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REPRODUCTIVE BIOLOGY OF TWO EDIBLE HONEYSUCKLES [*Lonicera edulis* Turcz. ex Freyn., *Lonicera kamtchatica* (Sevast.) Pojark.] IN THE CONDITIONS OF SOUTHWESTERN SLOVAKIA

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ABSTRACT

The formation and development of reproductive organs and fruits was examined for two edible honeysuckle species *Lonicera edulis* Turcz. ex. Freyn. and *Lonicera kamtchatica* (Sevast.) Pojark. 'Gerda' using cytological and embryological methods. We found out that the flower initiation has began during June in the conditions of southwestern Slovakia. Male and female archespores were differentiated before entering winter dormancy. In most cases we have observed normally developed tetrads, normal appearance of microspores, two-celled polen grains and mature pollen grain. In both species a sufficient amount of normally developed pollen grains was observed. Disturbances during female gametophyte development occurred occasionally, most mature ovules contained 7 cells female gametophyte. Our research pointed to fact that the species are protogynous. Flowering usually takes place in the first half of April. The fruits were mature in the second half of May. We have found that 10 to 11 fully developed seeds have evolved in the fruits of both representatives of *Lonicera* on average. The above results show the suitability of *L. edulis* and *L. kamtchatica* cultivation in SW Slovakia with a relatively low risk of fruit loss due to climatic conditions.

Key words: *Lonicera edulis, Lonicera kamtchatica* 'Gerda', male and female gametophyte, embryogenesis

INTRODUCTION

The edible honeysuckle has been studied as a very popular berry crop in respect of its nutritive value, especially polyphenols and ascorbic acid content [Pokorná-Juríková et al. 2007, Juríkova et al. 2012a, Gawroński and Kaczmarska 2018]. Among bioactive compounds in berries of edible honeysuckle, the phenolic acids and flavonoid substances predominat, especially anthocyanins, rutin, quercetin and isoquercetin have received the most attention due to its neuroprotective, antiinflammatory and anticancer effect [Gazdík et al. 2008, Gruia et al. 2008, Juríkova et al. 2012a, Sochor et al. 2014] although other flavonols are also presented [Juríkova et al. 2012a, b]. On the other hand, there are limited researches aimed on anatomy and reproductive biology of the edible honeysuckle species. Moreover, these species are not native in Central Europe but originated from the Russian Far East [Plekhanova 1998, Holubec et al. 2019].

Although several microscopic studies of the sexual reproduction of *L. caerulea* and *L. kamtchatica* were carried out, they concerned particularly areas in North America [e.g. Wilkinson 1948] and Asia

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[e.g. Wu et al. 2003, Ke et al. 2006, Guo et al. 2012]. The reproductive biology of edible honeysuckle in the temperate climatic conditions of Slovakia has not been studied yet. Therefore, the paper addresses microscopogenesis, microgametogenesis, macrosporogenesis and macrogametogenesis as well as flowers and fruits development. It is very important to know this process for successful introduction of the species into new, totally different conditions and to recognize the risk of successful fruit production before cultivation under large-scale conditions.

MATERIAL AND METHODS

Site description. An experimental area for the edible honeysuckle planting was established in the autumn 1994 in Nitra. Two *Lonicera* species – *Lonicera kamtschatica* (Sevast.) Pojark 'Gerda' 25 and *Lonicera edulis* Turcz. ex Freyn were planted in four repetitions. Planting was done at 2.0 × 1.5 m experimental plots using 2-years old seedlings. The experimental area was covered with black polyethylene mulch. The soil and climatic conditions of the site were as follows: open space, 130 m above sea level, corn processing area, clay – loam drift, pH 6.4, mould content 3.5% precrop fallow, average rainfall 564 mm per year, average temperature during the vegetation period 16.3°C [Ďurišová 2018]. Nomenclature of *Lonicera* taxa is in accordance to Poyarkova [1958].

Experimental procedures. The plant material for the experiments were collected from the January 2007 up to October 2014. Collected buds, flowers and seeds were fixed in FAA fixative (formaldehyde – acetic acid – ethanol) or Navashin's fixative. Customary methods of dehydration, infiltration, paraffin embedding, cutting and staining were based on Němec [1962] and Erdelská [1986]. Serial sections of anthers, ovaries and fruits were cut at the thickness of 5–12 microns in the rotary microtone CUT 4055 MICROTEC and stained with Heidenheim's haematoxylin or fast-green and safranin. The slides were examined by light microscope Olympus BX and photographed by Olympus E-520.

