally the amounts of phosphorus, potassium, calcium and magnesium were 23.78, 420, 175.5 and 37.5 ppm, respectively (Rouhi Moghadam, 2008). The cuttings were planted on February with one cutting in each pot. Weeding operation was done during the growing season. Three different irrigation intervals were applied: 7-day intervals, 14-day intervals and 21-day intervals. The amount of irrigation water at each time was 2000 mL for per pot.

Figure 1: Cuttings in plastic bags under different treatments

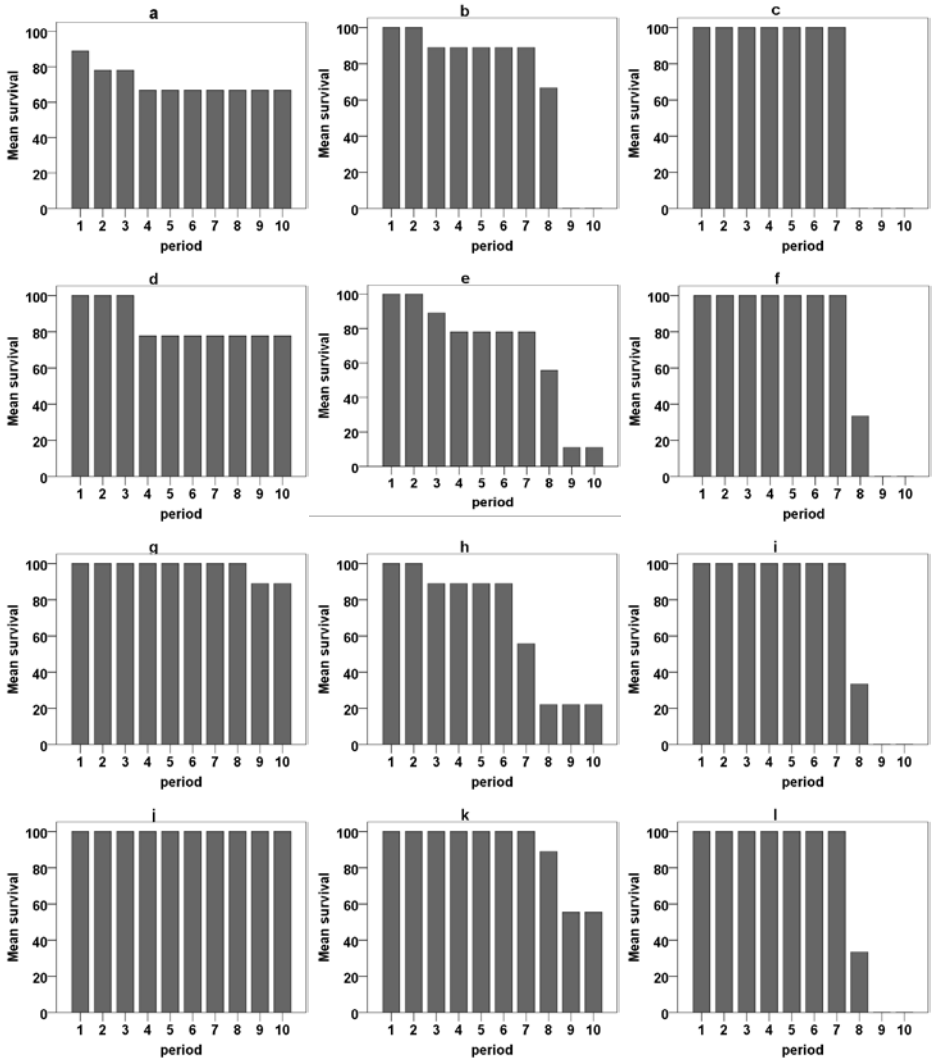
Measured objects and statistical analyses

In order to calculating the seedling height growth as an appropriate indicator for evaluating the effects of stress (Sadati *et al*. 2011) and survival under different treatments, every two weeks height and number of remaining cuttings were measured. Ten measurements were performed on April 27th, May 11th, May 25th, June 8th, June 22th, July 6th, July 20th, August 3rd, August 17th and August 31th.

Data were evaluated by Kolmogorov-Smirnov test for assessing the normality, two-way analysis of variance for analyzing the effects of applied treatments on height growth and survival, Duncan's multiple range test for measuring the differences between irrigation and period treatments. Statistical analyses were conducted using SPSS 21.0.

RESULTS AND DISCUSSION

Results showed (Figures 2 and Figure 3) the percentage survival of cuttings under five treatments (in total twelve treatments).



a: *P. alba* cuttings in 15 cm under 7-day irrigation, b: *P. alba* cuttings in 15 cm under 14-day irrigation, c: *P. alba* cuttings in 15 cm under 21-day irrigation, d: *P. alba* cuttings in 25 cm under 7-day irrigation, e: *P. alba* cuttings in 25 cm under 14-day irrigation, f: *P. alba* cuttings in 25 cm under 21-day irrigation, g: *P. caspica* cuttings in 15 cm under 7-day irrigation, h: *P. caspica* cuttings in 15 cm under 14-day irrigation, i: *P. caspica* cuttings in 15 cm under 21-day irrigation, j: *P. caspica* cuttings in 25 cm under 7-day irrigation, k: *P. caspica* cuttings in 25 cm under 14-day irrigation, l: *P. caspica* cuttings in 25 cm under 21-day irrigation

Figure 2. The percentage survival under different treatments during measured periods

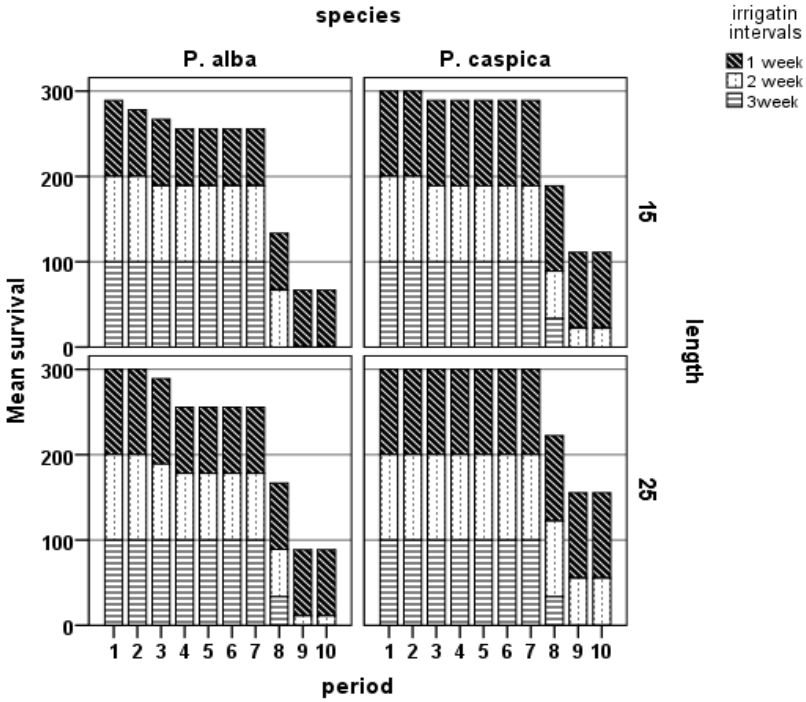


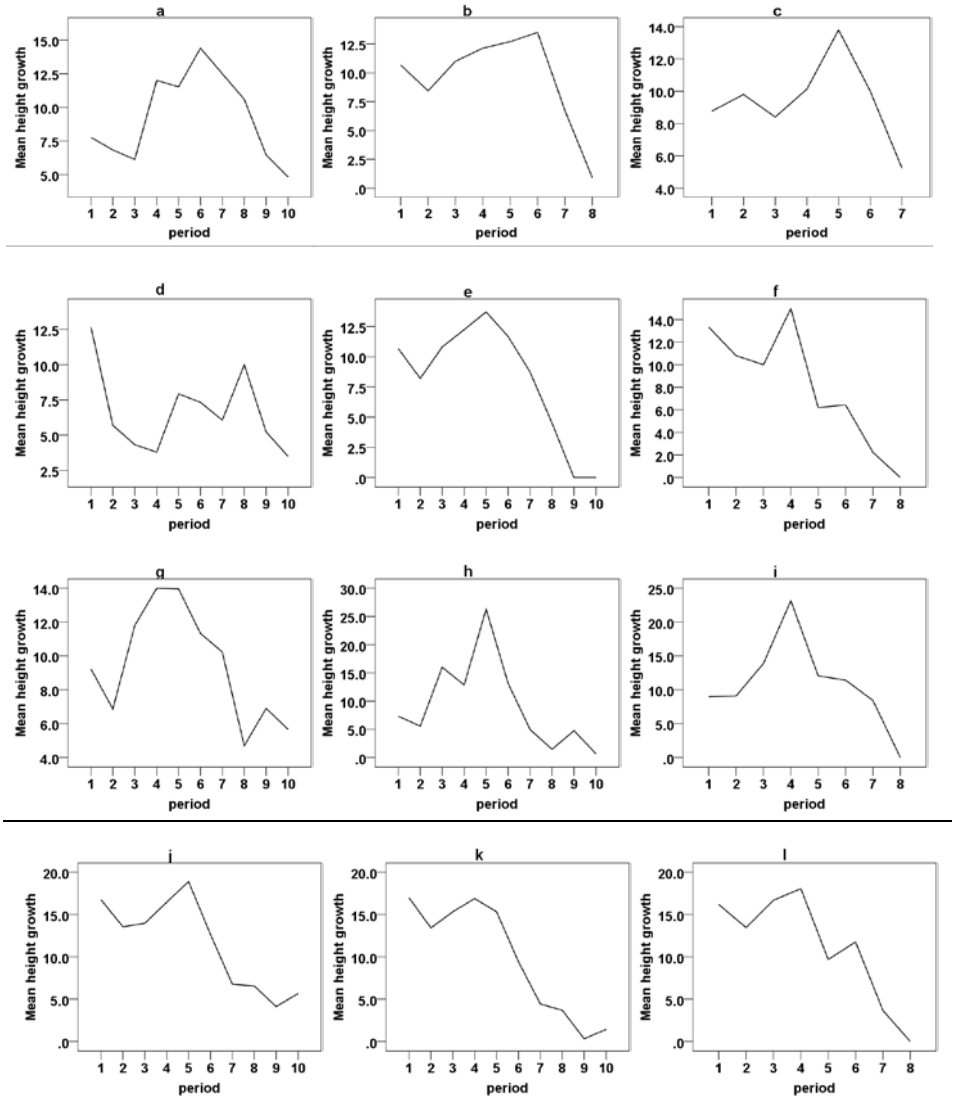
Figure 3. Survival of study species under three irrigation intervals and two sizes

The percentage survival was zero of which three treatments were related to P. alba include the cuttings in 15 cm under 14 and 21-day irrigation intervals and the cuttings in 25 cm under 21-day irrigation intervals and two treatments were related to P. caspica include the cuttings in 15 cm under 21-day irrigation intervals and the cuttings in 25 cm under 21-day irrigation intervals.At the end of periods there was no zero percent survival in any 7-day irrigation treatments but all cuttings under 21-day intervals (all treatments related to two species and two sizes) had died that four deaths had occurred at the ninth period and the *P. alba* cuttings in 15 cm under 21-day irrigation intervals had died at the eighth period. The only treatment with 100 percent survival at the end of periods was related to *P. caspica* cuttings in 25 cm under 7-day irrigation intervals.

The survival average at the end of periods for 7-day, 14-day and 21-day irrigation treatments were 83.33 %, 22.08 % and 0 %, respectively, for *P. alba* and *P. caspica* were 25.89 % and 44.39 %, respectively and for cuttings in 15 and 25 cm were 29.61 % and 40.66 % respectively.

The average height growth of *P. alba* and *P. caspica* were 7.44 cm and 9.62 cm. Furthermore, for 7-day, 14-day and 21-day irrigation intervals were 9.25 cm, 8.68 cm and 7.67 cm, respectively and the average height growth of cuttings in 15 cm and 25 cm were 8.50 cm and 8.56 cm (Figures 4 and Figure 5). The maximum height growth of four treatments occurred during the fourth period, for four others occurred during fifth period, two treatments during sixth

period had maximum height growth and two treatments during first period had maximum height growth (Figure 4).



a: *P. alba* cuttings in 15 cm under 7-day irrigation, b: *P. alba* cuttings in 15 cm under 14-day irrigation, c: *P. alba* cuttings in 15 cm under 21-day irrigation, d: *P. alba* cuttings in 25 cm under 7-day irrigation, e: *P. alba* cuttings in 25 cm under 14-day irrigation, f: *P. alba* cuttings in 25 cm under 21-day irrigation, g: *P. caspica* cuttings in 15 cm under 7-day irrigation, h: *P. caspica* cuttings in 15 cm under 14-day irrigation, i: *P. caspica* cuttings in 15 cm under 21-day irrigation, j: *P. caspica* cuttings in 25 cm under 7-day irrigation, k: *P. caspica* cuttings in 25 cm under 14-day irrigation, l: *P. caspica* cuttings in 25 cm under 21-day irrigation

Figure 4. The height growth under different treatments during measured periods

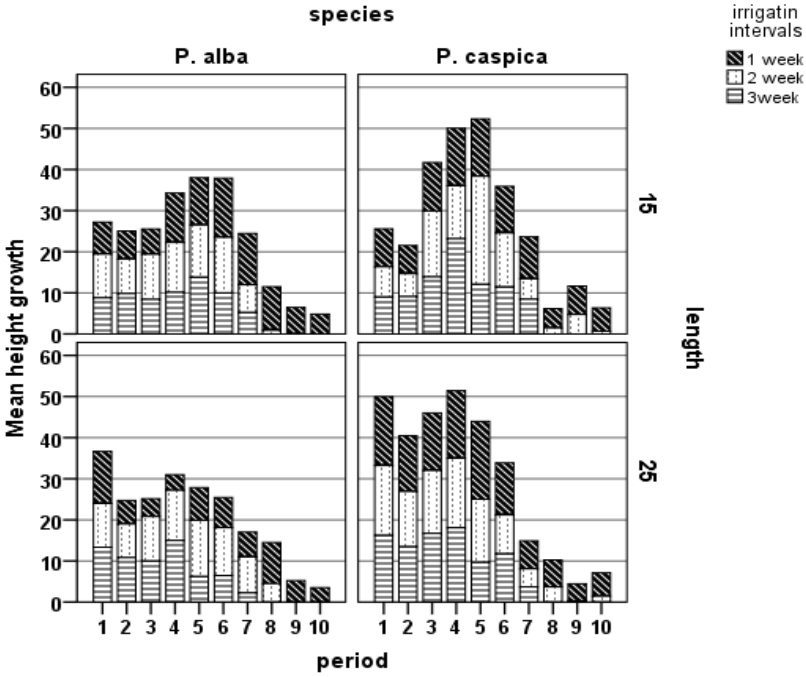


Figure 5: Height growth of study species under three irrigation intervals and two sizes

The results of two-way analysis of variance of the survival among cuttings (Table 1) indicated that the differences among cuttings were all marked under the four treatments (length, species, irrigation intervals and periods). Based on Duncan's multiple range test, irrigation intervals were classified in two separate groups: 21-day and 14-day irrigation treatments as first group and 7- day irrigation treatment as second group. Furthermore, periods based on multiple comparisons were divided into 3 groups (Figure 6).

Table 1: Two-way analysis of variance for survival of study species under irrigation and size treatments

Treatment	df	F	*p* Value
species	1	40.385	.000**
length	1	7.666	.006**
irrigation	2	26.754	.000**
period	9	75.899	.000**
species × length	1	.160	.690ns
species × irrigation	2	9.162	.000**
species × period	9	1.108	.357ns
length × irrigation	2	.939	.392ns
length × period	9	.521	.859ns
irrigation × period	18	18.170	.000**

Significance values are indicated as: * $P<0.05$, ** $P<0.01$ and ns non-significant

The results of two-way analysis of variance of the height growth among cuttings (Table 2) indicated that except under the size treatment, the differences height growth among cuttings were all marked under the other three treatments (species, irrigation intervals and periods). Based on Duncan's multiple range test, irrigation intervals were classified in two separate groups: 21- day irrigation treatment as first group and 14- day and 7- day irrigation treatments as second group. Furthermore, periods based on multiple comparisons were divided into 5 groups (Figure 7).

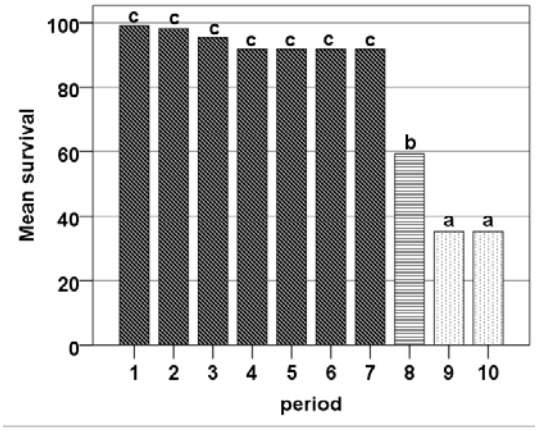


Figure 6: Grouping of periods based on mean survival

Table 2: Two-way analysis of variance for height growth of study species under irrigation and size treatments

Treatment	df	F	*p* Value
species	1	31.505	.000**
length	1	2.077	.151ns
irrigation	2	6.997	.001**
period	8	64.840	.000**
species × length	1	2.992	.085ns
species × irrigation	2	.398	.672ns
species × period	8	5.883	.000**
length × irrigation	2	.497	.609ns
length × period	8	2.990	.003**
irrigation × period	16	6.516	.000**

Significance values are indicated as: * $P<0.05$, ** $P<0.01$ and ns non-significant

Owing to limited water resources, irrigation interval is more important than irrigation volume in conventional and industrial poplar plantation systems (Bagheri *et al*. 2012). According to this our goal was to examine the more efficient irrigation regime for *P. caspica* and *P. alba* plantations. The results of applying three irrigation intervals during four months indicated that all cuttings

under 21-day irrigation intervals after about three months died. Furthermore the only 22 percent of the cuttings under 14-day irrigation intervals survived at the end of period, but 83 percent of cuttings under 7-day irrigation intervals survived, therefore it might be concluded that these irrigation regimes (14-day and 21-day) are not appropriate for poplar wood production. This conclusion is comparable with the work of Bagheri *et al* (2012) that showed all the clones of *P.euramericana*, *P.trichocarpa*, *P. alba*, *P. nigra* and *P. deltoides* have intensive growth reduction at the 12-day interval irrigation in comparison 4-day and 8-day regime, but there was no difference between 4-day and 8-day irrigation intervals. Moreover Hara (2004) suggested weekly irrigation during the dry months for high intensity production of poplar. Chaghaii (2016) believed in areas where there is no groundwater the irrigation intervals may be reduced to 10-day and soil should be completely full of water, but on windy sites with sandy soil the irrigation intervals may be reduced to 7-5 day. Some researchers suggest that the irrigation intervals should not be considered the same and according to local conditions should be given more flexibility on warm days (Bagheri *et al*. 2012, Zomer *et al*. 2007).

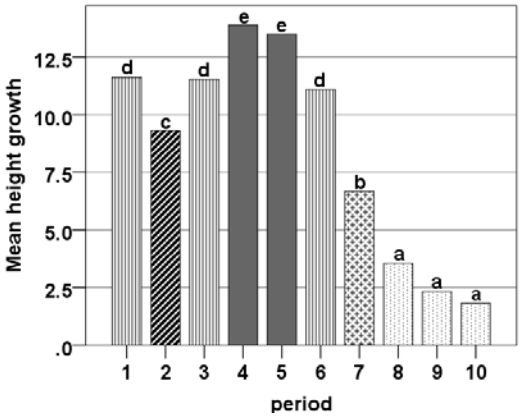


Figure 7: Grouping of periods based on mean height growth

Unlike this fact that *P. alba* is known as high drought tolerance species (Edward *et al*. 1994) the present result showed the survival average at the end of periods and average height growth of *P. alba* cuttings are significantly less than *P. caspica* cuttings and therefore *P. alba* than *P. caspica* are more sensitive to longer intervals of irrigation, but Bagheri *et al* (2012) showed *P. alba* clones than other study poplar clones are less affected by drought conditions and have the same performance at three irrigation intervals (4, 8 and 12-day).

