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HISTOLOGICAL AND CYTOLOGICAL ANALYSIS OF MICROSPOROGENESIS AND MICROGAMETOGENESIS OF THE INVASIVE SPECIES *GALINSOGA QUADRIRADIATA* RUIZ & PAV. (ASTERACEAE)

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Galinsoga quadriradiata Ruiz & Pav. is an annual weedy plant that can be found all over the world. It belongs to the Asteraceae family and is recognised as one of the invasive foreign plants in Poland, which are native to Central and South America. The aim of this study was to describe the reproductive features of *Galinsoga quadriradiata* focusing on the changes that occur during microsporogenesis and microgametogenesis along with the morphology of its pollen. As it is typical of the eudicot clade of Angiosperms, cytokinesis of *G. quadriradiata* is simultaneous. The pollen grains are tricolporate with spiny outer walls and the course of the microsporogenetic process is fairly typical of the Echinatae group of weed plants. The high viability of the pollen grains, which mature unequally in the inflorescences, and the proper course of meiosis determine whether a plant has the invasive character of *Galinsoga quadriradiata*.

Key words: *G. quadriradiata*, microgametogenesis, microsporogenesis, pollen grains, invasive kenophyte, weed plant

INTRODUCTION

The main process in Angiospermae that leads to production of pollen, which represents a male gametophyte, is meiosis. This process containing a series of tightly controlled events, which occur in the anthers, can be divided into two major processes – microsporogenesis and microgametogenesis (Bedinger, 1992). Microsporogenesis starts with the beginning of the meiosis of the diploid mother cells and ends with the formation of haploid microspores. When the microspores are released from the tetrads, microgametogenesis starts and after subsequent divisions, complete male gametophytes are formed.

Our studies concern the events and changes that occur during microsporogenesis and microgametogenesis that lead to formation of mature pollen grains in *Galinsoga quadriradiata* Ruiz & Pav. This species is an annual plant that flowers from June to late autumn. An eight- to nine-week-old plant can produce 3000 flower heads and a large number of seeds, up to 7500 (Kagima, 2000; Huffman, 2004). Seeds are able to germinate immediately upon contact with warm and moist soil, and therefore each growing season the plant can achieve two to three generations (Reinhardt et al., 2003). Seeds are dispersed mainly by the wind or by animals (Reinhardt et al., 2003).

G. quadriradiata is a weedy and invasive plant, which causes economic damage to crop cultures as it is a strong competitor in weedy plant communities. It takes up nutrients that are necessary for the growth of cultivated plants and because of its relatively large leaf surface area it may shade out the cultivated plants (Reinhardt et al., 2003). Moreover, according to Huffman (2004), *Galinsoga* species serve as alternate hosts for many insects, viruses and nematodes that affect crop species.

The genus *Galinsoga* Ruiz & Pav. comprises 14 species (Cannie, 1977), which are mainly confined to Mexico as well as Central and South America, except for *G. parviflora* Cav. and *G. quadriradiata* (Gopinathan and Babu, 1982). The native range of *G. quadriradiata* covers parts of South and Central America from Mexico to Chile, but due to human

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activity the species has spread far from its original range (Kabuce and Priede, 2010). In Poland, the species was first found near Wrocław in the second half of the 20th century, while the first herbarium records in Poland date back to 1876 (Tokarska-Guzik, 2005). It is now a common crop weed and ruderal plant here. *G. quadriradiata* is often found on arable lands, roadsides, railways and in gardens where it is highly competitive and spreads quickly often becoming the dominant species in a field (Kabuce and Priede, 2010). It is adapted to a warm climate and heavy, nitrogen-rich soils (Anonymous, 1996).