The number of seeds was calculated on the average sample of 100 mature fruits.

RESULTS

We found that buds initiations of assayed *Lonicera* species began in the first half of June in SW Slovakia. Flowers primordia appeared in the flower buds in late June. Differentiation of the reproductive organs continued during July (Fig. 1a). Meristematic protuberance of the ovules without apparent differentiation was presented in early August in flowers of both species (Fig. 1b). The formation of female archesporium was observed in the apical part of ovules before entering dormancy. There was a differentiation of the male

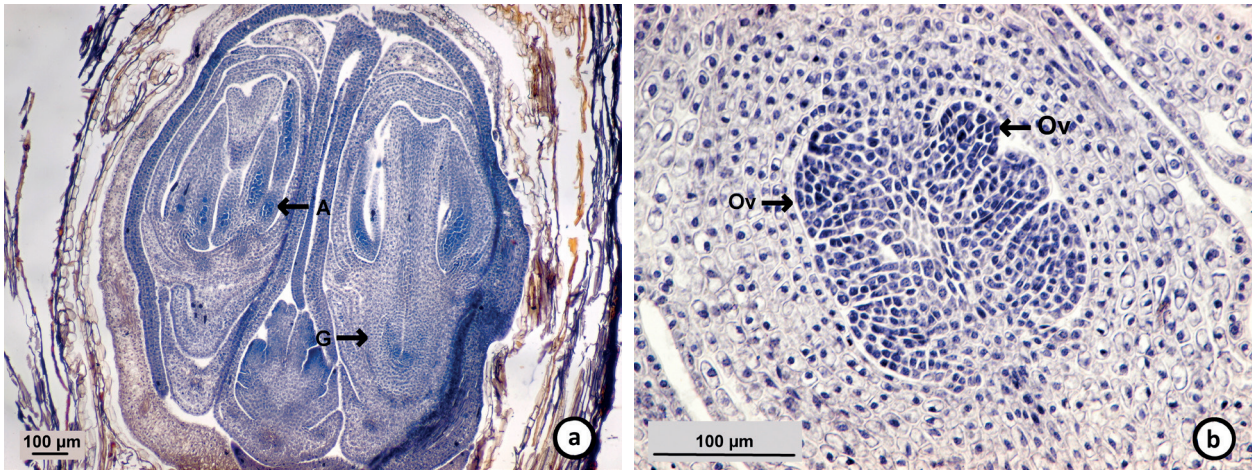

Fig. 1. *Lonicera kamtchatica*: **a)** buds with reproductive organs, 4.07.2007; **b)** meristematic protuberance of ovules 13.08.2007. A – anther, G – gyneceum, Ov – ovule

archesporium in the anthers also at the same time. If the winter came during this stage, the archesporium kept the reproductive organs during winter dormancy.

Microsporogenesis, microgametogenesis. Young anthers of *Lonicera* taxa were tetrasporangiate. Microsporangia wall consisted of three layers of parenchymatous cells in the early stages of development. At this developmental stage, male archespore was surrounded by a single layer of cells that subsequently develops the tapetum. The beginning of the differentiation processes in dormant buds was highly dependent on climatic conditions of a particular year, mainly temperature. Due to the mild winter, the differentiation of microsporocytes in anthers was already observed in mid-January (Fig. 2a). In such cases microsporogenesis was carried out in the second half of January (Fig. 2b). On the other side, the low temperatures during microsporogenesis had a negative effect on this process. The buds damaged under low temperatures contained the anthers with degenerated microsporangia or degenerated tapetum cells (Fig. 2c). During the last year of research, the microsporogenesis has been observed in early February. The dividing nuclei of tapetum cells was detected during the differentiation of the microsporocytes. There were two-nuclei cells most commonly. We found out that microsporogenesis was carried out simultaneously. Microspore tetrads were tetrahedral (Fig. 2d). No disturbances of meiosis were examined during microsporogenesis process. Tapetum of honeysuckle was ameboid. The formation of syncytium was observed shortly after of meiotic division. At this stage the whole microsporangium was filled with a homogeneous mass of periplasmodium. Separation of microspores from tetrads took place shortly after meiosis, too (Fig. 2e). Subsequently, the microspores enlarged and vacuoles development appeared. The first mitotic division of nuclei microspores was observed in conditions of SW Slovakia at the second half of February. Two-celled pollen grains were found in anthers in early March (Fig. 2f). The smaller generative cell was separated from the rest of the pollen grain with a thin wall. Second mitotic division, that was a division of generative nucleus, was observed from the end of March to the beginning of April. As a result of the divisions, 3-celled pollen grains occurred. Mature pollen grains were present in the anthers shortly before the opening of the flowers. Although the anthers with degenerated tapetum or whole microsporangia were observed, a sufficient amount of normally developed pollen grains was developed each year. We have rarely observed lagging chromosomes and chromosome bridges in anaphase I of the meiotic division. However, the proportion non-development pollen grains not exceed 5%.