The results showed the survival of cuttings in 25 cm length are significantly more than cuttings in 25 cm length, although there was no significant difference between height growths of cuttings in two lengths. These results are confirmed by some studies that showed the longer the cuttings, the higher are the survival (Singh Thakur *et al*. 1995, Rossi, 1991).

Since potential growth of poplar is highly dependent on the amount of applied irrigation (Shock *et al*. 2002) and soil moisture (Kharytonov *et al*. 2017) therefore the improvement of water use efficiency especially in arid areas with regard to limited water supply is a key objective to improve the sustainability of cultivated poplar (Rancourt *et al*. 2015).

Water-saving irrigation (Zhu *et al*. 2014) and increasing the intervals of irrigation (Bagheri *et al*. 2012) could be as economical practices for managing water use in such areas, As our goal was to examine the effect of different irrigation intervals on survival and height growth of two poplar species. Furthermore, management of irrigation intervals owing to water influence on pest management is very important. As the work of Tahriri Adabi *et al* (2013) showed high provided amount of water in 4 days interval in comparison to 8 and 12 days, make poplar species and clones more susceptible against pest. It is worth mentioning that today using treated wastewater for poplar irrigation is suggested as an alternative strategy for water supply problems in semi-arid to arid areas (Houda *et al*. 2016).

CONCLUSIONS

Our study revealed that 1) survival of study poplar was found to be dependent on irrigation intervals; 2) the best performance (survival and height growth) of study poplar was under 7-day irrigation interval; 3) *P. caspica* has relatively higher water-stress resistance than *P. alba*; 4) longer cuttings than shorter cuttings had higher survival.

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CHEMICAL CONSTITUENTS AND BIOLOGICAL POTENTIAL OF ESSENTIAL OILS OF *HELICHRYSUM ITALICUM* (ROTH) G. DON FROM MONTENEGRO

SUMMARY

The chemical constituents of essential oils isolated from the *Helichrysum italicum* (Roth) G. Don by steam-distillation were analyzed by GC-MS. The oils were predominantly composed of sesquiterpene hydrocarbons with 52.35%, with γ-curcumene as major constituent (14,11%), β-selinene (11,31%) and α-curcumene (10,42%). The antimicrobial activity of the essential oils was evaluated against Gram-positive and Gram-negative bacteria and fungi. Results showed that the oils exhibited antibacterial activities and that *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus subtilis* are very sensitive.

Keywords: *Helichrysum italicum*, GC-MS, antimicrobial activity

INTRODUCTION

The interest in herbal remedies has been significantly increased in the last few decades. In the worldwide, all the natural resources including medicinal plants, fungi and algae are screened for their biological activities (Enazi *et al.* 2018). Medicinal plants play an important role in the discovery and isolation of new drugs. The essential oils are known as a secondary plant metabolite which form part of naturopathic therapy, are widely known for their antimicrobial properties (Rapper *et al.* 2013; Hosseini *et al.* 2016). Various biological characteristics, such as digestive, anti-inflammatory, sedative, antioxidant, antimicrobial, antiviral, and also cytotoxic activities have been attributed to the essential oils (Carvalho *et al.* 2018).

Numerous members of the *Asteraceae* family are important crop species of cut flowers and ornamentals, as well as being medicinal and aromatic plants, many of which produce essential oils used in pharmaceutical industries (Abad *et al.* 2013). Helichrysum is one of the important genera for medical purposes. This genus is currently widely distributed in Africa, Madagascar, the Mediterranean basin, Macaronesia, central Asia and India (Juliano *et al.* 2018) includes more than a thousand taxa that have a higher occurrence in the Mediterranean areas of

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Notes: The authors declare that they have no conflicts of interest. Authorship Form signed online.

Europe (Viegas *et al.* 2014; Facino *et al.* 1988). Almost 25 species are native of Mediterranean area and the most widespread species is *Helichrysum italicum* (Roth) G. Don (Guinoiseau *et al.* 2013). *H. italicum* is a typically Mediterranean plant. It is a small aromatic shrub with yellow flowers, up to 40–50 cm high, growing on dry cliffs and sandy soil. It is widespread along the East coast and on the islands of the Adriatic sea (Mastelic *et al*, 2005). The genus Helichrysum has an important source of secondary metabolites such as flavonoids, phytocannabinoids, triterpenoids, diterpenoids, steroids, organic acids, phloroglucinol and acetophenone derivatives (Guinoiseau *et al.* 2013).

The aim of this study was to determine the chemical composition and antimicrobial activity of *H. italicum* oils from Montenegro.

MATERIAL AND METHODS

Plant material of *Helichrysum italicum* was collected from several sites in the south of Montenegro; Isolation of the essential oils the dried plant samples was subjected to steam distillation for 3 hours. Samples oils were dried over anhydrous sodium sulphate and stored at low temperature before analysis. The analyses were performed on a gas chromatograph-mass spectrometer, GC-MS QP 2010 plus, Shimadzu, equipped with split-splitless injector and a ZB-5MS capillary column (30m × 0.25 mm; 0.25 µm film thickness). The chromatographic conditions were as mentioned in the preceding paragraph. Injector was heated at 260 °C, detector (MSD) was heated at 260 °C, while the column temperature was linearly programmed from 35 to 270 °C (5.0 °C min-1). The EI MS spectra (70 eV) were obtained in the scan mode in m/z range 50–500.

Antimicrobial activity

To assess the antimicrobial properties of essential oil of *Helichrysum italicum*, nine strains of pathogenic microorganisms were used in the study (Table 1). All microorganisms were derived from the culture collection of the Institute of the Health of Montenegro and Department of Biology, Faculty of Sciences and Mathematics, University of Montenegro. Bacteria were subcultured from nutrient agar slopes into nutrient broth and in Sabouraud dextrose broth for *Candida albicans*. The resulting bacterial broth was used as the inoculum in microbial analysis. Cell numbers of the inoculum were standardized at 10^5 cell/mL. Incubation lasted 18 h at 37°C for bacteria and 48 h at 26°C for *C. albicans.*

Essential oils dilutions were prepared directly in the Mueller-Hinton broth and tested in concentrations range from final concentrations of 71, 35, 14, 7, 3.5, 1.4 µl/ml.

The minimal inhibition concentration (MIC) values were determined for the microbial strains to the essential oils of *Helichrysum italicum.* The MIC is defined as the lowest concentration at which the microorganism does not demonstrate visible growth. Tests were carried out in duplicate

Table 1. Microbial strains and reference number ATCC

Ref. number	**Microorganisms**
ATCC 25922	*Escherichia coli*
ATCC 25923	*Staphylococcus aureus*
ATCC 6633	*Bacillus subtilis*
ATCC 13076	*Salmonella enteritidis*
ATCC 19111	*Listeria monocytogenes*
ATCC 25933	*Proteus mirabilis*
ATCC 27853	*Pseudomonas aeruginosa*
ATCC 19433	*Streptococcus faecalis*
ATCC 10231	*Candida albicans*

RESULTS AND DISCUSSION

Chemical analysis

The results of the chemical analyses of essential oils of. *H. italicum* investigated are presented in Table 2.

Table 2. Chemical composition (% of compound) of the essential oils from *H. italicum*

	Compound	**RT**	**RI**	**%**
1.	alpha.-pinene	11.49	939	0.72
2.	beta-myrcene	13.65	991	0.03
3.	para-cymene	14.85	1026	0.23
4.	limonene	15.02	1031	0.72
5.	1,8-cineol	15.10	1033	0.22
6.	gamma-terpinen	16.03	1062	0.10
7.	linalool	17.41	1098	0.11
8.	beta-thujone	17.61	1114	0.62
9.	camphor	18.91	1143	0.16
10.	borneol	19.71	1165	0.10
11.	alpha-terpineol	20.41	1189	0.16
12.	neryl acetate	25.00	1365	3.91
13.	alpha-copaene	25.57	1376	3.68
14.	beta-elemene	25.91	1391	0.20
15.	alpha-cedrene	26.37	1409	6.25
16.	alpha-bergamotene	26.52	1415	0.92
17.	beta-caryophyllen	26.74	1418	5.36
18.	gamma-curcumene	28.17	1480	14.11
19.	alpha-curcumene	28.24	1483	10.42
20.	beta-selinene	28.52	1485	11.31
21.	alpha-selinene	28.69	1494	6.07
22.	delta-cadinene	29.20	1524	1.40
23.	caryophyllene oxide	30.77	1581	0.52

The main components in *H. italicum* oils are γ-curcumene (14,11%), β-selinene (11,31%) and α-curcumene (10,42%). From Table 2 it can be seen that in the essential oils from *H. italicum* dominates sesquiterpene hydrocarbons with 52.35%. Other components that are present in the essential oil are: α-selinene (6.07%), neryl acetate (3,91%) and α-copaene (3,68%). The monoterpene 1,8-cineole is represented in essential oil with 0,22%. The twenty three compounds were identified, representing about 67.32% of the total oil. According to the literature, essential oil of *H. Italicum* has significant chemical and biological potential. However, the composition of essential oils varies considerably depending on the method of extraction, time of maturation, soil composition and climatic conditions. Research work Paolini *et al*, 2006 reported that in composition of essential oils of *H. italicum* dominated monoterpenes such as neryl acetate, neryl propanoate and α-pinene. According research Morone-Fortunato *et al.* 2010 in essential oils of *H. italicum* are a large proportion of sesquiterpenes. The samples from Italian essential oils of *H. italicum* contained mainly γ-curcumene, β-selinene and α-selinene (Morone-Fortunato *et al.* 2010).

Antimicrobial potential

The data of the antimicrobial activity assessed by dilution method showed that in general the antimicrobial activities of the tested essential oil were varied on the type of microorganism (Table 3).

Table 3. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *H. italicum* tested Essential Oils against microorganisms using dilution method

Microbial strains	**MIC** (μg/mL)	**MBC** (μg/mL)
Echerichia coli ATCC25922	n.d.	n.d.
Staphylococcus aureus ATCC25923	<1.4	1.4
Bacillus subtilis ATCC6633	1.4	14
Salmonella enteritidis ATCC13076	n.d.	n.d.
Listeria monocytogenes ATCC19111	<1.4	1.4
Proteus mirabilis ATCC25933	n.d.	n.d.
Pseudomonas aeruginosa ATCC27853	n.d.	n.d.
Streptococcus faecalis ATCC19433	7.1	14
Candida albicans ATCC10231	n.d.	n.d.

∗ n.d. not detected from tested concentrations

It was demonstrated that *Staphylococcus aureus* and *Listeria monocytogenes* (with an MIC of <1.4 μg /mL), and *Bacillus subtilis* (with an MIC of 1.4 μg /mL) were the most sensitive bacteria. Investigation according Nostro *et al* (2001) reported that essential oil of *H. italicum* to inhibit the growth of *Staphylococcus aureus* in a concentration dependent manner, with no

difference in sensitivity between methicillin-resistant *Staphylococcus aureus* and methicillin-sensitive *Staphylococcus aureus* strains.

The antimicrobial activity of the essential oil of *H. italicum* from Montenegrian origin has been evaluated for the first time

CONCLUSIONS

Our results revealed that γ-curcumene, β-selinene and α-curcumene are the major components of *H. italicum* essential oil from Montenegro. The oils possess rather a significant activity against microorganisms *S. aureus*, *L. monocytogenes* and *B. subtilis.* Investigations by other authors have indicated that *H. italicum* oils from the Mediterranean region has significant biological potential, but also significantly differs from the area and ecological conditions. Since these first reports of the biological activity of *H. italicum* from the area of Montenegro research should be continued and extended to biotesting.

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TRICHODERMA-INDUCED SYSTEMIC RESISTANCE AGAINST THE SCALE INSECT (*UNASPIS MABILIS* LIT & BARBECHO) IN LANZONES (*LANSIUM DOMESTICUM* CORR.)

SUMMARY

Unaspis mabilis infestations have been causing huge economic losses to the lanzones industry in the Philippines since 2009. This study, therefore, seeks to determine the use of *Trichoderma* as myco-biocontrol agent to increase lanzones (*Lansium domesticum*) defense and resistance against this insect. Two experiments were designed to test for the protective and rescuing capacity of *Trichoderma* inoculation against the insect pest. For both experiments, leaf area, leaf count, scale insect populations and densities were observed over time. Additionally, to assess the phytochemical response of the plants, the concentrations of jasmonic acid, salicylic acid, total phenol content, and total flavonoid content were also measured in the rescuing capacity experiment. Results indicated that the plants inoculated with *Trichoderma* were performing better, showing lower populations and slower growth rate of the insect, than the uninoculated plants for both the protective and rescuing capacity experiments. Phytochemical analysis revealed the presence of tannins, phlobatannins, flavonoids, steroids, glycosides, and alkaloids in the leaves of *L. domesticum*. Total phenols and flavonoids showed decreasing concentrations as the infestation progressed, while the concentrations of jasmonic acid and salicylic acid in scale-insect-infested plants were found to be influenced by the inoculation of the plants with *Trichoderma*.

Keywords: biocontrol, phytochemicals, *Trichoderma*, scale insect, systemic resistance

INTRODUCTION

A new species of armored scale insect, *Unaspis mabilis* Lit and Barbecho, was identified in the Philippines in 2014. Reports of outbreak-level infestations were first recorded in North Cotabato in 2009, and since then in different parts of the Luzon and Visayas regions (Lit and Barbecho, 2014). However, other reports dated the infestations as early as 2004 (Provido, 2007) although the pest was

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Notes: The authors declare that they have no conflicts of interest. Authorship Form signed online.

misidentified as *Lepidosaphes ulmi* (Watson, 2015). The infestations were reported to have caused millions of pesos worth of losses in agricultural produce and hundreds of thousands of trees were already invaded in a municipality in North Cotabato alone. Methods of controlling the infestations, including spraying of commercially available chemical insecticides, have been proven ineffective. The research community in the Philippines has been seeking the use of alternative and biocontrol methods to solve or alleviate this problem, such as the use of scale insect-eating beetles *Chilocorus nigrita* and *Chilocorus circumdatus* (Brion, 2016; Watson, 2015; GMA News Online, 2008). This study explores the possible use of a fungal agent to induce plant resistance against the *U. mabilis* infestation.

Trichoderma spp. used as biocontrol agents against plant-infecting pathogens have been recorded since the 1920s. Earlier studies of the use of myco-biocontrol agents, such as *Trichoderma*, focused on increasing their efficiency to directly attack a fungal or bacterial plant pathogen, or even living insects, through competition for resources and space, parasitism and antibiotics production (Harman et al., 2008; Harman, 2006). Recent studies, however, argued that the direct effects of the use of *Trichoderma* as a biocontrol agent is as important, if not less, as its indirect effect to the overall plant defenses (Harman et al., 2004). This is due to the recent discoveries on the capacity of *Trichoderma* to elicit systemic and/or localized acquired or induced resistance, and therefore inducing the primed state of plants (Saldajeno et al., 2014). During the plant primed state, its defense system is immediately and/or more aggressively activated following exposure to either/both biotic and/or abiotic stress (Frost et al., 2008; Conrath et al., 2006).

This study aims to determine whether *Trichoderma* inoculation of seedlings can help protect against and/or alleviate scale insect infestation of lanzones. It is also of interest in this study to observe the relationships among the overall plant growth, jasmonic acid (JA), salicylic acid (SA), and some secondary metabolites concentrations, and the insect population in order to hypothesize on probable defense mechanisms.

MATERIALS AND METHODS

The Experimental Set-up

In order to evaluate the feasibility of using *Trichoderma* spp. as a myco-biocontrol agent against the lanzones scale-insect, *Unaspis mabilis*, two set-ups were utilized in this study. The first set-up was used to determine the protective effect of inoculating uninfested lanzones seedlings with *Trichoderma* spp. against the eventual attack of the scale insect. Meanwhile, the second set-up was used to directly establish the use of *Trichoderma* to rescue lanzones plants that are already infested with *U. mabilis.*

Protective Capacity: Eighteen (18) healthy uninfested one-and-a-half-year-old lanzones seedlings were randomly grouped into two treatments. The

first treatment was inoculated with *Trichoderma* microbial inoculant according to manufacturer's instructions, while the second treatment was not.

A month after the inoculation, both treatments were exposed to infestation of *Unaspis mabilis* by placing the seedlings inside an insect net together with plants that are already infested by the same insect. The seedlings were arranged inside the net in random order to minimize the effect of wind direction, and other dispersal parameters to the favorability of transfer of scale insect to the test plants.

After the one-month exposure of the healthy seedlings to the insect, the two groups were isolated by placing each group in separate nets to monitor the morphological changes in the plants, as well as the population changes of *U. mabilis.* The seedlings were inspected for the same parameters mentioned above every month for another three months.

All the leaves of the 18 seedlings belonging to the two treatments were photographed for morphological assessment. Leaf area of each of the leaves of the plants, count of adult female scale insect (FSIC) on each of the leaves, and the area covered by the male scale insect pupae (MSIC) were determined through assessment of photographs using ImageJ™. The leaf count of each of the seedlings was also noted. The density of the adult female scale insect (FSID) and percent cover of male scale insect pupae (MSID) were computed.

Rescuing Capacity: One hundred thirty-two (132) scale insect-infested one-and-a-half-year-old seedlings were randomly divided into six equal groups. Initial data on leaf count, leaf area, FSIC, FSID, MSIC, and MSID from 50 randomly selected leaves in each group were noted. In addition, phytochemical screening, and quantification of total phenols, total flavonoids, and phytohormones JA and SA were also assessed prior to treatment application.

After the initial data gathering, the six groups were randomly assigned under two treatments namely inoculated, and not inoculated with *Trichoderma,* in triplicates. Each treatment group was placed inside an insect net to avoid cross infestation of scale insect between replicates and treatments, and to avoid the introduction of outside pest population to the experimental set-up. The same set of infestation parameters, and morphological and phytochemical parameters were measured every two weeks for the next six weeks.

The *Trichoderma* microbial inoculant (BIOSPARK ™) used in this study was obtained from Dr. Virginia Cuevas of the Institute of Biological Sciences, University of the Philippines Los Baños. Five grams of the inoculant was used for each seedling following the established application protocol for BIOSPARK ™ administration.

Phytochemical Screening and Quantification

To determine some of the groups and classes of phytochemicals that are present in lanzones leaves which may be involved in the plant's defense mechanisms, an initial phytochemical screening was conducted wherein the presence of tannins, phlobatannins, saponins, flavonoids, steroids, and glycosides

were determined according to Evans (1989), while the presence of alkaloids and anthraquinones were tested according to Cock (2011).

Ten grams of leaves were randomly collected from each of the replicates of the two treatments. The leaves were cut into pieces approximately 2 cm^2 each. The pieces of leaves were mixed, and 7.0 g was randomly selected and then, it was extracted with 35.0 mL of methanol using a blender. Another gram of leaves randomly selected from the same group was extracted with 20.0 mL of distilled water. These extraction procedures were done separately for each replicate of the treatments. The methanolic extract was centrifuged at 3000 ×*g* for five minutes, while the aqueous extract was centrifuged at 10,000 ×*g* for ten minutes.

Jasmonic acid Extraction and Quantification: To extract the plant hormone jasmonic acid (JA) endogenous to the leaves sampled a modified method by Dhandukia and Thakkar (2008) was used for the methanolic extract described above.

Briefly, the extracts were acidified to pH 3.0 with 6N hydrochloric acid (HCl). The acidified solution was evaporated to dryness at 35° C. The dried products were re-dissolved in 1.0 mL of methanol-JA solution with a concentration of 100 µg/mL. This served as the internal standard of JA in the sample.