Because asexual reproduction has been detected in Asteraceae species (and connected with some abnormalities in pollen development), we wanted to check microsporogenesis and pollen morphology in *G. quadriradiata* Ruiz & Pav., especially since *Galinsoga* species were suspected in the case of apomixis (Pietrusiewicz et al., 2005). This seems to be valuable because of the interesting nature of this plant and the adaptations that support its invasive properties. This description of the development of the male gametophyte, cytological aspects and pollen morphology can constitute a compendium of the embryological events of this weedy and invasive plant together with female embryological studies (Kolczyk et al., 2014). Moreover, to the best of our knowledge, there is lack of such studies in the literature and the embryological aspects are very important since *Galinsoga quadriradiata* propagates only generatively (Jursik et al., 2003).

MATERIALS AND METHODS

The flower buds and flower heads of *Galinsoga quadriradiata* (Fig.1a) at different developmental stages were collected from plants in their natural habitats (wastelands, roadsides) in Kraków. For light microscopy, the plant material (the flower buds) were fixed in 5% buffered (0.1 M phosphate buffer, pH 7.2) glutaraldehyd for three hours at room temperature, washed four times in a phosphate buffer and then dehydrated in a graded ethanol series: 10%, 30%, 50%, 70%, 96%, 15 min each. Then the plant material was kept overnight in absolute ethanol and subsequently the samples were infiltrated for 1h each in 3:1, 1:1 and 1:3 (v/v) mixture of absolute alcohol and Technovit 7100 (2-hydroxyethyl-methacrylate) (Heraeus Kulzer). The samples were embedded in pure Technovit for 12 hours, followed by polymerisation of resin with addition of hardener. The plant material was sectioned to 7 μm on a rotary microtome (Microm, Adams Instrumenten). The sections were floated on water on a clean slide and dried to settle the sections onto a slide, then they were stained with 0.1% toluidine blue O (TBO) and mounted in Enthellan synthetic resin (Merck).

In order to detect insoluble polysaccharides, the sections were oxidised for 30 min in 1% periodic acid, rinsed in distilled water for 10 min, stained in Schiff's reagent for 30 min, washed twice in 0.5% potassium metabisulfite for 10 min each and rinsed under running water for 45 min.

For histological observations, the flower heads were also fixed in 96% ethanol and glacial acetic acid (v/v 1:3) and then stored in 70% alcohol at 4°C. After dehydration in an ethanol series and embedding in paraffin, the material was sectioned on a microtome (10 μm thick), stained with Heidenhain's hematoxylin with alcian blue and mounted in Enthellan synthetic resin (Merck).

The viability of the pollen grains was tested using the acetocarmine test and Alexander test. For both methods, young fresh inflorescences of *G. quadriradiata* were fixed in 96% ethanol and glacial acetic acid (v/v 1:3). In acetocarmine (1% acetocarmine) staining, the cytoplasm of viable pollen grains stains red while it remains transparent in non-viable pollen grains. Alexander's dye-stuff (a mixture of malachite green that stains the cellulose of the pollen wall green and acid fuchsin that dyes the pollen grains protoplasts red) shows viable pollen grains that are red while nonviable pollen grains stain green (Alexander, 1969).

The Technovit microscopy and paraffin sections were examined using a Nikon Eclipse 400 light microscope and photographed with a Zeiss Axio Cam MRe digital camera.

RESULTS

THE MICROSPORANGIUM WALL

The flower of *G. quadriradiata* possessed five stamens. Each stamen had four microsporangia that were arranged in pairs in the two symmetrical lobes (Fig. 1b), which were joined by a connective tissue with a centrally located vascular band. The anthers were elongated in the longitudinal sections (Fig. 1c) and each microsporangium was surrounded by a wall that consisted of four visible layers of the cells as follows: the epidermis as the external layer, a slightly deeper cell layer – the endothecium, a middle layer and the innermost tapetum (Fig.1b). Only anticlinal divisions were observed among the epidermal cells as well as within the endothecium cells. The middle layer mostly consisting of parenchyma cells, was very fragile and disappeared very quickly during pollen development and was no longer visible at the stage of young tetrads. The tapetum cells were much larger than the cells of the other layers of the pollen sac wall. The histological analysis revealed