We found out that mature anther wall consists of three layers: epidermis, endothecium and one ephemeral middle layer. Poorly developed fibrous thickening was present in the epidermal cells and well-developed in endothecium. Differentiation of the endothecium took place when microgametogenesis occurred in the microsporangia. Endothecium had one layer on the outside of the anthers and two layers by the connective.

Megasporogenesis, megagametogenesis. The ovules were anatropous, unitegmic, and tenuinucellar, the nucellus was presented in single layer. Integuments of ovules consisted of outer epidermis, 5 middle layers and inner epidermis was transformed to endothelium. We have observed that one archesporial cell was regularly presented in nucellus. This cell becomes the megasporocyte – megaspore mother cell (Fig. 3a). Differentiation of megaspore mother cell and megasporogenesis was recorded in early February (Fig. 3b). The result of meiotic division was linear tetrad of megaspores. Functional megaspore becomes chalazal, others were degenerated. In addition, we observed the ovules, in which all the megaspores were degenerated. We found out that megasporogenesis took place mostly in the first half of March, although we observed this process even at the end of March in some flowers. We determined that the development of the female gametophyte of studied *Lonicera* species followed Polygonum type (Fig. 3c, 3d). The mature female gametophyte was oval and consisted of 7 cells, the fusion of polar nuclei occurred before the process of fertilization (Fig. 3e). Egg apparatus was placed at the micropylar pole and consisted of egg cell and two synergids. There were three ephemeral antipodes on chalazal pole of the mature female gametophyte (Fig. 3f). The central nucleus was located nearby the egg apparatus. During maturation of the female gametophyte we observed accumulation of coloured substances in