Aluminum-backed silica gel 60 F_{254} TLC foils (14×10cm) with 200 µm layer thickness purchased from Sigma-Adrich Singapore were prepared also according to Dhandukia and Thakkar (2008). Thirteen lanes that are one cm apart were made in each TLC plate.

The re-dissolved dried extracts were loaded in the pre-conditioned TLC foils using a 10-µL pipette. One microliter (1.0µL) of the sample was loaded in triplicate. Two samples were loaded in each plate.

Known concentrations of the standard solutions of JA were made by dissolving 100.0 mg of jasmonic acid in methanol. Several lanes in each of the TLC plates were loaded with the JA solutions to achieve 0.1 µg/band (2 lanes), 0.2 µg/band (2 lanes), 0.4 µg/band (2 lanes), and 0.6 µg/band (1 lane). After loading the standards and samples onto the TLC plates, the plates were allowed to stand at 25° C for 5 to 10 minutes for drying before chromatogram development.

After drying, the TLC plates loaded with the appropriate samples and standards were placed inside a pre-saturated chamber with the mobile phase consisting of chloroform: ethyl acetate: acetone: acetic acid in 40:8:5:1 ratio (Ueda and Miyamoto, 1994). The average development time was 25 minutes.

After development, the plates were air-dried for 10 minutes. To visualize the chromatogram, 5.0 g of iodine crystals was placed in a cylindrical container with a height of 10.0 cm and a diameter of 10.5 cm, and the dried plates were then exposed to iodine vapor for 12 min.

After the exposure to the iodine vapor for visualization, the TLC plates were scanned to produce electronic image copies of the chromatograms.

Quantitative analyses were performed on the images of the chromatograms using JustTLC v. 4.0.3, a TLC analysis software by Sweday.

The band volume and the known concentrations of the standard were plotted to construct a standard curve. The concentrations of the JA bands in the samples were computed based on the standard curve produced.

Salicylic Acid Quantification: Salicylic acid quantification was performed on the aqueous extract described above according to Warrier, Paul, and Vineetha (2013). The measurement of the concentration of flavonoids in the sample was expressed as µg SA per gram leaves (µg SA/g leaves). Standard curves were made using known concentrations of salicylic acid.

Total Phenolics and Flavonoids Quantifications: The methanolic extracts were also quantified for total phenolics and flavonoids according to the protocols of Singleton, Orthofer, and Lamuela-Raventos (1999) and Kiranmai, Kumar and Ibrahim (2011), respectively. Standard curves of gallic acid and quercetin were also prepared. The concentration of the total phenolics of the sample was expressed as gallic acid units per gram leaves (GA/g leaves), while the concentration of the flavonoids in the same sample was expressed as quercetin unit per gram leaves (QU/g leaves).

Statistical Analysis

For the comparison of the two treatments every observation period for significance of difference, Shapiro-Wilk Test for normality and Mann-Whitney U Test were used. For the correlation analyses, Doornik-Hansen Multivariate Test for normality was used. Pearson's correlation was used when the data satisfied the assumption of normality, otherwise Spearman's correlation was used.

RESULTS AND DISCUSSION

***Trichoderma* Protective Capacity**

Assessments of the morphological characteristics of the seedlings belonging to the treatments with and without *Trichoderma* were done for the set up for the determination of the protective capacity of the *Trichoderma* spp.

Figure 1A shows that in the beginning of the experiment, there was no significant difference between the number of leaves of the seedlings in the two treatments. Meanwhile, seedlings inoculated with *Trichoderma* showed significantly higher leaf counts in all succeeding observation periods ($p = 0.040$, $p = 0.010$, and $p < 0 .0005$ for Months 2, 3, and 4, respectively).

The ability of the seedlings to produce more leaves reflects the capacity of the plant to grow even in the presence of an insect pest. Increased growth, specifically in biomass yield, in plants inoculated with *Trichoderma* has been demonstrated in a number of literature including Zarate et al. (2015), and Harman (2000) among other studies. This is particularly highly correlated to the ability of these plants with *Trichoderma* as their mycosymbiont to have a more efficient nutrient uptake and utilization (Harman et al., 2008; Harman, 2001, 2000), as well as the upregulation of genes supporting more rapid growth (Harman and Shoresh, 2007). It has also been demonstrated that the leaves have an increased

greenness that is hypothesized to be related to an increased photosynthetic rate thereby allowing the plants to create more resources for itself that is much needed for its own growth (Harman et al., 2008; Harman and Shoresh, 2007; Harman, 2000).

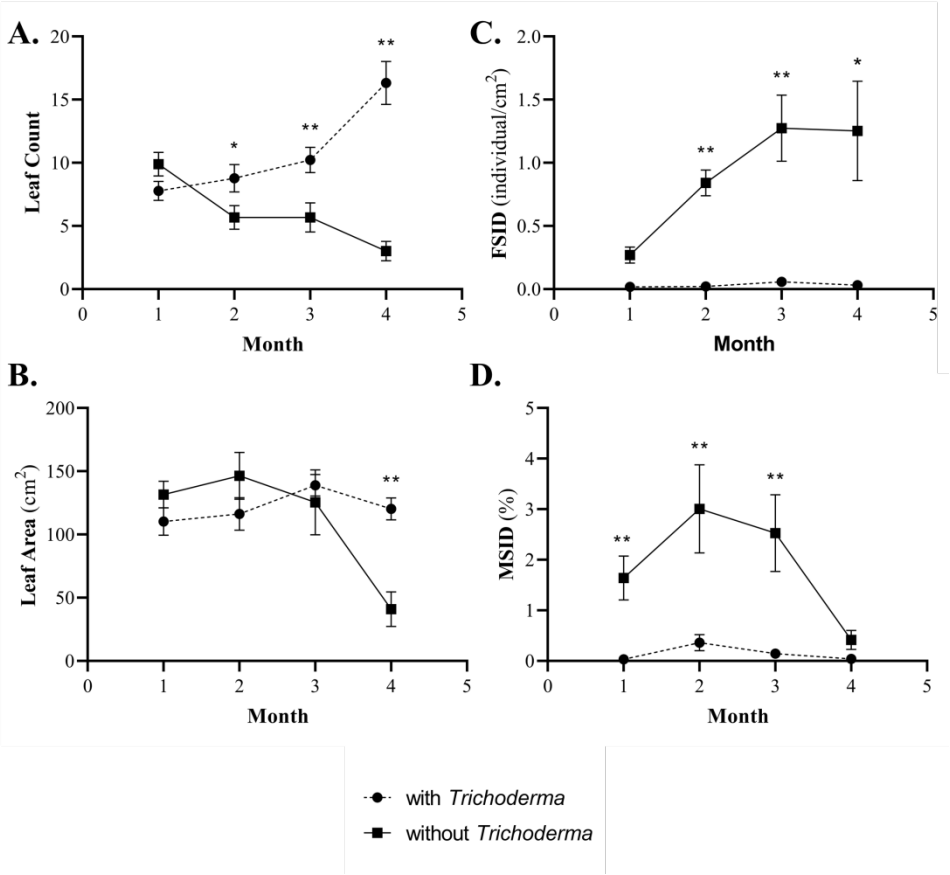

Figure 1. Comparisons between average leaf counts (A), leaf areas (B), female scale-insect densities (C), and male scale-insect densities (D) of the two treatments (with *Trichoderma* & without *Trichoderma*) over the four-month observation period **[**(Mean ± SEM); ***** shows that **p** ≤ 0.05**; **** shows that **p** ≤ 0.01 **]**

On the other hand, it has been observed that there was no significant difference in the area of the leaves (Fig. 1B) of the seedlings from the two treatments up until the fourth month (p = 0.004). However, on the sixth month, a highly significant difference was observed. This is due to the continuous loss of the older leaves of the seedlings in the treatment without *Trichoderma*. The much younger and smaller leaves are therefore the ones that were observed, thereby resulting in a significantly smaller leaf area.

Observation of the insect population showed that from the second up to the last observation period (Fig. 1C), FSIDs were observed to be significantly lower

for the seedlings with *Trichoderma* ($p < 0.0005$ for Months 2 and 3, and $p = 0.012$ Month 4). It is also noteworthy to mention that the FSIDs of the seedlings with *Trichoderma* did not significantly increase over the four months of observation ($p = 1.000$), while the seedlings without *Trichoderma* had a significant, generally increasing trend of FSIDs over the same period ($p = 0.01$).

Figure 1D shows that the treatment without *Trichoderma* has higher MSIDs from the first observation period up until the third month ($p < 0.005$). During the last observation period (Month 4) no significant difference were observed which may be attributed to the effect of drastic decline of both leaf count (Fig. 1A) and leaf area (Fig. 1B) of the uninoculated plants.

It is clear from the results that *Trichoderma* inoculation was able to protect the lanzones seedlings from damaging levels of infestation in the leaves even if it was inoculated only in the roots. This is probably related to the capacity of the *Trichoderma* inoculant to potentiate the plant for a faster and stronger defense hence preventing the rapid increase in pest population. It has been shown that *Trichoderma* inoculation stimulates the jasmonic acid (JA)–dependent defense responses (Contreras-Cornejo et al., 2018; Martinez-Medina et al., 2013; Contreras-Cornejo et al., 2011), which is partly responsible for the initiation of the cascade of biochemical defense responses of plants against a wide array of biotic stresses. The inoculation of *Lansium domesticum* seedlings with *Trichoderma* before the insect attack possibly increased the sensitivity and responsiveness of the seedlings to jasmonic acid, as demonstrated by Pieterse et al. (2000).

***Trichoderma* Rescuing Capacity**

Observations of the leaf counts of the seedlings belonging to the two treatments showed no statistical difference (Fig. 2A). However, leaf areas of the plants belonging to the treatment with *Trichoderma* are significantly broader during the fourth and sixth week of observation with $p=0.001$ and $p=0.034$, respectively (Fig. 2B). In addition, only the treatment with *Trichoderma* was also observed to exhibit a statistically significant increase in the average leaf area growth throughout the observation period ($p=0.006$).

In addition, Figures 2C and 2D show statistically significant reduction in FSID and MSID in infested plants inoculated with *Trichoderma* clearly showing the ability of the inoculant to alleviate the effects of the infestation. These observations alone demonstrate the advantage of *Trichoderma* inoculation to infested plants. In separate studies of Lo and Lin (2002), and Yedidia et al. (2001), leaf area of cucurbitaceous plants was shown to be larger in treatments inoculated with *Trichoderma.* This increase in leaf area is beneficial for the plants since it also increases the amount of intercepted light, thus increasing photosynthetic activity and, consequently, plant productivity. Increased productivity may provide the necessary “fuel” for increased production of metabolites for defense response and possibly compensate for losses incurred from insect feeding.

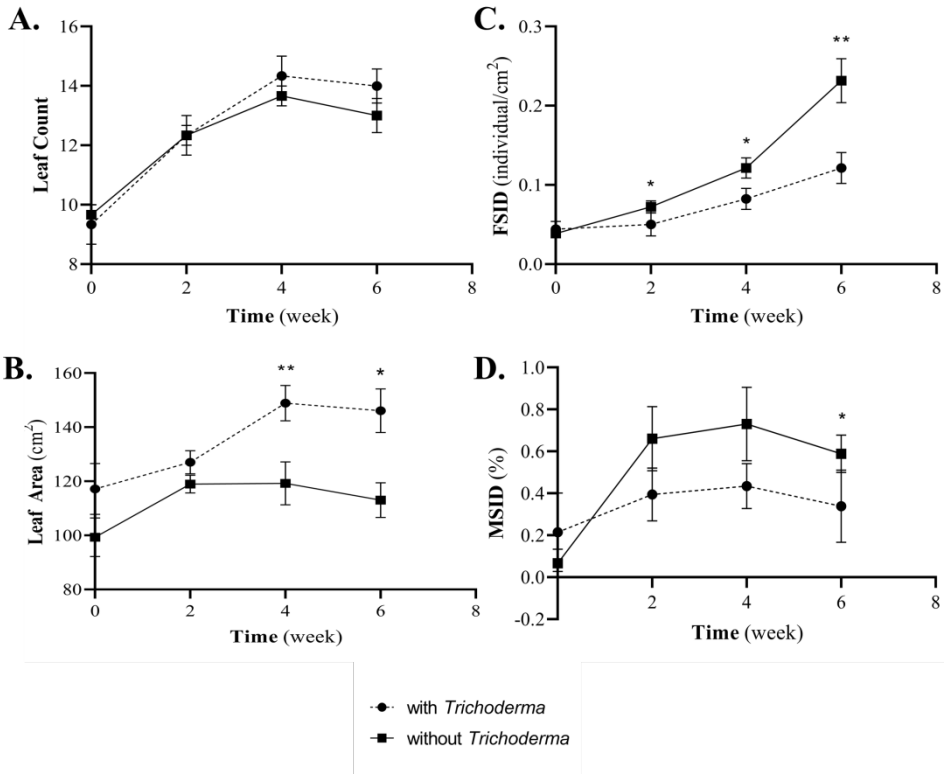

Figure 2. Comparisons between average leaf counts (A), leaf areas (B), female scale-insect densities (C), and male scale-insect densities (D) of the two treatments (with *Trichoderma* & without *Trichoderma*) over the six-week observation period **[**(Mean ± SEM); ***** shows that **p ≤ 0.05; **** shows that **p ≤ 0.01]**

To understand the mechanism behind these observed differences between the two treatments, phytochemical tests were also done. The phytohormones salicylic acid (SA) and jasmonic acid (JA) were quantified. Additionally, after assessing for the presence or absence of eight major groups of plant metabolites (Table 1), concentrations of flavonoids and phenols were quantified.

Table 1. Initial phytochemical screening of *Lansium domesticum* leaves

Alkaloids		Anthraquinones		Flavonoids	Glycosides	Phlobatannins	Saponins	Steroids	Tannins	Terpenoids
Mayer	Wagner	Ajayeoba	Kumar							
(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(+)

The qualitative screening showed presence of tannins, phlobatannis, flavonoids, steroids, glycosides, terpenoids, and alkaloids, which narrowed down the possible groups of *Lansium domesticum* secondary metabolites that can contribute to its general plant defense against the insect pest. Based on these results and the availability of methods and equipment that can be used for

quantification, it was decided that the total phenolic content and flavonoid content of the leaves of the plants be quantified.

Total phenolic content was chosen because this class of compounds is one of the most diverse of all the classes of plant secondary metabolites. This includes the tannins, quinone, and flavonoids, among other groups that can function as herbivore deterrent, or may either kill or retard the development of an herbivore (War et al., 2012; Harborne, Baxter, and Moss 1999). In addition, the flavonoid group was also chosen to be quantified as a subset of the phenol class that has the capacity to protect plants against pests by influencing the insect's behavior, growth, and development. Some flavonoids were also observed to have cytotoxic properties and they can interact with other enzymes by complexation (War et al., 2012).

Quantification of SA (Fig. 3A) showed that SA increased over time in both treatments. The coefficients of linearity for the trends of SA concentration was 0.907 ($p<0.0001$) and 0.515 ($p=0.086$) for with and without *Trichoderma*, respectively. Studies on the differences of expressions of defense-related genes showed that biotrophic pathogens and phloem-feeding insects primarily activate the SA-dependent defense pathway, while the pathogens and herbivores causing minimal tissue damage activate the JA/ET pathway (Thaler, Agrawal and Halitschke, 2010; Smith, De Moraes and Mescher, 2008; Kempema et al., 2007; Thompson and Goggin, 2006; Glazebrook, 2005; Kaloshian and Walling, 2005; Rodriguez-Saona and Thaler, 2005; Rojo, Solano and Sanchez-Serrano 2003; Moran and Thompson, 2001).

In this study, the observed increase of SA in lanzones plants as infestation of *U. mabilis* progresses over time demonstrates similarity to other studies on phloem-feeding insects activating the SA-dependent defense pathway. According to the "decoy defense hypothesis" by Zhu-Salzman et al. (2004) on their study of the green bug aphid (*Schizapis graminum*) in sorghum (*Sorghum bicolor*), certain insect pests take advantage of the antagonistic interaction between JA and SA in order to increase their establishment on their hosts. The "decoy defense" was likewise observed in studies on *Arabidopsis* infested with *Bemesia tabaci* type B or silverleaf whitefly (Kempema et al., 2007; Zarate, Kempema and Walling, 2007). In these separate studies, silver leaf whitefly leveraged the antagonistic interaction of SA- and JA- signaling pathways as it causes strong induction of the SA pathway to suppress the JA pathway that can launch a more effective defense response against the infestation. Walling (2008) explained that phloem-feeding insects are able to deceive the host and suppress effective defense. By taking advantage of the negative crosstalk between the SA and JA pathways, the insects are able to inhibit the more effective JA-controlled defenses by stimulating the less effective SA-controlled defenses that inhibit the JA pathway.

Meanwhile, consistent with Zarate, Kempema and Walling (2007) and Kempema et al. (2007), the concentrations of JA (Fig. 3B) in the uninoculated seedlings showed no significant increase during the observation period

(p=0.052). The association of JA concentration and time ($_{Q}$) in treatment without *Trichoderma* is 0.086 (p=0.79).

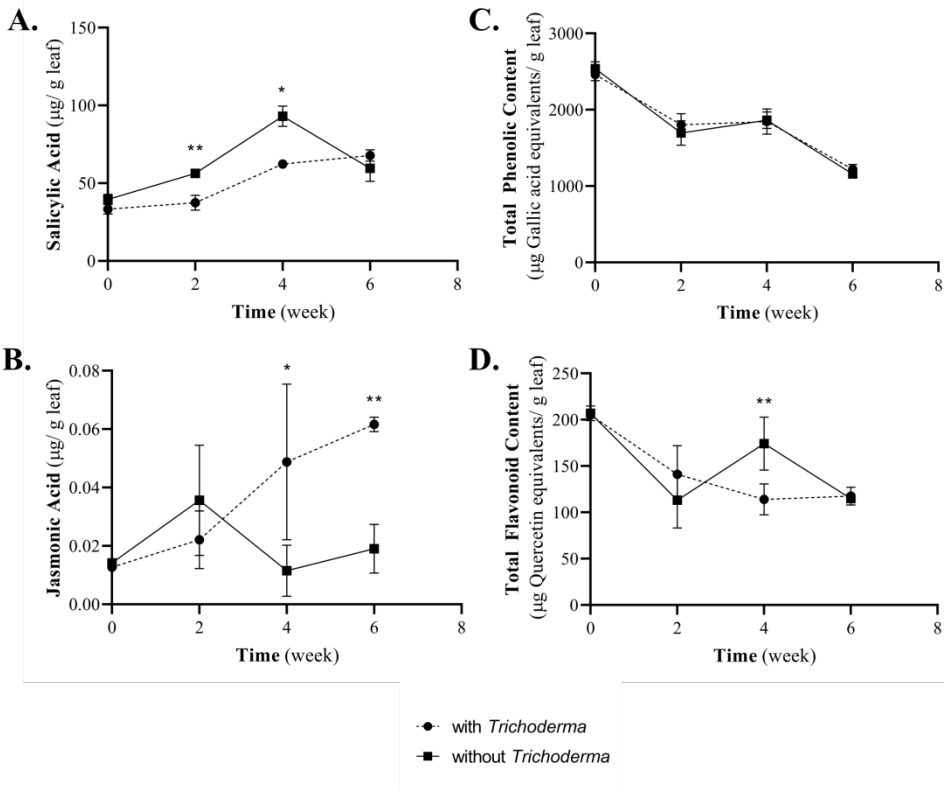

Figure 3. Comparisons between concentrations of jasmonic acid (A), salicylic acid (B), total phenolic content (C), and total flavonoid content (D) of the two treatments (with *Trichoderma* & without *Trichoderma*) over the six-week observation period **[**(Mean ± SEM); ***** shows that **p ≤ 0.05; **** shows that **p ≤ 0.01]**

In addition, it was observed that uninoculated plants had significantly higher concentration of SA during the second and third observation periods (p<0.0005 for Week 2, and p=0.040 for Week 4). Earlier study by Zhou et al. (2018) showed similar results. Lower SA concentration was observed in *Trichoderma gamsii* inoculated *Arabidopsis* plants that are subsequently infested with *Trichoplusia ni* than those that were not inoculated. Contreras-Cornejo et al. (2018) also reported reduced methyl salicylate in plants inoculated with *Trichoderma atroviride* and challenged with *Spodoptera frugiperda* as compared to the plants that were also challenged with the same insect but are uninoculated by the *T. atroviride.*

On the other hand, concentrations of JA for the seedlings with *Trichoderma* during the six-week observation increased significantly (p=0.001) with R=0.687 (p=0.0136). Specifically, JA concentrations were found to be

significantly higher in plants that were inoculated with *Trichoderma* compared to those that were uninoculated in Week 4 ($p=0.012$) and Week 6 ($p<0.001$). These observations suggest that in the absence of *Trichoderma* inoculation, SA accumulates while JA remains unchanged in infested lanzones plants. Meanwhile, relative to the treatment without inoculation, *Trichoderma*-inoculated plants accumulate SA slower and JA faster. Similarly, the observations of Contreras-Cornejo et al. (2018) indicate that, in their fungus-plant-insect model, SA-mediated defense responses are attenuated while JA concentration is increased. Drawing from other studies, the same authors (Contreras-Cornejo et al., 2018) noted that their observations, similar to the observations in this study, [can] "not be a coincidence but a demonstration of a similar defense time-course patter[n] of plants challenged with different species of *Trichoderma* (p. 8)." In extension, these observations suggest that *Trichoderma* inoculation in plants provides a counter mechanism against the "decoy defense" of some phloem-feeding insects hiding behind a "hacked" SA-mediated defense response.