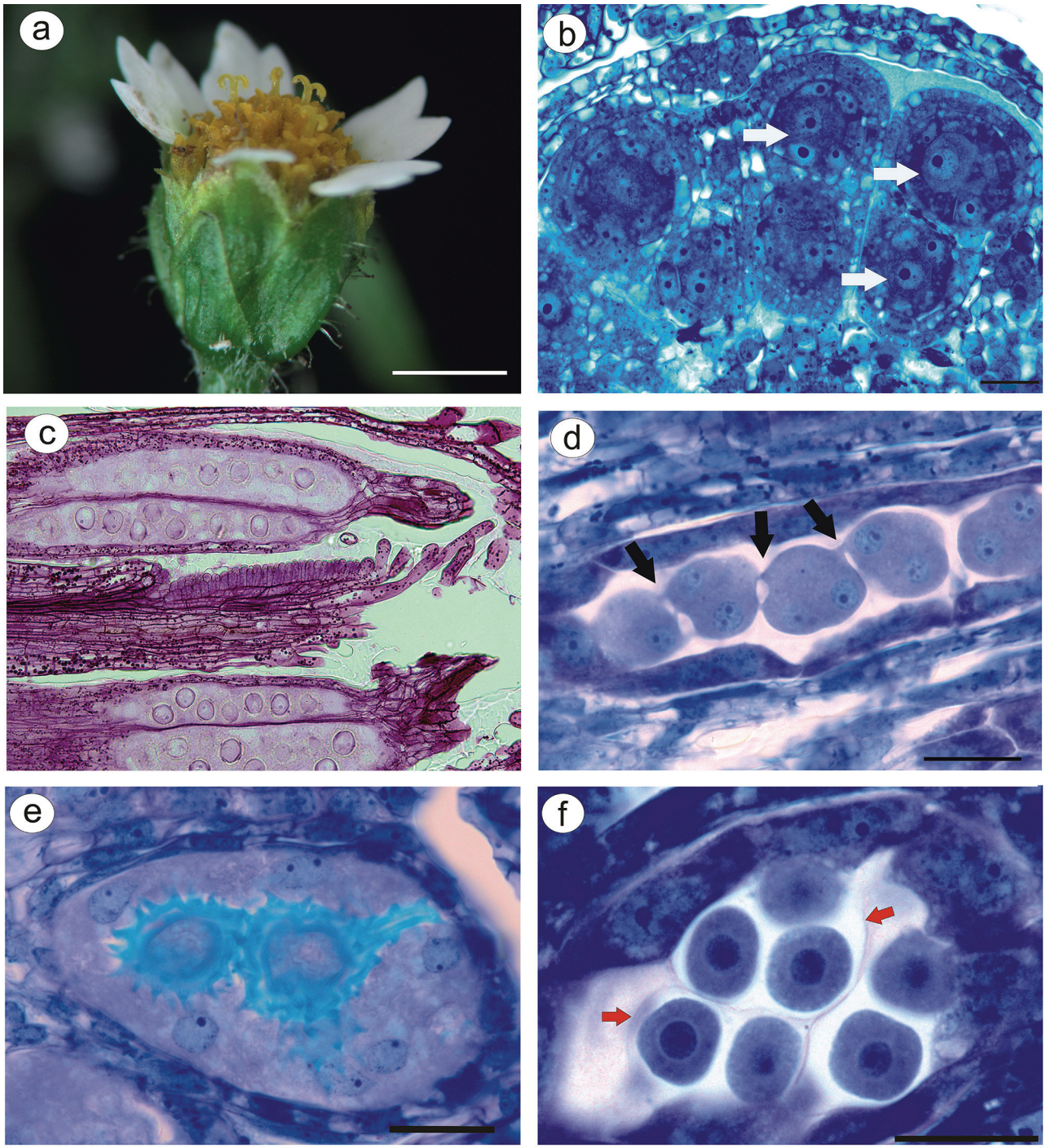

Fig. 1. General outline of inflorescence and several stages of pollen development of *Galinsoga quadriradiata* Ruiz&Pav. (**a**) A view of an inflorescence with compact arrangement of individual flowers with jutting stigmas of the pistils. Bar: 0.5 cm. (**b**) A sample of transversal section of pollen sacs with visible archesporial tissue and microspore mother cells (MMCs) marked with arrow. (Toluidine blue staining). Bar: 20 µm. (**c**) A singular flower section with clearly viewed 2 anthers containing microspores (PAS reaction). Bar: 50 µm. (**d**) A longitudinal section of an anther with good visible multinucleated tapetum cells, tetrads and the presence of cytoplasmic channels between them (arrows). (Toluidine blue staining). Bar: 20 µm. (**e**) Two microspores with specific spiny apertured walls visible on the cross section of a singular anther and peryplasmodium among them. (Toluidine blue staining). Bar: 20 µm. (**f**) A cross section of a pollen sac with visible callose layer around the tetrads (arrows). (Toluidine blue staining). Bar: 20 µm.