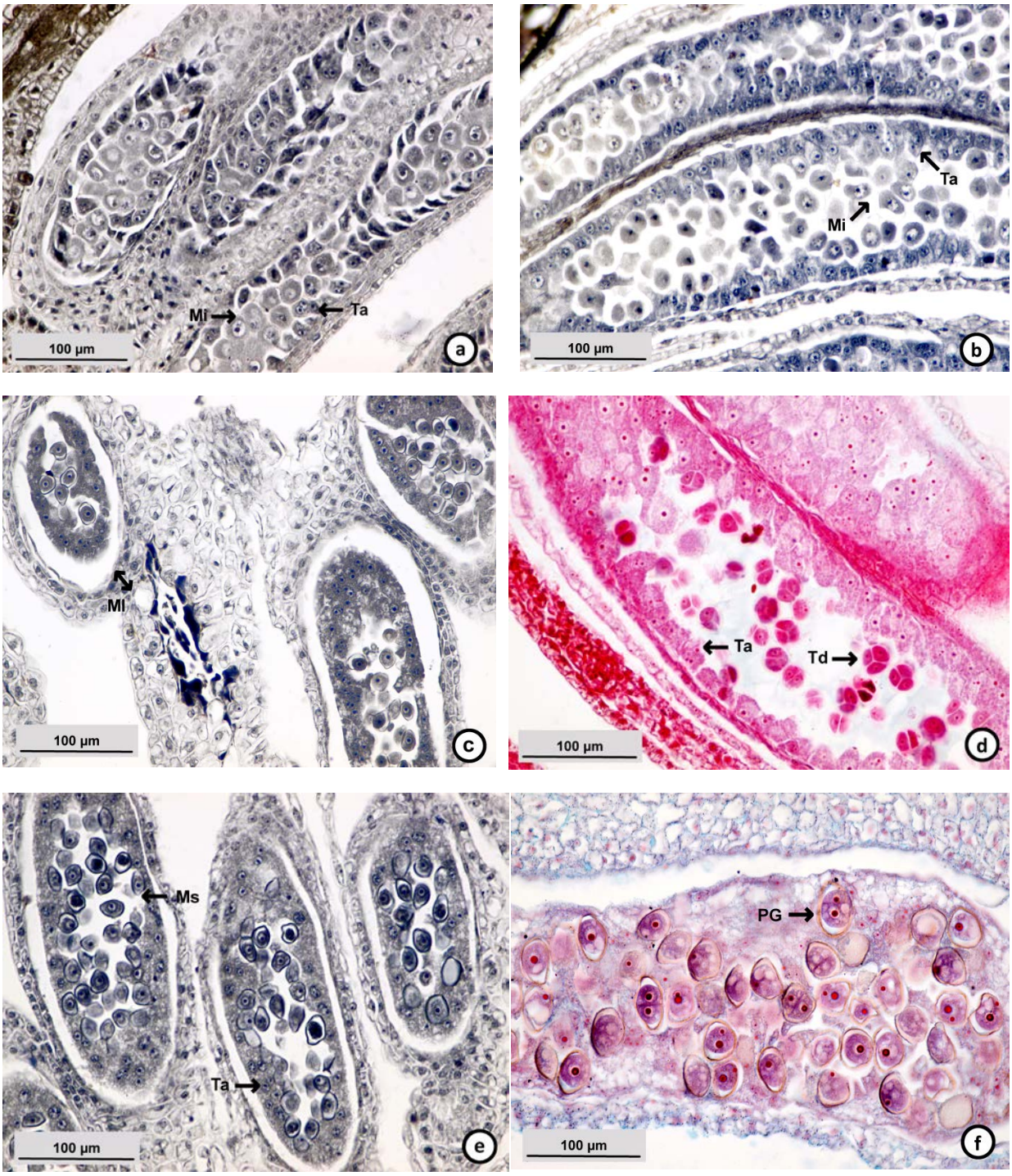

Fig. 2. Development of male gametophyte: **a)** anther with microsporocytes, *Lonicera edulis*, 23.01.2012; **b)** beginning of meiotic division of microsporocytes, *Lonicera edulis*, 29.01.2012; **c)** tetrahedral tetrads of microspores, *Lonicera edulis*, 6.02.2008; **d)** microsporangia with single microspores, *Lonicera edulis*, 29.01.2012; **e)** two-celled pollen grains, *Lonicera edulis*, 4. 3. 2008; **f)** damaged and normal developed microsporangia, *Lonicera edulis*, 29.01.2012. Mi – microsporocytes, MI – microsporangia, Ms – microspores, PG – pollen grains, Td – tetrads, Ta – tapetum