Quantifications of the total phenolic content of the leaves of the seedlings belonging to both treatments, meanwhile, show a very strongly decreasing linear trend over time (Fig. 3C).

Time and total phenolic concentrations of the leaves in set-ups with and without *Trichoderma* have correlation coefficient R values of -0.873 ($p=0.0002$) and -0.862 ($p<0.0001$), respectively. No significant difference in phenolic concentration between the two treatments in any of the observation periods was observed. In addition to the reduced SA concentration, Zhou et al. (2018) also observed no significant difference in total phenolic between *T. gamsii*-inoculated and uninoculated *T. ni*-infested *Arabidopsis* plants.

However, the total flavonoid concentrations over the six-week time period varied between the two treatments (Fig. 3D). During the third observation period (Week 4), the seedlings inoculated with *Trichoderma* had a significantly lower concentration of flavonoid ($p=0.005$). However, for first, second, and last observation periods, no significant difference was observed. In general, the flavonoid concentration was decreasing over time.

With the observations of decreasing concentrations of phenols and flavonoids in both treatments, it can be hypothesized that other defense response metabolites that are outside the phenol group may have been used by the lanzones plants and that a different set of metabolites was induced instead. In a paper by Xiao et al. (2019), they observed that herbivory by a phloem-feeding insect does not show any induction of the total phenolics and total flavonoids in the host plant unlike chewing herbivores that cause significant increases in total phenolics and flavonoids compared with control plants.

Alternatively, Close and McArthur (2002) suggested that perhaps there is a need to rethink the role of phenolics in plants, that they may be more affected by photodamage than by action of herbivores. The authors proposed that phenolics may be low because there is low risk to photodamage and therefore the

metabolites are not required and not because of defense against herbivores. Although it was observed that there is a moderate to very strong inverse correlation between the FSIC and the concentration of total phenol and flavonoids in the leaves of the *Lansium domesticum* plants (Fig 4A and 4B), this does not mean that one causes the other.

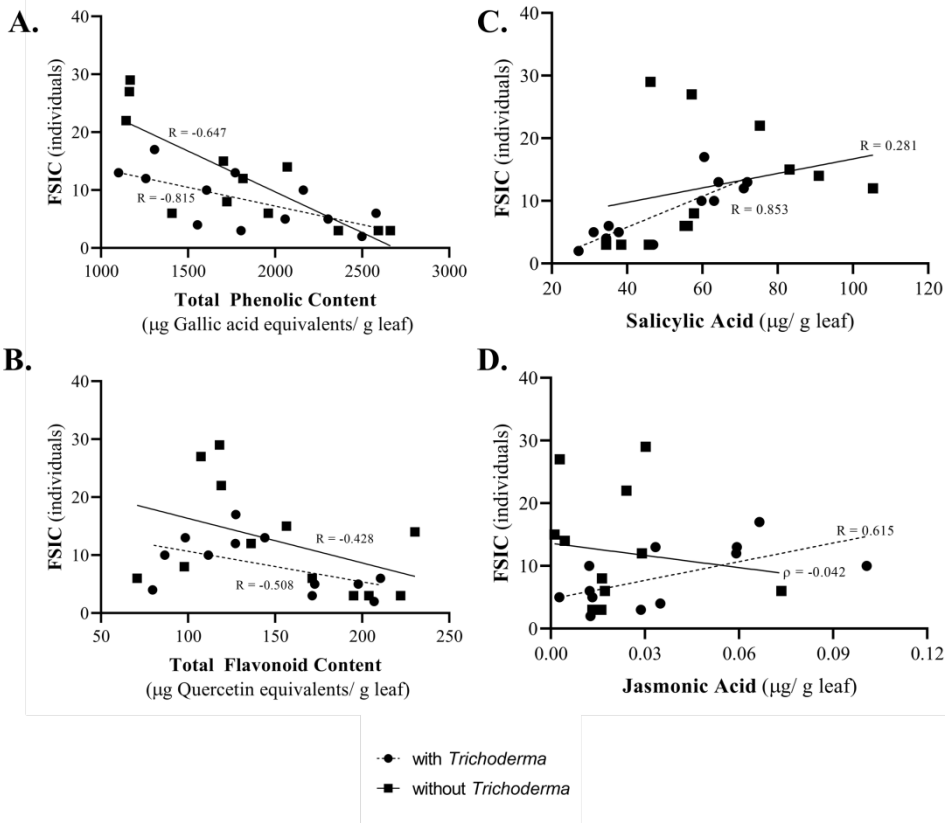

Figure 4. Comparisons of relationships between concentrations of total phenolic content (A), total flavonoid content (B), salicylic acid (C), and jasmonic acid (D) of the two treatments (with *Trichoderma* & without *Trichoderma*) over the six-week observation period

It is possible that total phenolic concentrations decreased through time because the plants were maintained under partially shaded conditions inside an insect net tent and that its negative correlation with FSIC is just coincidental. If this is true, then it makes sense that all plants, regardless of treatment, would exhibit similar patterns of decrease in phenolics through time as they are continuously maintained under non-photodamaging conditions.

The overall decrease of the concentration of the phenolic and flavonoid groups may also be viewed as a natural optimization response of the plant to environmental variations even while there is continuous insect attack (Pieterse et al., 2012). That is besides the toxicity of high phenolic compounds concentration

in the cell and the very resource-consuming whole range of adaptive responses that may be triggered due to the presence of the insect pests, often sacrificing even growth and development (Walters and Heil 2007).

Supporting the previously mentioned hypothesis that defense metabolites other than the members of the phenolic group is the example of the increased production of camalexin or 3-thiazol-2′-yl-indole that was shown to be induced by *Trichoderma* inoculation in *Arabidopsis thaliana* (Contreras-Cornejo et al., 2011). Camalexin is an example of indole derivatives that inhibit growth of pathogenic fungi like *Botrytis cinerea* and *Cochliobolus carbonum*, and the bacterium *Pseudomonas syringae*. Indole alkaloid camalexin has also been demonstrated to be involved in the resistance and defense mechanism of *A. thaliana* against *Myzus persicae*, a phloem-feeding green peach aphid (Kettle et al., 2013). Meanwhile, indole glucosinolates concentrations are also important in plants defense against plant herbivores.

For lanzones plants, the concentration of a specialized terpenoid found in lanzones trees called lansiolic acid that has been found to have a strong insect feeding deterrence activity (Mayanti et al., 2011; Ragasa, Labrador and Rideout, 2006; Arnason, Guillet, and Durst 2004) is a highly possible candidate for the secondary metabolite which could have had an increased production. Contreras-Cornejo et al. (2018) further noted the positive relationship of *Trichoderma* inoculation and the observed increase in JA levels and volatile terpene emission.

Since there is a lack of available tools to dissect and quantify all of the possible secondary metabolites which can/may be produced by the lanzones plant, the relationship of the hormones and the pest can be looked into instead for possible explanations. Figures 4C and 4D show the correlations of the FSIC and the concentration of the two commonly implicated plant hormones during insect herbivory.

Plant detection of phloem-feeding insects, like *Unaspis mabilis*, is similar to their detection of pathogens, since these phloem-feeding insects create minimal plant tissue injury. The jasmonic acid-dependent and the salicylic acid-dependent pathways regulate the plant's responses to this kind of attacker (Walling 2000). Figure 4D shows that there is a strong and significant relationship between JA and the FSIC (R=0.615, p=0.0332) for the seedlings treated with *Trichoderma*. The same can be observed with the relationship of the same treatment with the concentration of SA (R=0.8534, p=0.0004). On the other hand, the FSIC in seedlings that were not inoculated with *Trichoderma* showed a very weak and insignificant correlation with JA ($_0$=0.042, p=0.897), and a weak correlation with SA (R=0.281, p=0.376).

Since all the relationships between JA, SA, time, and FSIC are all weak and insignificant in the treatment without *Trichoderma*, while all the relationships between the same four parameters observed in the treatment inoculated with *Trichoderma* are strong, significant, and positive, it can be surmised that for lanzones plants infested with scale insects, the concentrations of both JA and SA are modulated by the inoculation of *Trichoderma* and that these

were contributory to alleviating the infestation. These observations support the earlier hypothesis that both SA and JA play key roles in scale-insect defense response in the lanzones plant. This is consistent with the results of the study of Contreras-Cornejo et al. (2011) that both JA and SA accumulation was observed in *A. thaliana* inoculated with *Trichoderma*. In addition, multiple studies have shown that jasmonic acid and its derivatives can induce the production and accumulation of other secondary defense metabolites including saponin (Hu et al., 2003), terpenoid indole alkaloid (Goldhaber-Pasillas, Mustafa and Verpoorte, 2014), and terpenoid-based resins (Martin et al., 2002), that like the lansiolic acid, are noted for their role in plants' defenses.

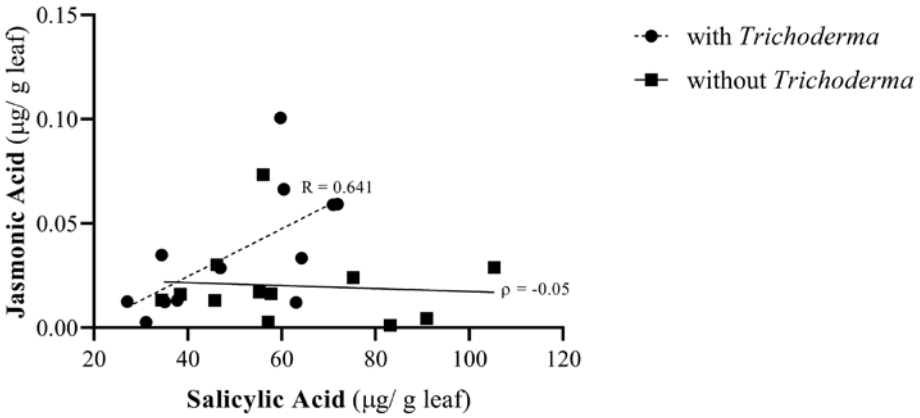

Figure 5. Correlation of salicylic acid and jasmonic acid concentrations in *Lansium domesticum* leaves infested with *Unaspis mabilis* in treatments with and without *Trichoderma*.

Figure 5 shows that there is a strong and significant correlation between SA and JA in plants inoculated with *Trichoderma* (R=0.641, p=0.0247). Meanwhile, a very weak and insignificant association was observed between SA and JA in plants that were not inoculated with the same fungus ($_{\rho}$=-0.056, p=0.863). These observations suggest a more refined tuning action of JA and SA in the presence of the *Trichoderma* inoculant. In these seedlings, the scale insect infestation declined through time and the seedlings were able to recover. This suggests that *Trichoderma*-inoculation was able to subvert the decoy defense of the insect by inducing the JA pathway thus allowing integration of both SA- and JA-mediated signaling pathways. This enabled the plant to launch effective defenses to antagonize insect performance.

While the *Trichoderma*-mediated subversion of the insect's "decoy defense" can effectively explain the observed concentrations of SA and JA in both the inoculated and uninoculated groups, possible SA-JA positive crosstalk may have still played an important role. This is because even with the "decoy

defense hypothesis," SA-dependent defenses still contribute, although weakly, to the plant's overall defense strategy (Zhu-Salzman et al., 2004). The significant positive correlation between the JA and SA concentrations shown in the present study for *Trichoderma*-inoculated seedlings (Fig. 5) may still suggest that there is positive crosstalk between these two pathways and that they may have cooperated in order to launch effective defense responses against the pest. It is still possible that the cooperation of these two pathways lead to the production of a wide array of defense metabolites that all contribute to resistance against the lanzones scale insect.

According to Martinez-Medina et al. (2013), reports of the necessity for JA in *Trichoderma*-induced systemic resistance are consistent in systems using *Arabidopsis thaliana* as model. Several other studies have been conducted that implicate the JA signaling pathway in the *Trichoderma*-mediated induced systemic resistance (TISR) in plants. In the study by Shoresh, Yedidia and Chet (2005), the JA signaling pathway was identified as the signal transduction pathway in the induction of systemic resistance by *Trichoderma asperellum* T203 in cucumber. The study by Segarra et al. (2009) likewise confirms the role of the JA pathway in *Trichoderma*-mediated ISR. In addition, Martinez-Medina et al. (2013) also reported that in tomato models, SA- regulated pathways are also required for a successful TISR development. Other studies show the capability of *Trichoderma* to induce resistance by triggering both JA- and SA-mediated defense response, and that the SA and/or JA/ET pathways may overlap depending on several conditions (Saldajeno et al., 2014; Yoshioka et al., 2012; Tucci et al., 2011; Segarra et al., 2007). Gallou, Cranenbrouck and Declerck (2009) also reported that the potato plants inoculated with *Trichoderma harzianum* when challenged with *Rhizoctonia solani* displayed a defense response that was dependent on both JA/ET and SA pathways.

Altogether, the results of the present study demonstrate that *Trichoderma* inoculation induced the JA pathway and played an important role in defense against lanzones scale insect. Furthermore, the interaction of the JA pathway with the SA-dependent pathway is fine-tuned in the presence of *Trichoderma*.

CONCLUSIONS

The capacity of *Trichoderma* spp. to be a protective agent has been clearly demonstrated. The healthy plants inoculated with the fungus have shown better resistance when challenged by *Unaspis mabilis* infestation. This was determined through the observation of morphological parameters of the plants tested to assess their general health condition, as well as the population changes of the scale insects during a four-month observation period. In general, the plants inoculated with *Trichoderma* have displayed higher leaf number, and lower insect population than that of the seedlings that were not inoculated with the myco-biocontrol agent.

The capacity of *Trichoderma* spp. to alleviate scale insect infestation, on the other hand, was tested through the simultaneous observation of the same

morphological parameters, and phytochemical parameters. It was observed that the plants inoculated with *Trichoderma* have shown better performance against the infestation of the *U. mabilis* since the population of scale insects was significantly lower and was growing more slowly.

The two groups of plant defense metabolites (phenols and flavonoids) concentration were observed to be decreasing over time. It was proposed that this could be due to other environmental cues (non-photodamaging conditions) and not just a response to insect attack. It is also probable that other compounds were involved or that it is not the total concentration of the groups that is important for an optimized defense but the characteristic and the blend of the specific compounds.

In addition, the phytohormones JA and SA, have been observed to be affected by the presence of the *Trichoderma* inoculation. Drawing from previous studies, three hypotheses were proposed to explain the effect of the *Trichoderma* inoculation on these two plant hormones in the case of *Trichoderma* - *L. domesticum* – *U. mabilis* interaction:

1.) the scale insects have the capacity to increase the production of SA inside the plants, thereby blocking the JA-dependent defense pathways, while the inoculation of *Trichoderma* to the plants causes an increased JA production to counter this SA increase;
2.) the inoculation of *Trichoderma* may have had a simultaneous effect on the concentration of SA and JA in order to utilize both the SA-dependent defense response and the JA-dependent defense pathways of the plant; or
3.) a combination of both.

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WINTER WHEAT YIELD AND QUALITY DEPENDING ON THE LEVEL OF NITROGEN, PHOSPHORUS AND POTASSIUM FERTILIZATION

SUMMARY

Tests were carried out on stationary field trial, soil type vertisol in the process of degradation. The land on which the view is derived is characterized by low pH (pH<5.0). The Dose of nitrogen was 120 kg N/ha, which was administered in combination with phosphorous and potassium fertilizer. The tests showed a significant grain yield. Nitrogen had a most significant impact on the yield of wheat. The average grain yield of all treatments in the 2008/09 growing season was significantly greater than in the following years, mostly as the result of highly favourable weather conditions at major stages of plant development. The average highest yield was achieved in the variant N120P100K60 (5.528 t/ha), although the high yield of more than 5 t/ha was obtained and the triple treatments NPK where nitrogen is applied in the maximum amount of 120 kg/ha, 60 kg/ha P_2O_5 and 60 kg/ha K_2O as well as variants NP in a quantity of 100 kg/ha P_2O_5 and 60 kg/ha K_2O. Also, the 1000-grain weight was highest in balanced fertilization with all nutrients NPK (40 g), in a quantity of 120 kg N/ha, 100 kg/ha P_2O_5 and 60 kg/ha K_2O. Averaged across years, significantly higher values for test weight were found in the control (76.91 kg/hl). Variance analysis showed statistically significant differences for grain yield and test weight between the vegetation seasons. Significant differences for 1000 grain weight between the variants of fertilization.

Keywords: fertilization, mineral nutrition, yield, wheat

INTRODUCTION

Wheat productivity and grain quality in Central Serbia are governed by a range of factors, notably climate, soil, genetics and crop nutrition. Soil acidity in wheat fields in Central Serbia has become a severe problem that leads to a significant decline in grain yield and quality of wheat (Đekić et al., 2017a, 2019;

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Notes: The authors declare that they have no conflicts of interest. Authorship Form signed online.

Jelic et al., 2015). The yield per unit area is the result of the action of factors of variety in interaction with environmental factors. The yield is largely dependent on the genetic potential and considerably vary primarily as a result of agro-ecological conditions during the growing season (Dodig et al., 2008; Hristov et al., 2011; Đekić et al., 2017b; Jelić et al., 2014; Djuric et al., 2018; Jordanovska et al., 2018; Popović et al., 2016, 2018; Terzić et al., 2018b).

Mineral fertilizers play a vital role towards improving crop yields but one of the main constraints in achieving proven crop potential is imbalanced use of nutrients, particularly low use of P as compared to N. The optimum rate of P application is important in improving yields of most crops (Jelic et al., 2013). Plant nitrogen nutrition has a great impact on the technological quality of wheat cultivars. Nitrogen, in interaction with other elements of mineral nutrition, plays a significant role in the grain yield and quality (Jelic et al., 2014; Đekić et al., 2014, 2017a; Popović et al., 2017; Terzic et al., 2018a). For high yield and grain quality, it is necessary to adopt nitrogen by plants during the whole vegetation period (Jelić et al., 2015). Understanding the fertilization, liming and rainfall effects have been a continuous endeavor toward improving farming technology and management strategy to reduce the negative impacts of these factors and to increase crop yield (Đekić et al., 2019; Jelić et al., 2016; Popović et al., 2017; Terzic et al., 2018a,b).

The objective of this study was to evaluate the effect of different fertilization systems on the grain yield and quality of wheat grown on a vertisol soil. The study was also aimed at optimizing fertilization for maximum profitability in the future wheat production of Central Serbia.