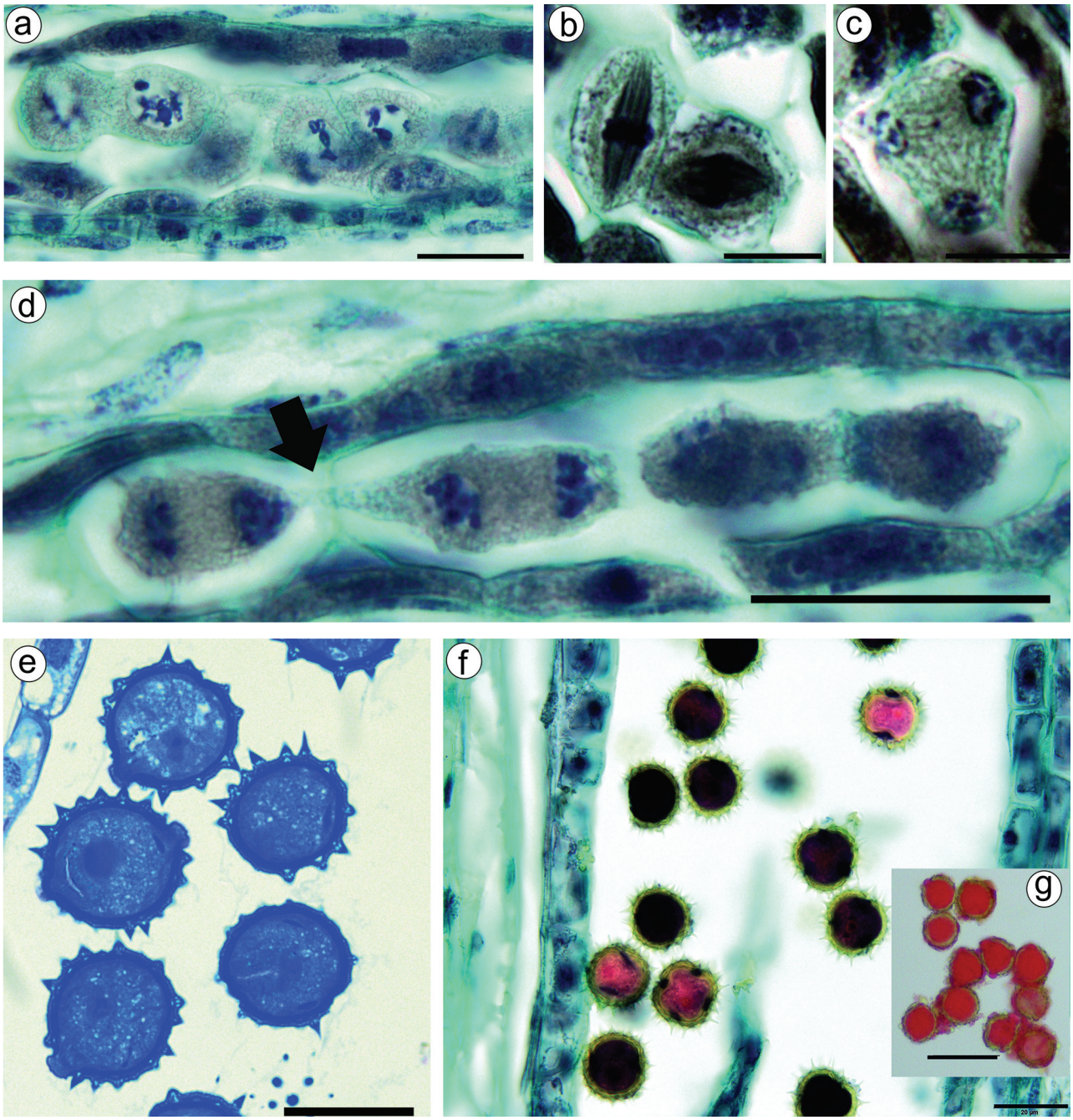

Fig. 2. Next development stages of microspores and pollen grains. (**a**) A section made in longitudinal cutting of a singular pollen sac with visible paring of chromosomes during meiotic division I in MMCs. (Haydenhain's hematoxylin and alcian blue). Bar: 20 μm. (**b**) A cross section of a pollen sac with viewed metaphase plates in MMCs. (Haydenhain's hematoxylin and alcian blue staining). Bar: 10 μm. (**c**) Microspores at telophase stage (Haydenhain's hematoxylin and alcian blue staining). Bar: 10 μm. (**d**) A longitudinal section of microsporangium at telophase stage with visible cytoplasmic channels (arrow) between young microspores. (Haydenhain's hematoxylin and alcian blue). Bar: 10 μm. (**e**) Mature pollen grains with stained nuclei, starch grains and spiny outer walls (methylene blue/ Epoxy Embedding Medium Kit). Bar: 20 μm. (**f**) Mature pollen grains with specific spiny outer walls (Haydenhain's hematoxylin and alcian blue). Bar: 20 μm. (**g**) Viable mature pollen grains tested with Acetocarmine. Bar: 20 μm.

high mitotic activity of tapetum nucleus at the early stages of microsporogenesis and the tendency of the tapetum cells toward endomitotic divisions. The presence of multinucleated tapetum cells was clearly visible, for instance, at the tetrad stage (Fig. 1d). With the beginning of meiosis in the sporogenous tissue, the cell walls of the tapetum cells began to disappear. Their protoplasts then developed an amoeboidal shape and initiated penetration between the microspores that had just been created. The protoplasts merged and formed peryplasmodium (Fig. 1e). Therefore, the type of the development of the tapetum *G. quadriradiata* is the amoeboid tapetum type. At the mature pollen grain stage, the peryplasmodium was fully absorbed and the anther wall only included epidermis and endothecium.

Starch was present in the tissues of the pollen sac wall during pollen development, mostly in the epidermis and endothecium cells until the mature pollen grain stage. There was no visible starch in the tapetum cells at any of the developmental stages of pollen (Fig. 1c).