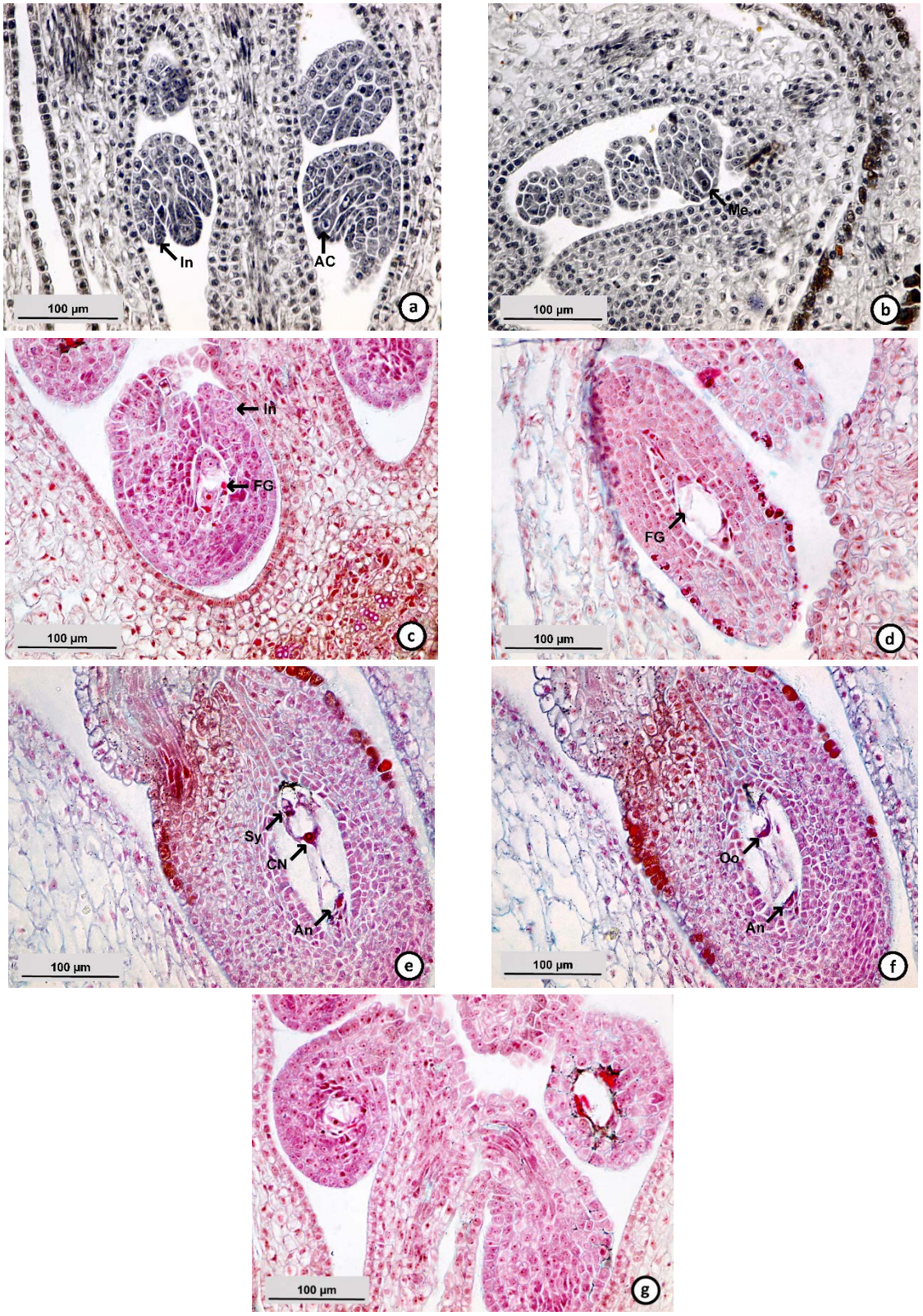

Fig. 3. Development of female gametophyte: **a)** ovules with archesporial cell, *Lonicera kamtchatica*, 6.02.2008; **b)** beginning of meiotic division of megasporocyte, *Lonicera kamtchatica*, 6.02.2008; **c)** two-nucleate female gametophyte, *Lonicera kamtchatica*, 11.03.2008; **d)** four-nucleate female gametophyte, *Lonicera kamtchatica*, 18.03.2008; **e)** mature female gametophyte with central nucleus, *Lonicera edulis*, 8.04.2008; **f)** mature female gametophyte with oosphere (egg cell), *Lonicera edulis*, 8.04.2008; **g)** two ovules: one with female gametophyte, other without female gametophyte, *Lonicera kamtchatica*, 11. 3. 2008. AC – archesporial cell, An – antipodes, CN – central nucleus, FG – female gametophyte, In – integument, Me – megasporocyte, Oo – oosphere, Sy – synergid