MATERIAL AND METHODS

Experimental design

The study was carried out in a stationary field trial involving fertilization over a three years period from 2008/09, 2009/10 to 2010/11. Trials were first set up in the experimental fields of the Small Grains Research Centre in Kragujevac in 1970. Plot size was 50 m^2. The trial was set up in a randomized block design with five replications. Fertilization was regular and followed a long-time scheme.

The rates of nitrogen application were 120 kg/ha N, and they were applied either individually or in combination with two phosphorus rates and a potassium fertilizer. A non-fertilized variant served as a control. The winter wheat cultivar used in the experiment was "Vizija", the dominant cultivar in the production region of Serbia. Eight variants of mineral nutrition N (120 kg/ha N), P_1 (60 kg/ha P_2O_5), P_2 (100 kg/ha P_2O_5), NP_1 (120 kg/ha N and 60 kg/ha P_2O_5), NP_2 (120 kg/ha N and 100 kg/ha P_2O_5), NP_1K (120 kg/ha N, 60 kg/ha P_2O_5 and 60 kg/ha K_2O), NP_2K (120 kg/ha N, 100 kg/ha P_2O_5 and 60 kg/ha K_2O) and untreated control were tested in the experiment. Total amounts of phosphorus and potassium fertilizers and half the nitrogen rate are regularly applied during pre-sowing cultivation of soil.

The crop was harvested at full maturity. Grain yield (t/ha) was harvested and reported at 14% moisture. Three parameters, namely grain yield, 1000-grain

weight (g) and test weight (kg/hl) were analyzed. Thousand grain weight was determined using an automatic seed counter. Test weight is the weight of a measured volume of grain expressed in kilograms per hectoliter.

Soil analysis

The trial was set up on a vertisol soil in a process of degradation, with heavy texture and very coarse and unstable structure. The humus content in the surface layer of soil was low (2.22%). The reduced humus content in field vertisols profiles suggests the necessity of involving humification when planning fertilization systems and soil ameliorative operations to be used to maintain and improve the soil adsorption complex. Soil pH indicates high acidity (pH in H_2O 5.19; pH in KCl 4.27), nitrogen content in soil is medium (0.11-0.15%), while the content of available phosphorus ranges from very low (1.7-2.9 mg 100 g^{-1} soil) in the N and NK trial variants to very high (26.9 mg P_2O_5 100 g^{-1} soil) in the NPK variants of fertilization. Available potassium contents are high, ranging from 19.5 to 21.0 mg K_2O 100 g^{-1} soil.

Statistical Analysis

On the basis of achieved research results the usual variational statistical indicators were calculated: average values and standard deviation. Experimental data were analysed by descriptive and analytical statistics using the statistics module Analyst Program SAS/STAT (SAS Institute, 2000) for Windows. All evaluations of significance were made on the basis of the ANOVA test at 5% and 1% significance levels. Relative dependence was defined through correlation analysis (Pearson's correlation coefficient), and the coefficients that were obtained were tested at the 5% and 1% levels of significance.

RESULTS AND DISCUSSION

Meteorological conditions

This study was conducted over a three-year period in the Sumadija region, Central Serbia, on a Vertisol soil, at Kragujevac location, (44° 22′ N, 20° 56′ E**,** 173-220 m a. s. l.), in a temperate continental climate having an average annual temperature of 11.76°C, typical of Sumadija district in Serbia and a rainfall amount of about 550 mm.

Table 1. Precipitation sum and average monthly temperature in Kragujevac, Serbia

Months	Mean monthly air temperature (°C)				The amount of rainfall (mm)			
	2008/09	2009/10	2010/11	Average	2008/09	2009/10	2010/11	Aver.
X	13.1	11.7	10.2	12.5	31.3	102.6	86.9	45.4
XI	8.5	8.8	11.4	6.9	30.6	77.5	27.9	48.9
XII	4.4	2.6	2.4	1.9	29.7	194.2	50.1	56.6
I	2.3	0.9	0.9	0.5	57.7	57.0	29.1	58.2
II	2.0	3.2	0.5	2.4	76.9	150.5	48.5	46.6
III	6.8	7.2	7.2	7.1	40.3	43.3	20.4	32.4
IV	13.4	12.1	12.0	11.6	16.8	142.2	20.8	51.9
V	17.8	16.5	15.8	16.9	46.0	116.7	65.8	57.6
VI	20.2	20.2	20.9	20.0	137.8	196.7	32.3	70.4

Aver.	9.83	9.24	9.03	8.87	467.1	1080.1	381.8	468.0

Meteorological conditions recorded high variability during year. The average air temperature was higher by 0.8°C in 2008/09, by 0.37°C in 2009/10 and was higher by 0.16°C in 2010/11 than the average of many years. The sum of rainfall precipitation was higher by 612.1 mm in 2009/10, where the sum of rainfall was lower by 0.9 mm in 2008/09 and was lower by 86.2 mm in 2010/11 than the average of many years and with a very uneven distribution of precipitation per months. During the April and May in 2009/10 it was 142.2 mm and 116.7 mm of rainfall, what was 90.3 mm and 59.1 mm more compared with the perennial average. During the June in 2009/10 it was 196.7 mm of rainfall, what was 126.3 mm more compared with the perennial average. Regard the high importance of sufficient rainfall amounts during the spring months, particularly. Namely, the total amount of precipitation is reflected on the multi annual average, but the distribution, especially at critical stages of development, is significantly disturbed in the 2009/10 year. In addition to the necessary reserve for the spring part of the vegetation, winter precipitation greatly influences the distribution of easily accessible nitrogen in the soil (Đekić et al., 2014, 2017b, 2019; Jelic et al., 2014, 2015; Milivojević et al., 2018; Popovic et. al., 2016, 2017; Terzić et al., 2018a,b).

Table 2. The analysis of variance for the tested parameters in Kragujevac, Serbia

Effect	df	Mean sqr Effect	Mean sqr Error	F	p-level
The analysis of variance for grain yield					
Year, (Y)	2, 117	23.437	2.708	8.656**	0.000
Fertilization, (F)	7, 112	38.949	0.813	47.931	0.000
Year x Fertilization, (YxF)	14, 96	0.7955	0.344	2.314**	0.009
The analysis of variance for 1000-grain weight					
Year, (Y)	2, 117	0.715	3.344	0.214	0.808
Fertilization, (F)	7, 112	7.213	3.056	2.360*	0.027
Year x Fertilization, (YxF)	14, 96	16.33799	1.167429	13.99485	0.000
The analysis of variance for test weight					
Year, (Y)	2, 117	279.970	4.938	56.698**	0.000
Fertilization, (F)	7, 112	11.567	9.435	1.226	0.294
Year x Fertilization, (YxF)	14, 96	2.659	4.787	0.555	0.893

ns non significant; * significant at 0.05; ** significant at 0.01;

Data in Table 2 for the investigated period (2009-2011) clearly indicate that the was found highly significant effect of year on the grain yield (F=8.656**) and test weight (F=56.698**). Furthermore, 1000-grain weight was significant among the fertilization. The use of different treatments induced a significant increase in 1000-grain weight. Furthermore, test weight was highly significant among the year x fertilization interaction. Considerable variation in yield depending on years of research has been established by Đekić et al. (2014; 2017a), Jelic et al. (2015) and Terzić et al. (2018a). The present results confirm

the opinion of many authors that the traits analyzed are genetically determined but are strongly modified by the nutrient status of the environment and weather conditions (Đekić et al. 2014, 2017a; Jelic et al., 2014, 2015; Milivojević et al., 2018; Popovic et. al., 2017; Stevanović et al., 2018; Ugrenović et al., 2018; Terzić et al., 2018a). Đekić et al. (2014) and Terzić et al. (2018a) find that the application of mineral fertilizers has a significant impact on the weight of 1000 grains, i.e. the grain weight is significantly higher in more intensively fertilized variants especially fertilized with nitrogen.

Table 3 presents average values for gran yield, 1000-grain weight and test weight across years and treatments during the study. The tests showed a significant grain yield and test weight. The highest values of grain yield were established in the year with moderate temperatures and high precipitation in the vegetation year 2008/09. The highest average grain yield of 4.611 t/ha of studied wheat treatments was recorded in vegetation season 2008/09 and it was significantly higher than the yield in 2009/10 (3.080 t/ha) and the yield in 2010/11 (3.827 t/ha). The 1000-grain weight of winter wheat significantly varied across treatments, from 38.25 g in treatment control to 40.00 g in treatment NPK, in a quantity of 120 kg N/ha, 100 kg/ha P_2O_5 and 60 kg/ha K_2O. The test weight in year 2010/11 (78.10 kg/hl) was significantly higher compared to 2008/09 and 2009/10 (76.28 kg/hl and 72.88 kg/hl). The highest grain yield had wheat application of NPK in a quantity of 120 kg/ha N, 100 kg/ha P_2O_5 and 60 kg/ha K_2O (5.528 t/ha).

Table 3. Mean values for grain yield, 1000-grain weight and test weight

Years	Grain yield (t/ha)		1000-grain weight (g)		Test weight (kg/hl)	
	$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S
2008/09	4.611^{a*}	1.673	39.12^{a}	2.259	76.28^{b}	2.531
2009/10	3.080^{c}	1.435	39.30^{a}	0.851	72.88^{c}	2.312
2010/11	3.827^{b}	1.807	39.05^{a}	2.051	78.10^{a}	1.750
Treatments						
C	1.292^{d}	0.339	38.25^{c}	2.411	76.91^{a}	3.046
N_{120}	3.750^{b}	0.777	38.29^{c}	1.448	75.42^{ab}	2.804
P_{60}	2.281^{c}	0.865	39.75^{ab}	1.831	76.00^{ab}	3.128
P_{100}	2.574^{c}	0.858	39.64^{abc}	2.127	76.82^{ab}	3.017
$N_{120}P_{60}$	4.932^{a}	1.006	38.55^{bc}	1.746	75.01^{ab}	2.966
$N_{120}P_{100}$	5.101^{a}	0.983	39.39^{abc}	1.574	74.31^{b}	3.729
$N_{120}P_{60}K_{60}$	5.258^{a}	1.155	39.39^{abc}	1.165	76.01^{ab}	3.392
$N_{120}P_{100}K_{60}$	5.528^{a}	0.991	40.00^{a}	1.332	75.54^{ab}	2.291

*Means within columns followed by different lowercase letters are significantly different (P<0.05) according to the LSD test.

Table 4 presents average values for grain yield, 1000-grain weight and test weight across years and treatments during the three vegetation seasons. During the first years (2008/09) grain yield significantly varied across treatments, from

1.671 t/ha in control to 6.305 t/ha in treatment $N_{120}P_{100}K_{60}$. During the second years (2009/10) grain yield significantly varied across treatments, from 0.951 t/ha in control to 4.438 t/ha in treatment $N_{120}P_{60}K_{60}$. During the 2010/11 years grain yield significantly varied across treatments, from 1.254 t/ha in control to 5.915 t/ha in treatment $N_{120}P_{100}K_{60}$.

Table 4. Mean values of yield and quality of fertilization and vegetation seasons

	Grain yield (t/ha)		1000-grain weight (g)		Test weight (kg/hl)	
2008/09	$\bar{X}$	S	$\bar{X}$	S	$\bar{X}$	S
C	1.671^{d*}	0.190	40.50^{ab}	0.962	77.44^{a}	2.942
N_{120}	4.410^{b}	0.583	37.03^{de}	1.507	75.82^{a}	2.257
P_{60}	3.341^{c}	0.463	41.62^{a}	0.691	75.83^{a}	4.029
P_{100}	3.185^{c}	0.216	42.00^{a}	0.418	77.91^{a}	1.873
$N_{120}P_{60}$	5.854^{a}	0.138	36.42^{e}	1.244	76.61^{a}	1.081
$N_{120}P_{100}$	5.971^{a}	0.280	37.86^{cde}	1.835	74.93^{a}	2.086
$N_{120}P_{60}K_{60}$	6.149^{a}	0.355	38.44^{cd}	0.573	75.42^{a}	2.866
$N_{120}P_{100}K_{60}$	6.305^{a}	0.460	39.07^{bc}	1.718	76.26^{a}	2.522
2009/10						
C	0.951^{e}	0.172	39.04^{bc}	0.639	74.01^{a}	1.552
N_{120}	2.936^{bc}	0.524	39.46^{ab}	0.555	72.37^{a}	1.180
P_{60}	1.606^{de}	0.284	39.12^{bc}	1.250	73.69^{a}	1.539
P_{100}	2.542^{cd}	1.228	38.22^{c}	0.415	73.57^{a}	2.748
$N_{120}P_{60}$	3.750^{ab}	0.641	39.16^{bc}	0.230	71.81^{a}	3.144
$N_{120}P_{100}$	4.054^{a}	0.602	39.96^{ab}	0.297	70.88^{a}	3.007
$N_{120}P_{60}K_{60}$	4.438^{a}	1.429	39.22^{b}	0.965	73.33^{a}	2.763
$N_{120}P_{100}K_{60}$	4.363^{a}	0.693	40.26^{a}	0.451	73.41^{a}	1.220
2010/11						
C	1.254^{e}	0.103	35.21^{d}	0.616	79.28^{a}	1.879
N_{120}	3.902^{c}	0.312	38.38^{c}	1.029	78.07^{abc}	0.656
P_{60}	1.896^{de}	0.409	38.51^{c}	1.702	78.48^{abc}	1.118
P_{100}	1.994^{d}	0.363	38.71^{bc}	2.216	78.98^{ab}	0.521
$N_{120}P_{60}$	5.192^{b}	0.469	40.02^{abc}	0.468	76.61^{c}	0.727
$N_{120}P_{100}$	5.277^{ab}	0.767	40.35^{ab}	0.858	77.12^{abc}	3.195
$N_{120}P_{60}K_{60}$	5.187^{b}	0.816	40.52^{a}	0.847	79.29^{a}	1.249
$N_{120}P_{100}K_{60}$	5.915^{a}	0.332	40.67^{a}	1.154	76.94^{bc}	1.323

*Means within columns followed by different lowercase letters are significantly different ($P<0.05$) according to the LSD test.

The 1000-grain weight of winter wheat significantly varied across years and treatments. During the 2008/09 years 1000-grain weight significantly varied across treatments from 36.42 g in treatment $N_{120}P_{60}$ to 40.50 g in control. During the 2009/10 years 1000-grain weight significantly varied across treatments from 38.22 g in treatment P_{60} to 40.26 g in $N_{120}P_{100}K_{60}$. During the 2010/11 years

1000-grain weight significantly varied across treatments from 35.21 g in control to 40.67 g in $N_{120}P_{100}K_{60}$.

The test weight of winter wheat significantly varied across years and treatments. Averaged across 2008/09 years, significantly higher values for test weight were found in the treatment P_{60} (77.91 kg/hl), in 2009/10 in the control (74.01 kg/hl) and in 2010/11 in the control and treatment $N_{120}P_{60}K_{60}$ (79.28 kg/hl and 79.29 kg/hl).

Table 5. Correlations between the traits analyzed by three vegetation seasons

Traits	Grain yield	1000-grain weight	Test weight
Correlations between the traits analyzed in 2008/2009			
Grain yield (t ha^{-1})	1.00	-0.562^{**}	-0.242^{ns}
1000-grain weight (g)		1.00	0.113^{ns}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in 2009/2010			
Grain yield (t ha^{-1})	1.00	0.450^{*}	-0.050^{ns}
1000-grain weight (g)		1.00	0.045^{ns}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in 2010/2011			
Grain yield (t ha^{-1})	1.00	0.711^{**}	-0.443^{*}
1000-grain weight (g)		1.00	-0.284^{ns}
Test weight (kg hl^{-1})			1.00

Table 5 presents the correlation coefficients between the years and examined traits. Test weight in 2008/09 was positively correlated with 1000-grain weight (r=0.113) and negatively but dependent significantly correlated with grain yield and 1000-grain weight ($r=-0.562^{**}$). Wheat yield in 2009/10 was positively and significantly correlated with 1000-grain weight ($r=0.450^{*}$). The correlative dependence of the grain yield in the 2010/11 vegetation season in was positively and highly significant correlation with the 1000-grain weight ($r=0.711^{**}$). Grain yield depends directly on the number of grains per spike and the 1000-grain weight (Hristov et al., 2011; Đekić et al., 2014, 2017a; Terzić et al., 2018a).

Table 6 presents the correlation coefficients between the treatments and examined traits. The correlative dependence of the grain yield in the treatment P_1 in was positively and highly significant correlation with the 1000-grain weight ($r=0.806^{**}$). Positive correlations were observed between grain yield and test weight in all treatments. Positively and medium significantly correlations were also found between grain yield and test weight in the N ($r=0.533^{*}$) and positively but strong significantly correlated were also found in the NP_1 ($r=0.846^{**}$) treatments. Negative correlation between 1000-grain weight and test weight has been established (Đekić et al., 2017c; Terzić et al., 2018a).

Table 6. Correlation coefficients for the traits analyzed across treatments

	Grain yield	1000-grain weight	Test weight
Correlations between the traits analyzed in the unfertilized control			
Grain yield (t ha^{-1})	1.00	0.343^{ns}	0.450^{ns}
1000-grain weight (g)		1.00	-0.344^{ns}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in the N			
Grain yield (t ha^{-1})	1.00	-0.463^{ns}	0.533^{*}
1000-grain weight (g)		1.00	-0.409^{ns}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in the P_1			
Grain yield (t ha^{-1})	1.00	0.806^{**}	0.169^{ns}
1000-grain weight (g)		1.00	-0.158^{ns}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in the P_2			
Grain yield (t ha^{-1})	1.00	0.501^{ns}	0.094^{ns}
1000-grain weight (g)		1.00	0.300^{ns}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in the NP_1			
Grain yield (t ha^{-1})	1.00	-0.481^{ns}	0.846^{**}
1000-grain weight (g)		1.00	-0.131^{ns}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in the NP_2			
Grain yield (t ha^{-1})	1.00	-0.458^{ns}	0.364^{ns}
1000-grain weight (g)		1.00	-0.111^{ns}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in the NP_1K			
Grain yield (tha^{-1})	1.00	-0.072^{ns}	0.167^{ns}
1000-grain weight (g)		1.00	0.675^{*}
Test weight (kg hl^{-1})			1.00
Correlations between the traits analyzed in the NP_2K			
Grain yield (t ha^{-1})	1.00	-0.094^{ns}	0.461^{ns}
1000-grain weight (g)		1.00	-0.288^{ns}
Test weight (kg hl^{-1})			1.00

^{ns}non significant; *significant at 0.05; **significant at 0.01;

CONCLUSIONS

Effects of mineral nutrition efficiency of wheat have been studied at the stationary field trial of the Institute of Small Grains Research Centre in Kragujevac (Serbia) for three years (2008/09, 2009/10 and 2010/11). Nitrogen had a most significant impact on the yield of wheat. The average grain yield of all treatments in the 2008/09 growing season was significant greater than in the following years. The highest average yields were gained by $N_{120}P_{100}K_{60}$ treatment (5.528 t/ha), $N_{120}P_{60}K_{60}$ treatment (5.258 t/ha) and $N_{120}P_{100}$ treatment

(5.101 t/ha) in three-year period. The analysis of variance indicated highly significant effects of year on the grain yield and test weight. Effects of fertilization on the 1000-grain weight were statistically significant. Interaction between the analysed factors (year x fertilization) shows a highly significant effect on 1000-grain weight in wheat. The average grain yield was significantly higher in 2008/09, while in 2010/11 of test weight was significantly higher.

In 2008/09 a negatively and medium-dependent significant correlation was found between grain yield and 1000-grain weight (-0.562**) as well as a significant positive correlation in 2009/10 (0.450*), while these positive and strong correlations in 2010/11 were highly significant (0.711**).