MICROSPOROGENESIS

The microsporangia of the *G. quadriradiata* had a circular shape in cross-section. The central part of the microsporangium was filled with archesporial tissue. On the anther cross-section, the microspore mother cells were well distinguished among the other cells of the pollen sac. They were of an isodiametric shape with a prominent and large nucleus that was embedded in dense cytoplasm that was centrally located without a vacuole. They were large in volume and tightly packed in the microsporangium (Fig. 1b). The histological analysis revealed that a large nucleus was noted and thin curly chromatin appeared in the microspore mother cells in prophase I of meiosis. During this time, the chromatin formed characteristic and common complexes that are called bouquets of chromosomes. During zygotene homologous chromosomes paired into bivalents (Fig. 2a). Subsequently, after the prophase I, the spindle fibers were created and bivalents were located in the equatorial plate in the metaphase (Fig. 2b). During anaphase I, the bivalents were regularly distributed at the opposite poles and after telophase I, the second meiotic division began (Fig. 2c). Analysis of microsporogenesis revealed the presence of cytomixis. Cytoplasmic connection channels, which represent a huge intercellular connections other than plasmodesmata, were present among the cells that underwent meiosis in the pollen sac; they were visible between two cells at the dyad stage and the adjacent tetrads were also connected by cytoplasmic channels (Fig. 1d and Fig. 2d). After the first meiotic division, a cell wall did not form between the telophase groups. The cytokinesis was of the simultaneous type, because the cell wall was created after the second division of meiosis. The studied tetrads were mostly of the tetrahedral type and still had a callose wall (Fig. 1f). At this stage, the microspores did not contain any starch grains.

MICROGAMETOGENESIS

After meiosis the callose wall surrounding tetrads gradually disintegrated and the microspore wall began to thicken. The young microspores that had been released from the tetrads had dense cytoplasm and a centrally located nucleolus with prominent nucleoli. The mononuclear microspores were round, vacuole-less and did not contain starch grains; however, starch was present in the surrounding tissues (Fig. 1c). The formation of the sporoderm began as the microspores were enclosed within the callose envelope of the tetrad. Subsequently, after the disintegration of the callose wall, the four microspores dispersed. The microspores, which were surrounded by the sporoderm, grew and became more highly vacuolated. They had a large central vacuole and the nucleus along with cytoplasm was pushed to the peripheral position. As a result of the unequal mitosis of the microspore, a large vegetative cell along with a smaller generative cell was formed, and both formed a bicellular pollen grain. The large vacuole in the vegetative cell disappeared and the cell became dense with cytoplasm. Moreover, in contrast to the microspore, a vegetative cell of bicellular pollen accumulated a large amount of starch grains (Fig. 2e). The generative cell had only a small amount of cytoplasm, was smaller than the vegetative one, was spindle-shaped and located near the pollen grain wall. The generative cell then underwent mitosis and a three-celled pollen grain was formed (Fig. 2e).

The histological analysis also revealed that at the stage of closed flower buds that was observed, the development of pollen grains is mostly synchronised in one flower within the anther's loculi. However, it was also observed that different stages of microsporogenesis were visible in one inflorescence, for example, meiosis in one flower and visible single microspores in another.

POLLEN MORPHOLOGY AND VIABILITY

The pollen grains of *G. quadriradiata* had a regular shape and the number of apertures in these pollen grains was usually three (tricolpate); however, four apertures (4-zonocolporate) were also observed but they were in the minority (Figs. 2f–g). The surface of mature pollen grains was not smooth (Fig. 2e). The exine created visible spines that were longer than a micron and that are referred to as Echinatea (Fig. 2f) and the tested pollen grains of *G. quadriradiata* were mostly of a similar shape and size.

It was also found that among the 1053 pollen grains which were tested, 935 were viable (88%) in the acetocarmine test (Fig. 2g) along with 1110 that were viable (87%) out of the 1269 grains in Alexander test. Both tests that were used showed a high degree of viability of the tested pollen grains of *G. quadriradiata* flowers .

DISCUSSION

Although *G. quadriradiata* is a weed and a plant of an invasive character all over the world, studies about this species are not numerous. Those that investigate the embryological aspects of this plant are also quite rare. Although Popham (1938) presented a comprehensive description of the female gametophyte of this genus, there is still lack of literature concerning the development of the male gametophyte. The size and viability of a Himalayan population of *G. quadriradiata* pollen grains described by Gopinatha and Babu (1982) are similar to the Polish one.