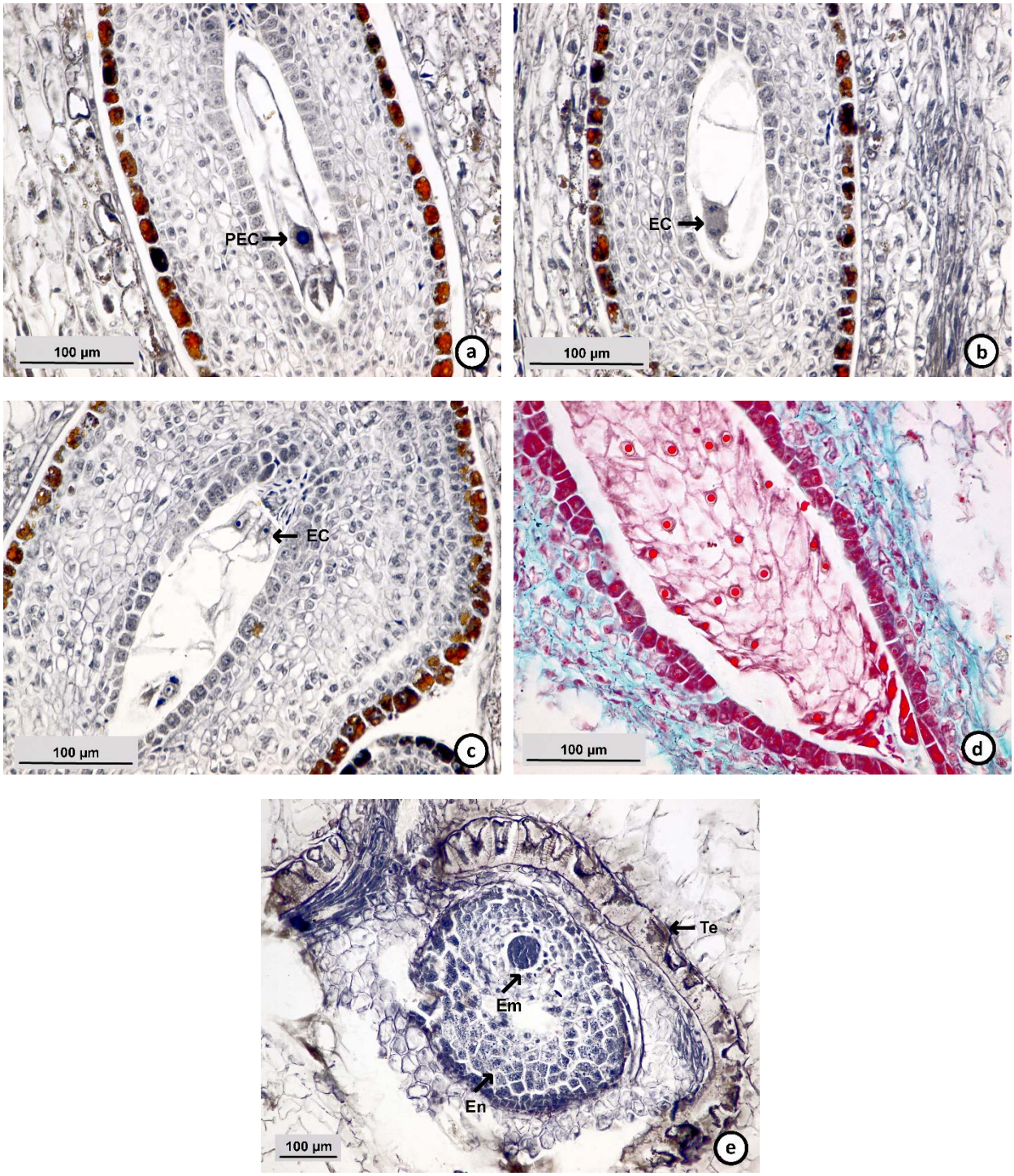

Fig. 4. Fertilization and embryogenesis: **a)** primary endosperm nucleus in fertilization ovule, *Lonicera kamtchatica*, 11.04.2008; **b)** transversal wall after first division of primary endosperm cell, *Lonicera kamtchatica*, 11.04.2008; **c)** early stage of endosperm development, *Lonicera kamtchatica*, 11.04.2008; **d)** advanced stage of endosperm development, *Lonicera kamtchatica*, 15.04.2008; **e)** globular embryo, *Lonicera kamtchatica*, 25.04.2008. EC – endospermal cells, Em – embryo, En – endosperm, PEC – primary endosperm cell, Te – testa