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BODY GROWTH, DEVELOPMENT AND NUTRITIONAL STATUES OF PUBERTY CHILDREN IN URBAN AND RURAL AREAS OF PODGORICA AND BERANE IN MONTENEGRO

SUMMARY

The puberty represents one of the most critical children and adolescents growth and development period, when considerable differences between age and sex may be observed. The aim of this paper was to determine body height, body mass, menarche and nutritional status in children aged 13 and 14 in Podgorica (urban area) and Berane (the rural area around Berane). This overview study, according to International Biology Program (IBP) instructions, was performed in 2018 in elementary schools in Podgorica and Berane, and includes children aged 13 and 14. In Podgorica, 216 students underwent analysis, while 214 of them underwent analysis in Berane. Boys from Podgorica had a slightly higher average body height, body weight and body mass index (BMI) in examined years compared to boys from Berane, but there was no statistical significance. Girls aged 14 years from Podgorica are slightly higher average body height and BMI and statistically significant ($p < 0.05$) higher mean body weight compared to girls from Berane. An analysis of the nutritional status of children aged 13 and 14 in Podgorica and Berane shows that the highest percentage of boys (68.5% from Podgorica and 68.4% from Berane) and girls (75.7% from Podgorica and 74% from Berane) are in the category of normal weight. Girls in Podgorica have a menarche with 12.15 ± 0.84 and girls in Berane with 12.51 ± 0.68 years, which is a statistically significant difference ($p=0.03$). The girls in Podgorica have been puberty earlier than their peers in Berane. Higher obesity rate in girls in urban areas, leads to a conclusion that the attention should be particularly directed towards the diet and origin of food, especially in child development period.

Keywords: *body height, body weight, BMI, nutritive status, menarche.*

INTRODUCTION

Growth and development represent the basic parameters of children age, that develop according to genetically determined rules and under the influence of the environment. The processes are mutually conditioned, and defined by the

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changes in body height, weight, proportions and body form, as well as physiological functions. The most critical period during these processes is definitely the puberty, when most significant changes in growth and development occur, as well as the most significant differences in sexual maturity. The puberty is connected to the increased growth, fat weight and obesity. The most reliable indicator of the puberty in girls is the menarche. Monitoring growth and development of children and adolescents is based on the evaluation of morphological parameters that enable the comparison of populations living in different external conditions, as well as different periods. Since parameters are changeable both in terms of time and space, it is significant to ensure their constant evaluation. Body height is one of the significant indicators of growth and nutritional status at children. Another, but not less important anthropometrical parameter is body weight, especially its relation to the age. Deviations in this relation indicate disorder in nutritional condition. Body weight is a basic parameter of the level and pace of bodily development, however it is considered a so – called dynamic – changeable dimension, being subject to influence of the environment and may demonstrate huge variations, even within a day (Vujmilović, 2012). Even though the body weight is closely connected to physical and motor development, significantly more stable indicator of growth and development is body height (Podstawski and Borysawski, 2012). It is evident that the development of standard as well as irregular nutrition cause greater occurrence of obesity – modern day epidemic. Obese children and adolescents have greater chances of being obese as adults (Wang and Lobstein, 2006). Often the literature connects increased body weight with reduced motor skills connected to physical activities (Delaš *et al.* 2008). Children who are obese or have increased body weight are more prone to reduced physical activity, or this reduced physical activity is the cause of increased body weight (Planišec and Matejek, 2004). Therefore, anthropometric parameters are also significant in evaluating motor abilities with children (Ceylan *et al*. 2014).

In 2014, World Health Organization (WHO) adopted a Global Strategy on Diet, Physical Activity and Health, with its primary aim of enhancing the health with proper diet and physical activity. Having in mind a modern lifestyle, with inadequate nutrition and little physical activity, along with genetics, a lot attention has been recently paid to the increase of BMI, hence this body index has been included in all the research related to evaluation of children and adolescents' development. Nowadays, obesity is considered to occur as a consequence of several factors – genetic traits, phycological, sociological (Tatar, 2014).

Different locations were not chosen randomly, but with an intention to determine potential differences in the level of physical development based on morphological characteristics of the examinees, having regard to differences in geographical, climate – wise, nutritional, socio – economic terms, and many more. There are numerous studies concerned with ecological and other characteristics of specific regions of Montenegro. Numerous researchers have

done studies on organic food production, autochthonous species and other characteristics of rural, northern region of Montenegro (Mirecki, 2011; Tesovic *et al*. 2012; Bozovic and Jacimovic, 2012; Sebek, 2013). All of these studies, might help understand potential developmental and morphological differences of the children in urban compared to rural areas.

The aim of this paper was to determine body height, body mass, menarche and nutritional status in children aged 13 and 14 in urban and rural areas in Montenegro.

MATERIAL AND METHODS

A sample of this cross – sectional study consist of students aged 13 and 14, with 216 (105 boys and 111 girls) examined in Podgorica, and 214 students (114 boys and 100 girls) examined in Berane. The survey was conducted in September 2016 by means of a random test sample, covering elementary schools students in Podgorica, and elementary schools students from the rural area around Berane.

Podgorica is an administrative center, the capital and the biggest town located in southern part of the Republic, with 44,5m altitude. It has Mediterranean climate with a considerable influence of the adjacent sea, with extremely hot summers where temperatures during July and August exceed 40 degrees Celsius. Most recent census recorded around 250 000 population. On the other hand, Berane is a town located on the north – east of Montenegro, with 703 m altitude, having moderate continental climate with strong impact of mountain climate. Average annual temperature of Berane is 9, 4C°. 2003 Census recorded population of 11 776. Municipality of Berane accounts for one of the poorest municipalities in Montenegro. Different locations were not chosen randomly, but with an intention to determine potential differences in the level of physical development based on morphological characteristics of the examinees, having regard to differences in geographical, climate – wise, socio – economic terms, and many more.

Decimal ages on the date of the survey and date of birth have been calculated for each examinee. Data for the menarche have been taken via status quo method, while the age of the occurrence of menarche with girls has been calculated from the date of birth and the date of the occurrence of menarche. Standard anthropological instruments have been used to calculate body height and body weight. Body height has been calculated by means of an anthropometer with the precision of 0.1 cm. Body weight has been calculated by means of a digital scale with the precision of 0.1 kg. In order to evaluate the nutritional status, BMI has been calculated as a ratio between body weight in kilograms and body height in square meters for age and sex. Percentile values for BMI have also been calculated, based on which the evaluation of the nutritional condition in children was performed, as recommended by NHANES I (Must et al. 1991), according to which: BMI <P5 are under malnourished: from P5 to P15 are

moderately malnourished; from P15 to P85 normally nourished; P85 to P95 - moderately obese (overweight), while obese are those with >P95.

Difference between average values (body height, body weight and BMI) of the students from surveyed locations, as well as sexual differences have been tested by independent t-test.

This survey has been conducted in line with Helsinki Declaration with the consent of all parents, school principals and the Dean of the Faculty of Science and Mathematics. This survey was approved in advance by the College of Natural Sciences and Mathematics and all the directors of elementary schools in Podgorica and Berane. Each participant voluntarily provided written informed consent before participating.

RESULTS

Average values of height, weight and BMI of boys from Podgorica and Berane and total sample by age are shown in Table 1.

Table 1. Average values of height, weight and BMI of boys from Podgorica and Berane

	Age (years)	Podgorica			Berane			Total			p value‡
		N	Mean	SD	N	Mean	SD	N	Mean	SD	
Height (cm)	13	52	170.86	8.02	55	168.49	8.90	107	169.68	8.46	0.32
	14	53	173.36	8.09	59	171.17	9.53	112	172.27	8.81	0.39
Weight (kg)	13	52	62.32	11.71	55	57.51	10.5	107	59.92	11.11	0.12
	14	53	63.59	11.66	59	60.52	13.04	112	62.06	12.35	0.39
BMI (kg/m^2)	13	52	21.26	3.29	55	20.19	3.13	107	20.73	3.21	0.23
	14	53	21.14	3.50	59	20.52	3.41	112	20.83	3.46	0.53

‡ t-test independent ; N-number; SD-standard deviation; BMI-body mass index

The height of the boy's increased with an average of 169.68 ± 8.46 to 172.27 ± 8.81 cm and was higher in both boys in Podgorica. Body weight also increased with age and amounted to 59.92 ± 11.11 kg in 13 years and 62.06 ± 12.35 kg in 14 years. Boys from Podgorica are larger body masses in both examined years than boys from Berane. Average BMI values are similar in both examined years (20.73 ± 3.21 kg / m2 in 13 years and 20.83 ± 3.46 kg / m2 in 14 years) and are higher in boyfriends from Podgorica (Table 1).

Average values of height, weight and BMI of girls from Podgorica and Berane and total sample by age are shown in Table 2.

In girls in the overall sample, the height of the body is approximately the same in the examined ages (165.16 ± 6.31 cm in 13 years and 165.62 ± 6.86 cm in 14 years), while the average body weight values (58.55 ± 9.68 kg in 13 years and 55.01 ± 8.46 kg in 14 years) and BMI (21.45 ± 3.27 kg / m2 in 13 years and 20.03 ± 2.63 kg / m2 in 14 years) decreased. Girls aged 13 years from Berane had higher average body height, body weight and BMI.

Table 2. Average values of height, weight and BMI of girls from Podgorica and Berane

	Age (years)	Podgorica			Berane			Total			
		N	Mean	SD	N	Mean	SD	N	Mean	SD	p value‡
Height (cm)	13	55	164.36	5.53	49	165.95	7.08	104	165.16	6.31	0.34
	14	59	166.65	7.68	51	164.58	6.03	107	165.62	6.86	0.33
Weight (kg)	13	55	56.79	8.00	49	60.31	11.35	104	58.55	9.68	0.17
	14	59	57.94	8.40	51	52.08	8.52	107	55.01	8.46	0.03*
BMI (kg/m^2)	13	55	20.98	2.67	49	21.91	3.87	104	21.45	3.27	0.28
	14	59	20.82	2.49	51	19.24	2.77	107	20.03	2.63	0.06

‡ t-test independent ; N-number; SD-standard deviation; BMI-body mass index; *p<0.05

Girls aged 14 years from Podgorica are slightly higher average body height and BMI and statistically significant ($p < 0.05$) higher mean body weight compared to girls from Berane (Table 2).

The results presented in Tables 1. and Tables 2., which relate to gender differences, showed that boys from Podgorica in both examined years were of higher values of all tested parameters compared to girls, while girls from Berane age 13 had a higher body mass and BMI in relation to boys.

The results of the t-test of independence showed statistically significantly higher body height and body mass in 13-year-old boys from Podgorica ($p < 0.05$) and 14-year-old boys from Berane ($p < 0.01$) compared to girls. There were no significant gender differences in BMI.

Table 3. presents the nutritive status of boys and girls from Podgorica and Berane.

Table 3. Nutritive status of boys and girls from Podgorica and Berane % (N)

	Podgorica		Berane	
	Boys (105)	Girls (111)	Boys (114)	Girls (100)
Underweight	4.8% (5)	3.6%(4)	7%(8)	8%(8)
Moderate underweight	12.4%(13)	6.3%(7)	14.9%(17)	9%(9)
Normal	68.5% (72)	75.7%(84)	68.4%(78)	74%(74)
Overweight	9.5%(10)	10.8%(12)	5.3%(6)	6%(6)
Obesity	4.8%(5)	3.6%(4)	4.3%(5)	5%(5)

An analysis of the nutritional status of children aged 13 and 14 in Podgorica and Berane shows that the highest percentage of boys (68.5% from Podgorica and 68.4% from Berane) and girls (75.7% from Podgorica and 74% from Berane) are in the category of normal weight. A higher percentage of boys (9.5%) and girls (10.8%) in Podgorica had an overweight compared to boys (5.3%) and girls (6%) in Berane. A slightly higher percentage of obese boys

(4.8% compared to 4.3%) was observed in Podgorica, and girls (5% compared to 3.6%) in Berane. Fewer and moderately malnourished boys and girls were more in Berane than in Podgorica (Table 3).

Present menarche mean of girls from Podgorica and Berane were 12.15 ± 0.84 and 12.51 ± 0.68 years, respectively, p = 0.03, so it this parameter was significantly higher in group from Berane. Menarche medians for female subjects aged 13 and 14 in Podgorica and Berane were 12.00 ± 0.12 and 13.00 ± 0.10 years respectively (Table 4).

Table 4. Menarche of girls from Podgorica and Berane (N=180)

	Podgorica (N=93)	Berane (N=87)	p value‡
Mean ± SD (years)	12.15 ± 0.84	12.51 ± 0.68	0.03*
Median (years)	12.00	13.00	
Standard error (years)	0.12	0.10	

‡ t-test independent, SD- standard deviation; *p<0.05

DISCUSION

Mean values of height, body weight and BMI in boys from Podgorica are higher if compared in same parameters with peers in Berane, however statistically significant differences have not been proven. At girls, only in the age of 14 in relation to body weight the values are slightly higher in Podgorica, with significance (166.65±7.68 cm compared to 164.58±6.03 cm, p=0.33). Similar ratio of parameters was achieved in the analysis related to comparison of puberty population in Pljevlja and Podgorica (Scepanovic, 2001), for both sexes.

Results of the survey performed recently in Nikšić within fourteen – year old children compared to their peers in European countries, indicated that examinees in Montenegro have higher values in all three parameters when compared to Belgium, Spain, Slovakia, Lithuania, Estonia and Albania (Radulovic and Krivokapic, 2013). Mean values of the examined parameters with boys in Podgorica and Berane are higher than the same ones in Nikšić, which is not the case with girls from Berane. Results achieved indicate the need for further research of the children living in different regions, and for the research of the impact the environment, along with all its characteristics, has on children growth and development.

The analysis of the results achieved through anthropometrical measurements indicates that boys from Podgorica and Berane, in terms of weight and body height, as well as BMI, go ahead of the girls in both regions, which is expected for this age. Based on different research (Ivanović, 1996; Rakic, 2009; Bozic-Krstic *et al*. 2004), it has been known that boys of 13 and 14 years exceed girls in terms of height, as well as other parameters, which has been confirmed in these surveys as well.

Additionally, based on BMI values and the level of nutrition it can be concluded that majority of children are normally nourished. Overweight has been

detected in 9.5% boys and 10.8% girls in Podgorica, slightly higher than the case with Berane (5.3% boys and 6% girls). On the other hand, there are more malnourished boys and girls in Berane. Comparing the total obesity of surveyed population (14.30% boys and 14.40% girls in Podgorica, and 9.6% boys and 11% girls in Berane) with the surveys in the neighborhood (Mirilov and Mirosavljev, 2004) it may be stated that children in Berane feature less percentage of total obesity, while Podgorica population almost fits the recent research in Novi Sad, where the frequency of complete obesity according to BMI is 14.20% in boys and 12.60% in girls. If the fact that in recent years the tendency for increased obesity and overweight in Europe for ages 13 to 17 reached 15% both in boys and girls (Lobstein, et al. 2004), it is clear that such a trend is being present in our population, with a somewhat more favorable situation in Berane. The results definitely indicate an increased level of obese children in developed and bigger cities, which may be connected to earlier puberty. Results of numerous research also showed that the time of menarche is considerably connected to obesity (Salces et al. 2001; Laska-Mierzejewska, 1996; Rao et al. 1998, Scepanovic, 2013; Rakic, 2009).

Fat mass is being increased especially in girls, since body fat represents a significant energy deposit, which is need for normal function of female gonads (Phillips et al. 2003).

Also, postmenarchal girls are characterized by an increased fat weight (Rakic et al. 2013). That can explain the results obtained in this paper, where girls who are more obese from Podgorica get their menarche prior to the girls from Berane. The results show that Podgorica girls get their menarche at the age of 12.15 ± 0.84, with medians 12.00 ± 0.12 and in Berane 12.51 ± 0.68, with medians 13.00 ± 0.10, which has proven as statistically considerable difference. Monitoring of sexual development of primary – school girls in the region showed that the menarche in Vršac and other bigger towns in Vojvodina is obtained with 12.51 age on average, and medians is 12.50 age (Rakic, 2009), which is approximate to the values obtained within this paper. On the other hand, girls from Niš are characterized by later maturity, because the data say medians is 13.66 age (Cvetković *et al*. 2012).

However, recent studies in different European countries show different results. Therefore, girls from Podgorica mature earlier than girls from Turkey (Ekerbicer et al. 2007) and the Netherlands (Mul et al. 2001), and insignificantly later than girls from Greece (Papafimitriou *et al*. 2008) and Portugal (Padez and Rocha, 2003).

It is believed that better life conditions cause a better starting point for sexual development of girls. Parameters taken in consideration are nutrition, genes, but mother's education as well. Numerous studies deal with the issue of puberty onset in relation to life and environment conditions (Cole, 2000; 2003, Cukic; Hurbo 2008; Pavlica *et al*. 2012), which supports the statement that there are many different factors that condition growth and development of puberty population in different manners.

CONCLUSIONS

Compared populations of children aged 13 and 14 in urban and rural areas do not statistically differ in terms of body height, body weight and BMI, except in girls from Podgorica aged 14 in terms of body weight when compared to their peers in Berane. Additionally, girls in Podgorica mature in sexual terms earlier.

Results obtain indicate the need for further research of the children living in different regions, in order to achieve better insight into the changes of morphological parameters, especially indicators of sexual development.

Higher obesity rate in girls in urban areas, leads to a conclusion that the attention should be particularly directed towards the diet and origin of food, especially in child development period.

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INVESTIGATING THE HYDROLOGICAL RESPONSE AND NUTRIENT LOSS IN RAINFED LANDS IN NORTHEAST OF IRAN USING RAINFALL SIMULATOR

SUMMARY

Topsoil is the richest part in terms of the amount of organic matter and nutrients. Therefore, its lost is more harmful compared to the subsoil. During the erosion process, soil nutrients are either lost as dissolved or absorbed to the soil particles. The present study was aimed to investigate the effects of rainfall intensity and slope gradient on infiltration, runoff, soil and nutrients (N, P, K) loss in rainfed lands of Pishkamar region, NE of Iran. To achieve the study purposes, four rainfall intensities of 33, 64, 80 and 110 mm h^{-1} were simulated using Kamphorst rainfall simulator in three slope gradients of 6, 12 and 25%. Two slope aspects of north and south were also considered for the slope gradient of 25%.

The results showed that the effects of rainfall intensity and slope gradient on infiltration and runoff were significant, while the effect of slope aspect (north and south) was not. Taking into account the linear trend, the plot hydrological response threshold of sediment concentration was about 50 mm h^{-1} of rainfall intensity and 9% of slope. In the case of total soil loss, however, higher thresholds were obtained for rainfall intensities (about 72 mm h^{-1}) and slope gradients (about 18%). The effect of rainfall intensity and slope gradient on soil loss were significant individually, but no interaction was observed among study factors. The results of nutrient losses showed considerable loss for Potassium in the runoff and Phosphorus and Potassium in the sediment.

Keywords: Average infiltration rate, Average runoff coefficient, Soil and sediment granulometry, Soil erosion, Soil fertility

INTRODUCTION

Soil erosion is one of the environmental treats to the ecosystem that leads to soil and water quality degradation and washes away the fertile topsoil and reduces agricultural production (Chalise et al., 2019). The natural and accelerated

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soil erosion is one of the major hydrological hazards in Iran (Sadeghi et al., 2013). Soil erosion depends on many parameters such as soil and topographic condition (e.g. antecedent soil moisture content, slope gradient and aspect) and rainfall characteristics (e.g. rainfall intensity and duration) (Arnaez, 2007; Assouline and Ben-Hur, 2006; El Kateb, 2013; Huang 2013). Study the factors that affect soil erosion is essential for soil and water conservation planning (Khaledi Darvishan et al., 2017; Spalevic et al., 2015, 2017a, 2017b). Many researches have been investigated the different aspects of soil erosion and some of them have been conducted to study the effect of rainfall intensity and/or slope gradient in different land uses on soil erosion. Some studies have been also investigated the effects of surface seal induced variations on infiltration, runoff and erosion processes (Bradford et al., 1987; Mualem et al., 1993; Foley and Silburn 2002; Assouline, 2004; Liu, 2015; Khaledi Darvishan et al, 2015).