As has been observed in our research, the course of pollen development as well as the anther structure of *G. quadriradiata* are typical of those that have been described for the *Heliantheae* (Pullaiah, 1981) and for those that have been reported for *Galinsoga parviflora* (Pullaiah, 1981).

The structure and development of the anther wall are consistent with the typical characteristics of Angiospermae that have been described for many genera, e.g., *Chrysanthemum morifolium* Ramat. (Li et al., 2010) as well as *Aster subulatus*, *Kalimeris indica*, *Heteropappus arenarius*, *Erigeron annuus* (Ao et al., 2009). The differentiation of the tapetum and its high mitotic activity that results in multinucleate cells in which the nuclei are positioned in a row are typical of the *Solidago* genera (Musiał, 1989) or *Valeriana officinalis* (Skalińska, 1958) as well as of *Taraxacum*, in which a high polyploidy level is achieved, namely = 36n (Małecka, 1961, 1971). At about this time, the pollen mother cells (PMC) create the dyads and the next isobilateral tetrads, which is a common pattern not only for Asteraceae but is also observed, for example, in *Brachypodium dystachyon* in Poaceae (Sharma et al., 2014) and *Asphodelus aestivus* (Filiz et al., 2013).

The function of the meiotic bouquet is to make meiotic prophase much faster and more efficient (Harper et al., 2004), their presence in *G. quadriradiata* can be correlated with the proper course of meiosis and as a consequence with the assurance of the development of normal pollen grains. Although the male meiocytes are surrounded by the cell wall and then during microsporogenesis by the callose deposition, a connection exists between these cells. This connection is provided by cytoplasmic/cytomictic channels that were also observed between meiocytes and between tetrads in *G. quadriradiata*, which can additionally facilitate the symplastic connectivity. They represent a cell wall channel type that is different from plasmodesmata, have no internal structure, such as desmotubules and – compared with them – have relatively large apertures that vary from 50 to 500 nm (Baquar and Husain, 1969; Heslop-Harrison, 1966; Mursalimov et al., 2010). In addition, they may occupy over 25% of the cell surface (Heslop-Harrison, 1966). Mursalimov et al. (2013) proposed the scheme of cytomictic channels formation in angiosperm microsporogenesis. According to these authors, microsporocytes entering meiosis, surrounded by a primary cell wall, are connected by plasmodesmata. Next, at zygotene-pachytene stage, cells are connected by primary cytomictic channels, either formed of plasmodesmata or independently of them. When the primary cell wall is replaced by callose, firstly created connections become closed and the secondary channels can be formed in the callose wall with the help of spherosome-like vesicles, containing the enzyme callase. Under the influence of the released callase enzyme, the callose wall could be locally destructed and formation of the cytomictic channel is possible, during all meiotic stages, including the late stages. The connections observed in the tested *G. quadriradiata* material, between tetrads, could be the secondary cytomictic channels, but this suggestion must be thoroughly checked and needs further investigation. These connections join the cell protoplasts across the cell wall and coordinate the development and growth (Wang et al., 2006), play a critical role in plant morphogenesis (Lucas et al., 1995; Sessions et al., 2000), as well as in the defense responses (Voinnet et al., 1998) by controlling intercellular communication (Mourrain et al., 2000). Furthermore, according to Wang and coauthors (2004), the cytoplasmic channels provide for the intercellular trafficking of water, ions, nutrients, various macromolecules and metabolites in the response of plants to environmental stimuli.

Although in the past cytomixis was considered to be an anomaly, either pathological (Morisset, 1978) or induced by fixation or by traumatic injury (Takats, 1959), it is now usually considered to be a normal, though infrequent, cytological phenomenon (Bellucci et al., 2003). According to Mursalimov et al. (2013), the absolute majority of the instances of cytomixis have been noted in the microsporogenesis of angiosperms and have been described in over 400 plant species belonging to 84 families. Among the Asteraceae family this process has been observed in 13 genera, e.g. *Artemisia* (Malik et al., 210), *Helianthus* and *Rudbeckia* (Whelan, 1974),

Heliopsis, *Ambrosia*, *Achillea* (Cooper, 1952); however, this is the first report about cytomixis in *Galinsoga*.