the epidermal cells of the integument. Accumulation of these substances began in the micropylar area. At the stage of the mature female gametophyte all epidermal cells of integument contained vacuoles with colouring substances. We assumed that this may signal the maturity of the female gametophyte or these substances may have a protective role for female gametophyte. In addition, we occasionally observed ovules without female gametophyte, such ovules were formed only by integument (Fig. 3g). Gametophyte absence was the result of disturbances during megagametogenesis. Normally developed ovules with mature female gametophyte were without a nucellus, because the cells of nucellus were gradually degenerated. Degeneration of the nucellus began at the chalazal end of ovule and continued toward the micropyle. As a consequence, mature female gametophyte came into direct contact with the integument of the ovule. We found that female gametophyte matured earlier than the male gametophyte. At this time, mature 7-celled female gametophyte was present in the ovules, observed pollen grains with a dividing nucleus of the generative cell. Our observations showed that studied species of genus *Lonicera* are protogynous. In our conditions, the maturation of the female gametophyte took place in early April.

Pollination, fertilization, embryogenesis. We have observed that in our conditions, both *Lonicera* species bloomed in early April. In addition to normal flowering in the spring, we repeatedly recorded this phenomenon in the summer. Our microscopic observation showed that the stigma was captured with a large number of germinated pollen grains at the time of full flowering. We had also observed the growth of pollen tubes through the style of ovary. The process of double fertilization was observed at the first in the ovules placed in the upper and middle part of the ovary. In the ovules placed at the bottom of the ovary this process was delayed by fusion of gametes. Our research showed that endosperm of studied taxa was of cellular type. We recorded the first division of the primary endosperm nucleus shortly after fertilization (Fig. 4a). We have found that the formation of the walls after the first two divisions of the primary endosperm cell took place transversely to the female gametophyte (Fig. 4b). The following division of endosperm nuclei and forming of the walls was carried out in all directions (Fig. 4c). The intensive development of the endosperm occurred in the first half of April (Fig. 4d). The division of the zygote occurred later. The beginning of embryogenesis has been seen in mid-April. Globular embryos were observed in the second half of April and torpedo embryos in early May (Fig. 4e). The endosperm cells accumulated storage material during embryogenesis, mainly starch and protein. The mature embryo was commonly observed in first decade of May.

We found that in mature fruit of *L. edulis* there were of 10 (4–14) fully developed seeds an average, fruits *L. kamtchatica* contained of 11 (5–19) seeds an average.

DISCUSSION

We observed that in conditions of SW Slovakia both of the studied species bloom in the first half of April, while in southeastern Poland, *L. kamtschatica* flowering occurred between April 20 and the middle of May [Bożek 2007]. In both investigated species, earlier development of the female gametophyte was observed. It is in accordance with observation of Bożek [2007], which concluded that flowers of *L. kamtschatica* are protogynous. Romanyuk [1990] found out that the protandry was observed in *L. xylosteum* and was clear in 4 more species, while progyny was observed in *L. hispida*, *L. involucrata*, *L. dioica* and the edible-fruited species of subsection *Caeruleae*.

The anthers of representatives of the *Caprifoliaceae* familie are tetrasporangiate [Davis 1966]. The anther wall comprises the epidermis, fibrous endothecium and ephemeral middle layer [Johri et al. 1992], which is in agreement with our observation. We also confirmed the ameboid tapetum during our study; it is typical to genus *Lonicera* [Davis, 1966, Johri et al. 1992] and to *L. edulis* [Wu et al. 2003] and *L. caerulea* [Guo et al. 2012], respectively. Periplazmodium of ameboid tapetum is formed at the uninucleate pollen [Jakovlev 1981, Johri et al. 1992], which was also evident in the assayed species of honeysuckle.

Johri et al. [1992] reported that the simultaneous cytokinesis in the microspore mother cells of the *Caprifoliaceae* family followed meiosis and the microspore tetrads were tetrahedral and decussate. We observed only tetrahedral tetrads of the microspores in both species. This type of tetrads in *L. edulis*

was noticed by Wu et al. [2003] as well. We found out that pollen grains of studied *Lonicera* species were 3-celled when shed, which is consistent with the observations of other authors [Wu et al. 2003, Bożek 2007, Guo et al. 2012]. We have found that the dehiscence anthers and pollen shedding occur shortly after the opening of the flowers that was also observed by Bożek [2007] in *L. kamtchatica.* Bożek [2007] stated that anthers start to shed pollen a few hours after the opening of the corolla and the process of pollen release lasted until the second or even third day of flower life, depending on the time of flower bud opening and the weather conditions. She found that pollen grains of two cultivars *L. kamtchatica* are suboblate and considering their size, they can be classified as large ones with mean values of polar axis 47.55 µm and equatorial axis 60.37 µm, their viability is high – about 95%. Guo [2010] recorded that pollen grains germinated at 1 hour after pollination and pollen tubes reach the base of style at 30 hours after pollination.