Few studies have been investigated soil erosion in different rainfall intensity and slope gradients in rainfed lands. Rainfed lands accounts for about 82% of the world's total cropland (FAOSTAT 2005). Rainfed lands usually carry out in semiarid condition which no irrigation water is available. The area of rainfed lands in Iran is about 10 million hectares (Siadat 1998) located in the areas which its ecosystem is very sensitive to weather uncertainties and climate change. Vegetation cover change leads to the changes of hydrologic response, soil erosion and sediment dynamics characteristics (Kavian et al., 2018; Nikolic et al., 2018). The effect of vegetation cover change is more important in semi-arid regions because of the sensitivity of vegetation cover and relative higher intensity and shorter duration of rainfalls in these areas.

One of the main consequences of soil erosion is the loss of soil fertility and the depletion of nutrients such as Nitrogen, Phosphorus and Potassium (Kauffman et al., 1993; Miller, 1995). These three elements are the most important elements of plant nutrition (Hanson, 1967).

The topsoil usually contains more organic matter, which improves infiltration and increases water holding capacity (Pimental et al., 2004). It is the first part of soil that exposed to erosion. The most effective factors in soil permeability are organic materials that play an important role in soil water retaining and soil permeability. Splash erosion which depends on rain drop size distribution and velocity, is an important process (Khaledi Darvishan et al., 2014) and can significantly affect the soil surface. In general, the most sensitive time to erosion in arable land is the periods in which the plant does not exist and when the rainfalls are erosive (Walter and Dwight, 1978). Therefore, the rainfed lands in the plowing stage before cultivating until the plant is still insufficient to protect soil surface (spring and summer) are in the most sensitive condition. The rainfalls in these two seasons have also high intensity and short duration, so that the highest amount of soil and nutrient loss can occur.

Many of previous studies have been carried out to determine the effects of slope gradient and rainfall intensity on hydrological response variables such as infiltration and runoff (Peoson 1984, Romkens et al 2002, Grander and Gerard

2003, Marquez et al 2005, Sadeghi et al 2013, Huang et. al, 2013, El Kateb et. al, 2013, Khaledi Darvishan 2015, Liu et al 2015) and sediment concentration and soil loss (Fox and Brayan 1999, Poulenard et. al 2001, Grander and Gerard 2003, Assouline et.al 2006, Arnaez et. al 2007, Romkens et. al 2002, Elkateb et. al 2013, Zhao et.al 2015, Liu et. al 2015). The results of previous studies generally showed that infiltration and runoff were significantly affected by slopes and rainfall intensities. Because of the importance of rainfed lands especially in the northeast of Iran, despite the results of previous researches, it is still very important to quantify the effects of slope gradient and rainfall intensity on hydrological response as well as soil nutrient loss in these lands.

In rainfed lands located in Golestan province, heavy rains usually occur in summer with consequent high flood events. Therefore, it's very important to study soil erosion and its parameters in rainfed lands of Iran. The present study has been conducted to know the individual and interaction effects of rainfall intensity and slope gradient on soil and nutrient losses in rainfed lands in northeast of Iran.

The results of the present study can be used to distinguish the critical of slope gradient and rainfall intensity in case of their effects on hydrological response in rainfed lands.

MATERIALS AND METHODS

Study area

The field experiments were conducted using a small simulator (Kamphorst, 1978) in Pishkamar (37º 31´ N- 55º 35´ E) which is located in Golestan province, NE Iran (Figure 1). Pishkamar has an average elevation around 1000 m above sea level with mean annual precipitation of 592.8 mm with maximum and minimum monthly amounts in January (80.5 mm) and July (28.4 mm), respectively. The maximum and minimum seasonal precipitation is commonly occurring in winter and summer, respectively. The major soil of Pishkamar is silty-loam (26% Clay, 66% silt and 8% sand) with 44% porosity. Most of the slopes are in the range between 6 and 25% gradient. The major land uses in the study area are rangeland, forest, rainfed and irrigated lands.

Layout of experiments

The studied soil covered by erosion control blanket to protect soil from direct hitting of rain drops and splash erosion occurring and sprinkled 5 liter of water for each 0.5×0.5 m using a handy sprinkler to soaking soil until 5 cm depth after 24 h, in order to achieve the same antecedent soil moisture content equal to field capacity in all the study plots. Three slope gradients of 6, 12 and 25% and four rainfall intensities of 30, 60, 80 and 120 mm h^{-1} were applied. The rainfall duration was 15 min for all experiments because of limited volume of water reservoir of rainfall simulator. In the present study the Intensity-Duration-Frequency curves of the nearest synoptic weather station of Kalaleh (shown in Figure 1 as a red triangle) were used to determine the relationship between rainfall

intensity, duration and return period in the study area. In this regard the return period of 15-20 years for the given rainfall intensities was considered.

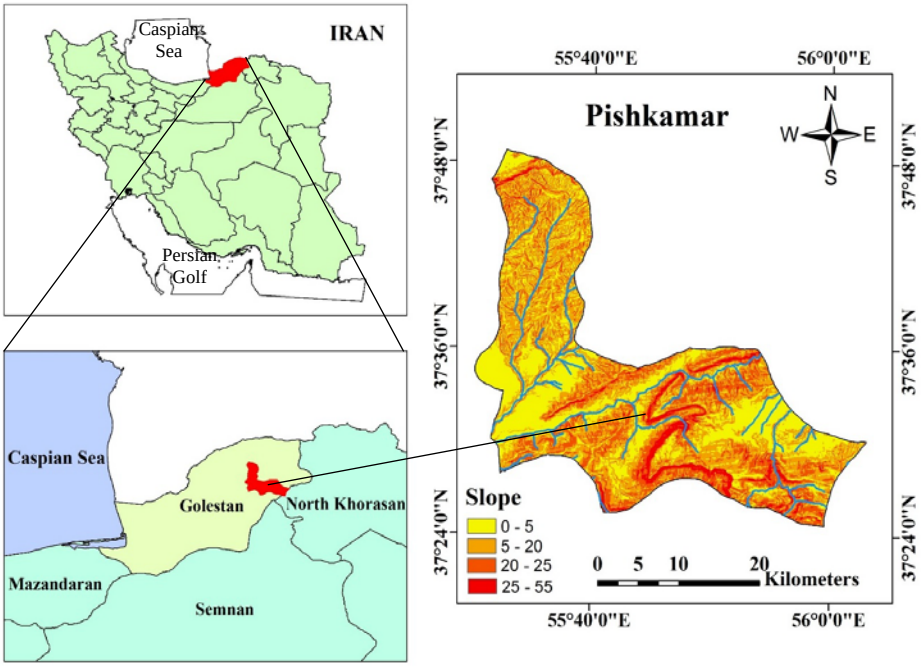

Figure 1. Location and general landscape of the study area

The selected duration of 15 min was corresponding with the high rainfall intensities, so that it considered for all given intensities of 30, 60, 80 and 120 mm h^{-1}. The Sprinkling on jute bags to achieve antecedent soil moisture content equal to field capacity and a Kamphorst rainfall simulator and clinometer are shown in Figure 2.

Data collection and analysis

During rainfall simulation in each treatment, runoff samples were collected in special bottles for immediately transport to laboratory to avoid preventing bacteria's effects on soil nutrients (carter, 1993). Measurable runoff was recorded for each experiment and then the infiltration rate was calculated as the difference between the amount of rainfall on the plot area and the runoff output at the same

time. The sediment concentration was measured using decantation procedure, oven dried at 105°C for 24 h (Khaledi Darvishan el al., 2016) (Figure 2).

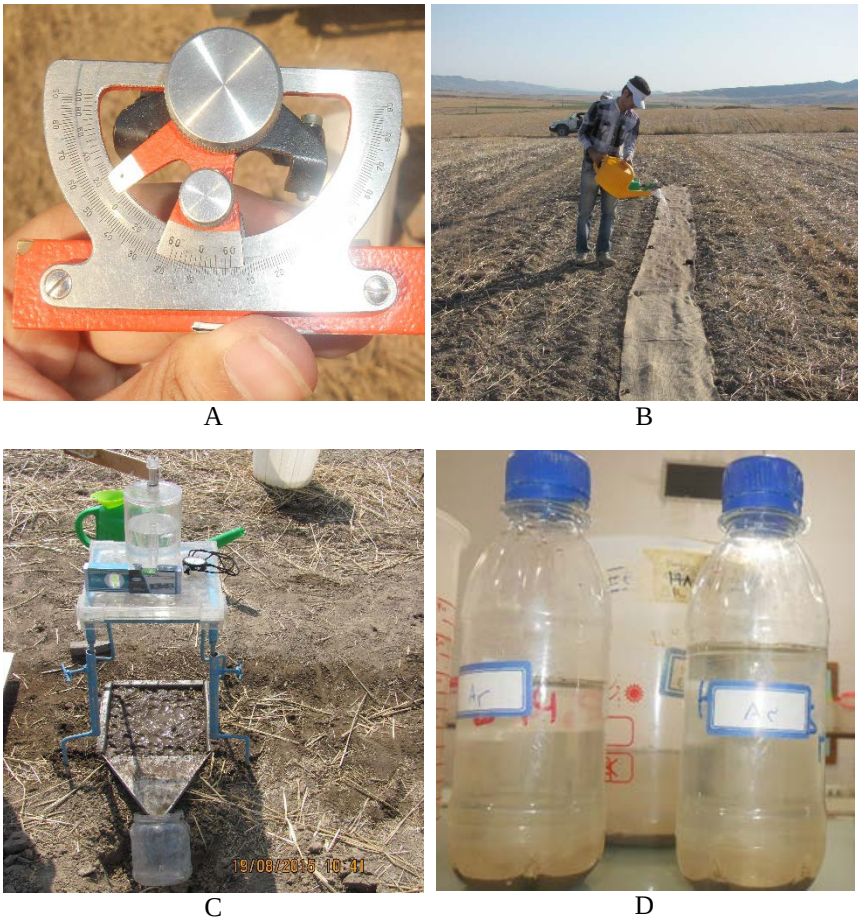

Figure 2. Find intended slope with clinometer (A) Sprinkling on jute bags to achieve antecedent soil moisture content equal to field capacity (B), rainfall simulation and collecting runoff (C and D)

In the end of measuring samples sent to the laboratory and other variables such as soil nutrients in original soil, sediment samples and runoff samples were measured. The methods that used for measuring Nitrogen, Phosphorus and potassium in laboratory were Kjeldahl, Olsen and Photometry respectively (Bremner, 1960; Watanabe and Olsen, 1965; Reitman and Frankel, 1957). As runoff and sediment were not enough to determine soil nutrients in some samples, samples were mixed together.

Statistical analysis

The experimental design of spilt plots and factorial experiments with four slope gradient-aspect conditions (6%-north, 12%-north, 25%-north and 25%-

south) and four rainfall intensities (33, 64, 80 and 110 mm h^{-1}) were performed as statistical tests. The normality test was done for all variables of infiltration, runoff and soil and nutrient loss. The un-normal variables were transformed to logarithmic form to achieve normality distribution, because parametric tests on normal data seems to be more powerful to detect the differences than the nonparametric tests on non-normal data (Townend, 2002). The analysis of variance (ANOVA) with considering the split plots design (Bihamta and Zare Chahouki, 2011) was performed to describe the individual and interaction effects of rainfall intensity and slope gradient on the study variables including infiltration, runoff, soil and nutrient loss. The statistical analysis was performed using SPSS 21 software.

RESULTS AND DISCUSSION

Study soils

The results of physical and chemical characteristics of soil samples have been shown in Table 1.

Table 1 Physical & Chemical properties of the studied soil and Amount of organic matter and bulk density in various studied slopes

Physical properties	Unit	Amount	Chemical properties	Unit	Amount
Bulk density	g cm^{-3}	1.50	pH	---	8.54
Density	g cm^{-3}	2.68	EC	$ds.m^{-1}$	1.38
Porosity	%	44	CaSO4	Percent	0.005
Clay	%	26	SAR	—	0.48
Silt	%	66	Lime	Percent	30.97
Sand	%	8	Carbonate	$Meq.l^{-1}$	00
Soil texture	---	silty-loam	Bicarbonate		5
Soil sample	Bulk density (g cm^{-3})	Organic matter (%)	Chlorine		4.5
			Sulfate		7.7
Slope 6%-North	1.454	1.017	Calcium solution		10.0
Slope 12%-North	1.239	1.059	Magnesium solution		5.2
Slope 25%-North	1.369	1.073	Sodium solution		1.9
Slope 25%-South	1.391	0.975	Potassium solution		0.15

Because of the more importance and variability of organic matter (OM %) in various slope gradient and aspects, the soil was sampled in each studied slope separately and then the OM were measured and shown in Table 3.

Infiltration and runoff

The results of statistical analysis to investigate the individual and interaction effects of rainfall intensity and slope gradient on infiltration and runoff variables are shown in Figures 3 and 4 and Table 2. As shown in Figure 3, in rainfall intensity of 33 mm h^{-1} the rainfall has not intense enough to generate measurable runoff in all studied slopes. The effects of slope gradient and rainfall

intensity on mean infiltration coefficient and mean runoff coefficient were significant (P≤0.01). The interaction effect of slope gradient and rainfall intensity on mean infiltration coefficient and mean runoff coefficient were also significant in P≤0.01 and P≤0.05, respectively (Table 2).

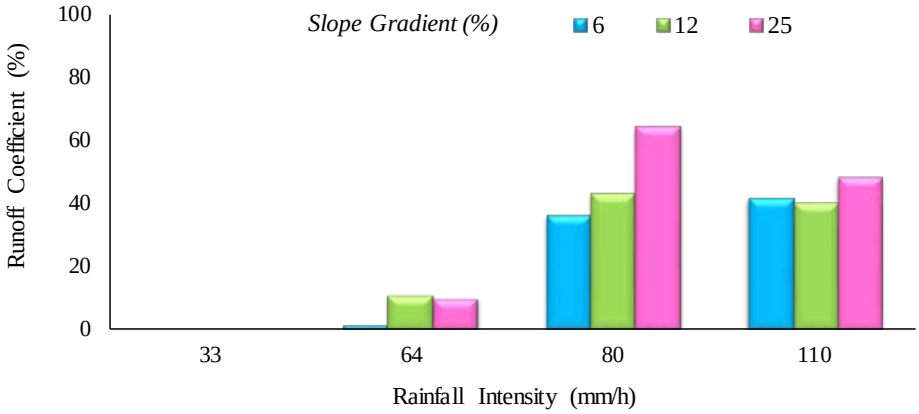

Figure 3. Effect of rainfall intensity and slope gradient on runoff coefficient

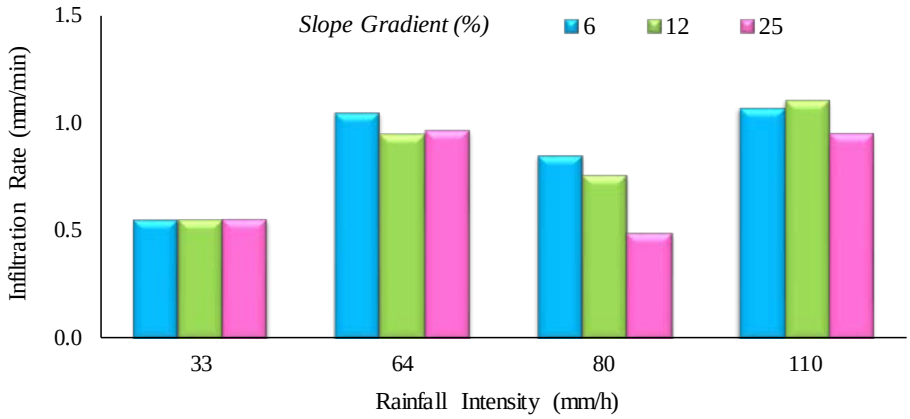

Figure 4. Effect of rainfall intensity and slope gradient on mean infiltration rate

After realizing that the effect of rainfall intensity and slope gradient on mean infiltration coefficient rate and runoff coefficient is significant, Determination of significant differences between studied rainfall intensities and slope gradients using post hoc analysis (Duncan test) was carried out (Table 3).

The purpose of using post hoc analysis was to determine significant changes between the levels of treatments (slope gradient and rainfall intensity) which leads to understand the turning points in hydrological response in the study conditions.

Table 2. The results of the effects of slope gradient and rainfall intensity on infiltration and runoff variables

Dependent Variable	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Infiltration rate (%)	Slope	0.178	2	0.089	7.237	0.002
	Rainfall intensity	1.974	3	0.658	53.435	0.000
	Slope × Rainfall intensity	0.193	6	0.032	2.615	0.033
Depth of runoff (mm)	Slope	39.497	2	19.749	7.174	0.002
	Rainfall intensity	1262.405	3	420.802	152.866	0.000
	Slope × Rainfall intensity	42.891	6	7.184	2.597	0.034
Mean runoff coefficient (%)	Slope	1.082	2	0.541	11.183	0.000
	Rainfall intensity	2.358	3	0.795	16.432	0.000
	Slope × Rainfall intensity	4.631	6	0.772	15.951	0.000
Soil loss ($kg.h^{-1}$)	Slope	157.327	2	78.664	6.844	0.000
	Rainfall intensity	645.511	3	215.170	18.721	0.000
	Slope × Rainfall intensity	141.750	6	23.625	2.056	0.083
Sediment concentration ($g.l^{-1}$)	Slope	225.742	2	112.871	5.828	0.006
	Rainfall intensity	895.727	3	298.576	15.416	0.000
	Slope × Rainfall intensity	142.466	6	23.744	1.226	0.316

As shows in Table 3, mainly two groups have been created, slope gradient 6 and 12% located in one individual group and 25% separated in another group. In case of rainfall intensity, 33 and 64 mm h^{-1} were in one group, while 80 and 110 mm h^{-1} were in another group. It means that there are significant changes in hydrological response between slope gradients of 12 and 25% as well as between rainfall intensities of 64 and 80 mm h^{-1}. Therefore, it can be concluded that the critical slope gradient and rainfall intensity are located somewhere between 12-25% and 64-80 mm h^{-1}, respectively.

These results show that mean infiltration rate intensity of 64 and 100 have not significant difference and they located in one subset and we have three subsets. So we have significant difference between 33, 80 and 64_110 mm h^{-1} intensities.

The results showed that as rainfall intensity and slope gradient increased, the mean infiltration rate and mean runoff coefficient were also significantly increased ($p \leq 0.01$).

The results of post hoc analysis for rainfall intensity and slope gradient using Duncan test showed that, in case of the effects of rainfall intensity and slope gradient on infiltration and runoff, two turning points are distinguishable in studied conditions; one between rainfall intensities of 64 and 80 mm h^{-1} and another between slope gradient of 12 and 25%. In other words, at the rainfall intensity more than about 70 mm h^{-1} and at the slope gradient more than about 18%, the runoff and infiltration were significantly decreased and increased,

respectively. This results emphasized on land use management via avoiding change from rangelands to rainfed lands especially when the slope is more than 18%.

Table 3. Determination of significant differences between studied rainfall intensities and slope gradients using post hoc analysis (Duncan test)

Source \ Subsets	Subset 1	Subset 2	Subset 3
Rainfall intensity (mm h^{-1})	Mean infiltration rate (%)		
	33	80	64, 100
	Runoff (mm)		
	33, 64	80	110
	Mean runoff coefficient (%)		
	33, 64	80, 110	-
	Sediment concentration (g l^{-1})		
	33	64, 80, 110	-
	Soil loss (kg h^{-1})		
	33, 64	80, 110	-
Slope (%)	Mean infiltration rate (%)		
	25	6, 12	-
	Runoff (mm)		
	6, 12	25	-
	Mean runoff coefficient (%)		
	12, 25	6	-
	Sediment concentration (g l^{-1})		
	6	12, 25	-
	Soil loss (kg h^{-1})		
	6, 12	25	-

Sediment concentration and soil loss

Sediment concentration and soil loss were affected by both slope gradient and rainfall intensity as well as by their interactions. The results indicated that sediment concentration increased with increasing rainfall intensity in all slope gradients. The effect of rainfall intensity and slope gradient on sediment concentration and soil loss was significant. The results of subgrouping with Duncan test showed that between the rainfall intensities of 33-64 mm h^{-1} and between the slope gradients of 6-12%, the sediment concentration was significantly changed. In case of soil loss, the significant change was happened between the rainfall intensities of 64-80 mm h^{-1} and the slope gradients of 12-25%, respectively. In other words, with assuming a linear relationship changes, at a given plot scale the critical condition in sediment concentration (detach and arrival to transport agent) was the rainfall intensity of around 50 mm h^{-1} and slope gradient of around 9% but for soil loss (soil transport), it was around 72 mm h^{-1} and 18%, respectively.