At the bottom of the corolla of *L. kamtchatica* we observed nectariferous tissue, which was also described by Weryszko-Chmielewska and Bożek [2008], consisting of two layers of epidermal formations: short papillae and about 3× longer unicellular trichomes.

The stigma had a lot of papilla cells. Guo [2010] pointed out that effective pollination period is 5 days at least and stigma *L. caerulea* cv. Berel loses most of its receptibility in 50 hours after hand pollination. In the nucellus, each ovule differentiates into one archesporial cell [Guo et al. 2012]. For most *Caprifoliaceae* species early degeneration of nucellus is typical [Batygina 1994]. We also recorded this process in the two assayed species. Degeneration of the nucellus was complete at the end of megagametogenesis.

Cytokinesis accompanied by meiosis and the chalazal megaspore of the linear tetrad developed into a Polygonum type embryo sac. Mature embryo sac is seven-cell [Wu et al. 2003, Guo et al. 2012]. Ovules in the middle part of ovaries need 9 days to develop from macrospore mother cell to mature embryo sac [Guo 2010].

We have observed that at the stage of full maturity of the female gametophyte colouring substance integument was accumulated in the outer layers of vacuole epidermis. Jiang et al. [2007] claimed that idioblast in the specialized connectives of *Lonicera* contains phenolic compounds protecting the developing vascular bundles from damage in anthers. Thus the normal development of microspores and normal pollination could be ensured. However, following this stage, phenolic compounds have begun degrading. We suppose that coloured vacuoles in the epidermal cells of integument play a similar protective role during development of the female gametophyte.

The mature ovules of *Lonicera* species are anatropous, unitegmic and tenuinucellate. Usually 20 ovules can be found in each ovary of *L. caerulea* [Guo et al. 2012]. Development of ovules proceeds unevenly, upper ovules have earliest developmental stage.

Embryogeny in *Lonicera* conforms best to the Asterad type and is somewhat irregular [Davis 1966]. Miyashita et al. [2009] determined that in *L. caerulea* var. *emphyllocalyx* division of the primary endosperm nucleus was initiated 3 days after pollination (DAP). At 14 DAP endosperm was composed of more than 100 cells, at 21 DAP endosperm occupied the entire ovule and at 35 DAP endosperm cells were rich in starch grain.

The division of the zygote occurs later than the division of the primary endosperm nucleus. [Miyashita et al. 2009] observed that zygotes did not exhibit cell division activity until 7 DAP. He found that at 14 DAP embryos comprised of 5 to 9 cells, at 21 DAP globular to heart shaped embryos were formed, at 35 DAP torpedo shaped embryos were present.

It seems the number of seeds per fruit is very variable in edible *Lonicera* species. For example the number of seeds per fruit for *L. caerulea* vary from 4.2 to 20.63 in published studies [Bożek 2012, Boyarskikh 2017, Gawroński and Kaczmarska 2018], it was strongly depending to genotype and selfpollination resulted in a significant decrease in the number of seeds in the fruits. Our results confirmed this large variabilty, we found 10 fully developed seeds an average in *L. edulis* fruits (min. 4, max. 14) and *L. kamtchatica* fruits contained of 11 seeds an average (min. 5, max. 19).

CONCLUSIONS

The edible honeysuckle is considered a successful berry crop in the territory of Slovakia. It represents the very plastic and non-demanding species with the high

nutritional value of berries, especially bioactive components – polyphenols and ascorbic acid. The results of our experiments proved that the edible honeysuckle is capable of generative reproduction which is important for the maintenance of selected genotypes of *Lonicera s*p. Despite of the warmer climatic conditions of southwestern Slovakia compared to the area of the original occurrence, the reproductive process and fruit set formation of edible heneysucle was successful.

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