Soil nutrients (NPK)

The results of soil nutrients including Nitrogen, Phosphorus and Potassium in two slope gradients of 6 and 25 % are shown in Table 4. The differences between soil nutrients N, P and K in two extreme slope gradients (6 and 25%) are not considerable, it means that there is no need to sample and measure soil nutrients in slope gradient of 12%.

Since there were no considerable differences between N, P and K in two extreme studied slope gradients of 6%-North and 25%-North, it can simply reveal that the effect of slope gradient in the given range in the study area on N, P and K is ignorable. But in case of the effect of slope aspect on studied variables, the soil sampling in the slope gradient of 25%-South was also done and nutrients were measured and compared with those in the slope gradient of 25%-North and the results are shown in Table 4.

Table 4. Comparison between the results of soil nutrients and organic matter in two slope gradients of 6%-North and 25%-North and 25%-South

Organic matter and soil nutrient of slope 25%-North				Organic matter and soil nutrient of slope 6%-North			
Nitrogen (%)	Potassium (ppm)	Phosphorus (ppm)	Organic matter (%)	Nitrogen %	Potassium (ppm)	Phosphorus (ppm)	Organic matter (%)
0.10	297.20	2.36	1.073	0.10	265.40	2.56	1.017
				Organic matter and soil nutrient of slope 25%-South			
				Nitrogen %	Potassium (ppm)	Phosphorus (ppm)	Organic matter (%)
				0.13	233.5	6.35	0.953

The results presented in Table 5 indicated that Nitrate, Ammonium and Phosphorus contents of runoff in the experiments under rainfall intensity of 80 mm h^{-1} in the slope gradient of 12% in North aspect had the significant difference with those under rainfall intensity of 110 mm h^{-1} in the slope gradient of 25% in South aspect, while in case of Potassium the differences were not significant. These results were in agreement with previous studies such as Alberts (1981).

Because of non-significant changes in N, A and P in the runoff of the given extreme experiments (lowest slope gradient and rainfall intensity against highest ones-Table 4), the measurements were not carried out for other samples, so that only the results of 12%-North and 25%-South slopes are presented in Tables 5 and 6.

Table 5. Comparison between the results of nutrients and organic matter in the runoff of the plots located in two slope gradients of 12%-North and 25%-South

Slope	Rainfall intensity (mm h^{-1})	Rainfall depth (mm)	Concentration of nutrients in runoff			
			Nitrate	Ammonium ions	Phosphorus ions	Potassium ions
12%-North	80	20	0.89	0.14	0.13	4.56
25%-South	110	27.5	0.00	0.36	0.02	2.63

Table 6. Comparison between the results of nutrients and organic matter in the soils and sediments in the plots located in two slope gradients of 12%-North and 25%-South

Slope	Rainfall intensity (mm h^{-1})	Rainfall height (mm)	Soil				Sediment			
			Nitrogen %	Potassium (ppm)	Phosphorus (ppm)	Organic matter (%)	Nitrogen %	Potassium (ppm)	Phosphorus (ppm)	Organic matter (%)
12% North	80	20	0.1	206.1	3.59	1.059	0.07	344.7	12.47	0.975
25% South	110	27.5	0.1	155.4	2.42	0.975	0.13	233.5	6.35	0.953

The comparison between the results of nutrients and organic matter in the soils and sediments in the plots located in two slope gradients of 12%-North and 25%-South have been shown in Table 6.

Although as shows in Table 6, the differences between Nitrogen and also organic matter in the soil (before experiment) and the sediment at the plot outlet was not considerable, in higher slope (25%) the amount of Nitrogen in sediment was more than soil, while the amount of organic matter in sediment samples was lower than the original soil in both 12% and 25% slopes.

Since the amount of organic matter is highly related with the amount of organic Carbon in the soil, the results showed that Nitrogen is carried out with sediment a little bit more than Carbon. It can be one of the main sources of increasing C/N ratio in rainfed lands and one of the main reasons for burning straws after harvest by the local farmers. The results also indicated that the amount of Phosphorus and Potassium in original soil were clearly lower than those in sediment samples.

CONCLUSION

The main objective of the present study was to investigate the individual and interaction effects of slope and rainfall intensity on infiltration, runoff, soil and nutrient loss in rainfed lands in Golestan Province, northeast of Iran. According to the results:

- The individual effects of slope gradient and rainfall intensity on infiltration and runoff were significant.
- The interaction of the slope gradient and rainfall intensity on infiltration and runoff was significant.
- The individual effect of rainfall intensity and its interaction with the rainfall intensity on sediment concentration were significant.
- The effect of slope and rainfall intensity on soil loss was significant individually, but their interaction on soil loss was not significant.
- The amounts of OM in original soil of the studied slopes was more than the sediment in all studied slope gradients.
- The amounts of N in original soil of the studied slopes was less than the sediment in higher slope gradient.
- The amount of Phosphorus and Potassium in original soil were clearly lower than those in sediment samples.

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SIGNIFICANCE OF THE PROTECTION OF HONEY BEES FROM BRAULOSIS (WITH A SPECIAL FOCUS ON MONTENEGRO)

SUMMARY

The causative agent of bee braulosis is ectoparasite, bee louse *Braula coeca* Nitzsch (Insecta: Diptera: Braulidae). Harmful effects are caused by larvae of bee louse which are found in the honeycomb and its adult forms that parasitize on the honeybee *A. mellifera*. Adults bee louse live on the bee body, feeding on nectar, honey and royal jelly from the bee's mouth. A strong invasion can lead to the weakening of the entire bee's society and its death. Damage of honey and wax caused by larvae of bee louse leads to economic losses. There have been no systematic studies in this disease in Montenegro, nor has it been diagnosed in Montenegro so far. Legislation in Montenegro does not prescribe measures for the prevention, suppression and eradication of this disease. Regular diagnostics of this disease should be carried out in order to prevent and eradicate it in the event of its occurrence. In order to detect braulosis, it is necessary to have a regular control of the queens bee and frames with the honeycomb. Braulosis is most often suppressed by the use of thymol or camphor. Good beekeeping practice can significantly influence the prevention of disease.

Keywords: *Braula coeca*, braulosis, *Apis mellifera*, Montenegro

INTRODUCTION

Braulosis can cause five species of bee louses of genus *Braula*, family *Braulidae*: *Braula coeca* Nitzsch, *B. orientalis* Örösi-Pál, *B. schmitzi* Örösi-Pál, *B. kohli* Schmitz, *B. pretoriensis* Örösi-Pál and subspecies *B. coeca ssp. angulata*. *Braula coeca* Nitzcsch, *B. orientalis* Örösi-Pál and *B. schmitzi* Örösi-Pál were found in the Palaearctic region, while *B. kohli* Schmitz, *B. pretoriensis* Örösi-Pál and the subspecies *B. coeca ssp. angulata* found in the Afrotropical region. *B. coeca ssp. angulata* was also established in Italy. The most important causative agent of honey bee braulosis is ectoparasite bee louse *Braula coeca* Nitzsch (Dobson 1999). *Braula coeca* is not real louse, but a fly without a wings. It is classified in the phyllum *Arthtropoda*, class *Insecta*, order *Diptera*, suborder *Brachycera*, superfamily *Ephydroidea*, family *Braulidae* and genus *Braula*. In the literature, it was first mentioned in 1740 by Réaumur and in 1818 was systematized by the German zoologist Christian Ludwig Nitzsch (Phillips, 1925). Bee louses of genus *Braulidae* were found in all parts of the world: Africa, Europe, Australia, North America, South Africa (Gemechu *et al*., 2013,

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Kulinčević et al., 1991, Kulinčević, 2006). With the advent of varoosis, braulosis has become less significant, because by treating the varoe also comes to the destruction of bee louse (Alfallah and Mirwa, 2018). There have been no systematic studies in this disease in Montenegro, nor has it been diagnosed in Montenegro so far (Bojanić Rašović, 2019a): However, it should be kept in mind that this disease can occur and spread due to non-implementation of measures of good beekeeping practice, uncontrolled trade and import of bees, bee honey, wax, beekeeping equipment, clothing, bee products, escaping and swarming bees etc. The disease spreads faster when the bee colonies are close together (Gemechu *et al*., 2013, Bojanić Rašović, 2019a).

Harmful effects are caused by larvae of honey bee louse and its adult forms that parasitize on the honeybee *A. mellifera*. Adults live on the bee body, feeding on nectar, honey and royal jelly from the bee's mouth - which they previously irritate with their claws or during social feeding (*trophallaxis*) - when bee workers feed the queens bee or when they feed bee larvae. That's why they are most often found near the beehive's mouthpiece. Bee louse binds to the mouth of the bee and irritates its upper lip, causing the secretion of drops of honey, nectar or royal jelly with which bee louse feeds. It has not been determined that food of the bees louse are honey from the honeycomb cells, but only from the mouth of the bee. Bee louse are most often found on the queens bee, because they prefer to feed royal jelly, but can also be found on bee workers and drones (Figures 1 and 2). They are most commonly found between the thorax and the abdomen of the queen bee (Ćerimagić *et al.*, 1986, Lolin, 1991, Alfallah and Mirwa, 2018). Over 100 adult forms of bee louse can be found at the queen bee (Martin and Bayfield, 2014). The infested bees are weak, the queens bee are exhausted, they lose the ability to lay eggs and often die. The productivity of the bee colony is decreasing (Gidey *et al*., 2012). A strong invasion can lead to the weakening of the entire bee's colony and its death. Especially sensitive are the weaker bees colonies that do not have enough food. Damage of the honey and wax caused by larvae activity of bee louse influences the appearance and market value of wax and honey, which causes significant economic losses (Gemechu *et al.*, 2013). The source of infection are sick bees - with adult parasites and honeycomb - infected with eggs and larvae of bee louse. Braulosis most effectively infects bees in late summer and autumn. Bee workers can not recognize and remove bee louse, because the composition of carbohydrate of cuticle of the bee louse is almost identical to the composition of carbohydrate cuticle of the bees (Martin and Bayfield, 2014).

Basic morphological and biological characteristics of the bee louse *Braula coeca*

Bee louse has a rounded red-brown body (Figure 3); The length is usually 1.5 mm, and the width is 0.9 mm. The body is segmented and covered with small hairs. Torax and abdomen are not clearly separated; The mouthpiece is suited for sucking. They have atrophied eyes that are located just above the antennas; The

antennas are hidden in grooves. It has three pairs of legs whose segments are trapezoidal; The legs are short, firm and protrude above the body.

Fig. 1. Bee louse on the thorax bee worker
http://beeaware.org.au/archive-pest/braula-fly/#ad-image-0

Fig. 2. Bee louse is on the head of an adult bee
https://bugguide.net/node/view/976856/bgref

Fig.3. Bee louse (*Braula coeca*) - an adult
https://www.insectimages.org/browse/detail.cfm?imgnum=5463594

The last segment of the leg - tarzus is reminiscent of the comb and is important for the fixation and holding for the hairs on the body of bees (Alvarez López, 2016) (Figures 4 and 5).

Fig. 4. End of leg - tarzus of bee louse in the form of comb
https://www.insectimages.org/browse/detail.cfm?imgnum=5463600

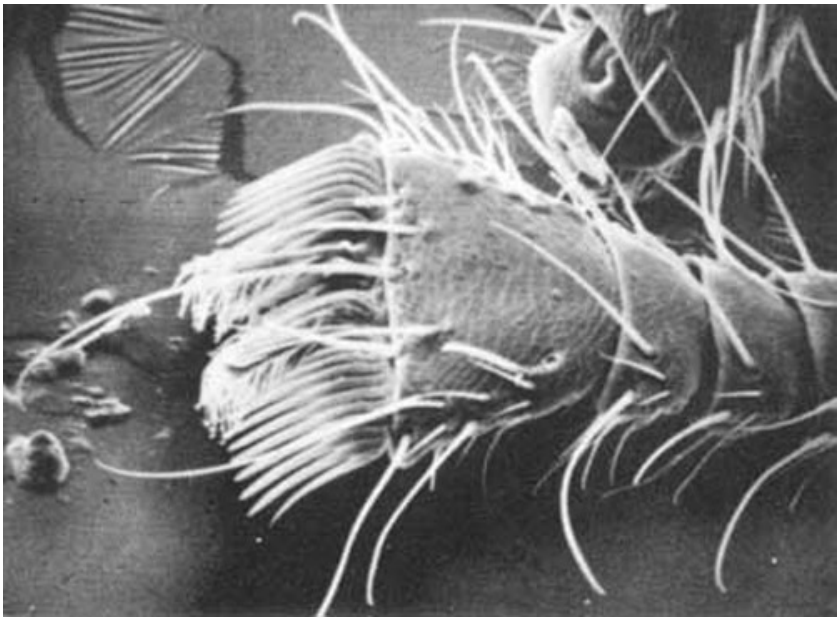

Fig 5. Dorzal side of the tarzus that bee louse holds for the hair on the bee body *A. mellifera* (SEM) http://entnemdept.ufl.edu/creatures/misc/bees/bee_louse.htm

Bee louse has no wings. As it moves quickly, it easily and quickly moves from bee on a bee, but also from one bee colony on the other - through despoilment, bee strangers, mergers of bee colonies, switching the queen bee etc. Due to its similarity to the outer appearance with the varoa, it should be kept in mind that adult bee louse has three pairs of legs, while the adult varoa has four pairs of legs; the adult braula have a rounded appearance while the varoa is flattened and oval-looking (Ćerimagić *et al*., 1986, Kulinčević *et al*., 1991) (Figure 6). However, since both parasites are very small, it is difficult to distinguish them with the naked eye, but a safe diagnosis should be made after observing the parasite under the microscope. The results of some authors have shown that only 28% of beekeepers distinguish bee louse from the varoa (Alfallah and Mirwa, 2018).

The mating of male and female bee louse is taking place in all parts of the bee hive, most often on the honeycomb frame or on the thorax of bees and lasts for a few minutes to an hour. Reproduction starts in the spring and ends in autumn. Adult mature bee louses lay eggs in all parts of the bee hive, but larvae develop only from eggs that are laid on the inside of the waxy coverlids of honeycomb cells with honey, immediately before closing cells (Gemechu *et al*., 2013). Eggs are oval - ellipsoidal, white, 0.84 mm in length and 0.42 mm in width; have two lateral edges. From them, the translucent white larvae develop in 2-7 days - depending on the temperature. The larvae are vermiform, with a flattened rear and a pointed front end - with two buccal hooks. Bee louse larva can reach a length of 2.25 mm; since they are very small, it is difficult to spot

them with the naked eye (Tomašec, 1955, Lolin, 1991). Therefore, the suspicion of braulosis is most often based on the appearance of a damaged honeycomb.

Fig. 6. *Braula coeca* - bee louse (up), *Varroa destructor* - mite (right), *Tropilaelaps spp*. - mite (down below the center), *Mellitiphis alvearius* - mite (left); dorsal - back side (OIE terrestrial Manual 2018)

After the larvae hatch from the eggs, they are drilled tunnels in the honeycomb cell and wherein the food is honey, wax and pollen. Making tunnels, before going to the stage of the puppet, they pass through three larval phases, which last for 7-11 days. On honeycomb it can be seen with naked eye whitish, branched tunnels, about 1 mm in diameter, made by bee louse larva. Tunnels that make larvae in wax give it a cracked and fragile look, which is the basic characteristic of bee louse presence in bee's colony.

On this path the larvae of bee louse damaged also larvae of bees and thus can lead to the complete destruction of the bee's brood. Due to the absorption of water vapor, the honey is of poorer quality, liquid, foamy and leaking from the honeycomb (Lolin, 1991). In the tunnel widening takes place the development of a larva in a puppet which has a flat body white to yellowish, with a length of 1.4-1.7 mm and a width of 0.5-0.75 mm. The puppet stage lasts 1-3 days after which the adult form develops. An adult bee louse must quickly find an adult bee for which it will be hooked and from which it will take food. Without the host - without feeding adult bee louse living longest 6h. The whole development of bee louse from egg to adult shape lasts 10-23 days, which depends on the temperature of the environment (Alvarez López, 2016). Bee louse can live without a bee brood, but it can not live without adult bees from which is taken honey, nectar

and royal jelly. Bee louses can survive the winter on adult bees feeding with honey and after the conditions of the environment become again favorable, the females lay eggs and the life cycle continues (Coffey, 2007).

Measures to combat braulosis

For the purpose of detecting braulosis, it is recommended regularly to control the queens bee and frames with the honeycombs. In order to determine the degree of infestation of bee colonies with bee louse a control treatment with tobacco smoke is carried out. To this end, a white oil-coated paper is placed on the bottom of bee hives. After inserting smoke, the paper is pulled out and bee louse count. If bee colonies has 5 bee louse per 100 bee workers infection is considered low, up to 15 of bee louse middle and over 15 bee louse - strong infection. The best time for suppression the bee louse is start of the summer season. Braulosis is most often suppressed using a thymol oil (Kulinčević, 2006). Mechanical methods consist of removing the infected honeycomb and bee brood and removing adult bee louses from the queens bee using a wooden stick coated with honey. If there are several parasites on the queen bee, they can be removed by a pointed stick dipped in honey; if more parasites are present, the queen bee should be placed in a cage and smoked with tobacco smoke. Dazed bee louses, after smoking fall from the queens bee. Also, bee louse from the queens bee and bee workers can be removed using camphor; The camphor is put on paper covering the entire bottom of bee hive, in the amount of 10-20 g. After releasing the camphor, bee louses are dazed and then fall on the paper. This procedure should be repeated several times (Plavsa and Pavlovic, 2018). Freezing of wax lasting at least 48h will kill all life stages of bee louse. It is very important regularly to change the old honeycomb in the bee hive. When extracting honey, in a certain number are removed and bee louse larvae.

In some countries, such as Australia (where braulosis is present in Tasmania), beekeepers are obligated to report suspicion or presence of this parasite at their apiary to veterinary service. It is also prohibited to buy bee colonies that are infected with braulosis and quarantine is required - the isolation of bee colonies during the sale, until it is proven that the colony is free from the this parasite (beeaware.org.au).

Measures to combat braulosis in Montenegro

Legislation in Montenegro does not prescribe measures for the prevention, suppression and eradication of this disease (Bojanić Rašović, 2019a). Bearing in mind the potential opportunities for the emergence of braulosis in Montenegro, measures to combat it should be timely. Regular diagnosis of bees on this disease must be carried out in order to prevent and eradicate it in the event of its occurrence. Good beekeeping and good hygiene practice can significantly influence the prevention of disease (Bojanić Rašović 2018a, 2018b, 2018c, 2018d, 2018e, 2018f, 2018g, 2018h, 2019a, 2019b, 2019c, Bojanić Rašović *et al.* 2018a, 2018b).

CONCLUSION

A strong invasion of bee colonies with bee louse *Braula coeca* can lead to significant economic losses due to damage to honey and wax and the weakening and death of the whole bee colony. There have been no systematic trials in this disease in Montenegro, nor has it been diagnosed in Montenegro yet. Legislation in Montenegro does not prescribe measures for the prevention, suppression and eradication of this disease.

However, it should be kept in mind that this disease can occur and spread due to non-implementation of measures of good beekeeping practice. Regular diagnosis of this disease should be carried out in order to prevent and eradicate it in the event of its occurrence. It is necessary to ban the sale of bee companies that are infected with braulosis and is obliged to quarantine bee companies when buying.

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