The occurrence of the callose around the microsporogenous tissue in Angiosperms during microsporogenesis is not unique feature because it was also found in Gymnosperms in *Larix* (Tretyn et al., 1987). The callose deposition has an influence on the proper development of pollen, but also the timing of callose activity is critical. The degradation of callose is accomplished by the callase that is secreted by the tapetum (Steiglitz, 1977) and premature or delayed callase activity is believed to be responsible for pollen abortion in some cytoplasmic male-sterile lines (Frankel et al., 1969; Warmke and Overman, 1972). During the microsporogenesis of *G. quadriradiata*, the callose surrounded the microspore mother cells then held together the microspores and started to hydrolyse when the construction of sporoderm of the released microspores had begun. This situation seems to be similar to other plants (Heslop-Harrison et al., 1986), where the correct callose distribution ensures the development of fertile pollen. Such occurrence of callose deposition can support the thesis of the normal course of meiosis of *G. quadriradiata*. Moreover, Furness and Rudall (2004) confirmed that the presence of callose after the first meiotic division is an important prerequisite for the normal successive division. Additionally, these authors noted that the callose deposition is implicated in both cell division and aperture formation in pollen grains (Furness and Rudall, 2000).

As has been revealed, the cytokinesis of *G. quadriradiata* is of the simultaneous type and in this way represents the one that is typical of the eudicot clade of Angiosperms, which is a kind of cytokinesis (Furness, 2008). In this type of cytokinesis, the nuclei and spindles of the second meiotic division can interact and the nuclei usually take up positions as far away from each other as possible, thus leading to the formation of tetrahedral tetrads (Blackmore and Crane, 1998), which is similar to our observations in *G. quadriradiata*. Furthermore, this type of cell division is correlated with the formation of tetrahedral tetrads (Blackmore and Crane, 1998) and related to their tricolpate characteristic (Furness and Rudall, 2004), which was also visible in *G. quadriradiata*. The position of the apertures during development suggests the existence of a close relationship between the aperture pattern definition and the cytoplasmic compartment that completes meiosis (Blackmore and Crane, 1998).

Each of the five *G. quadriradiata* stamens had four microsporangia. During microsporogenesis in one flower, meiotic divisions proceeded in a mostly synchronous manner, although the development of pollen in different flowers among the same inflorescence did not. This phenomenon can be regarded as an advantage for this species because the asynchronous process of pollen development is important for plant reproduction and is one of the reasons for the strong invasive ability and long flowering period, which is similar to another invasive plant – *Ambrosia artemisiifolia* (Liu et al., 2012).

According to Walker and Doyle (1975) and Stuessy (2009), the pollen features, e.g., grain shape, the number of apertures and sporoderm layers are important for phylogeny and taxonomy. The suitable size and high viability of *Galinsoga* pollen grains may be factors that enhance their potential of invasiveness. The pollen viability of *G. quadriradiata* of Polish populations that were tested represented 87% and 88%, while the pollen viability of Himalayan populations varies from 92 to 100% (Gopinathan and Babu, 1982). These values appear to be similar. This high viability of *G. quadriradiata* pollen grains indicates the high level of reproducibility of this plant. The observations of this great generative potential could be supported by the presence of four apertures, which is not observed very often in eudicots as eudicots are usually tricolporate. The increasing number of apertures, which was also observed in *Galinsoga*, as Furness and Rudall (2004) suggest, offers a potential selective advantage because it increases the number of prospective germination sites. What is more, these authors are convinced that this is the general trend in angiosperms. The increased number of pollen apertures suggests that this direction is under strong selection pressure. The shape and size of Polish *G. quadriradiata* pollen grains appear to be similar to the pollen of *G. quadriradiata* from Portugal that was described by Coutinho and Paiva (2003).

CONCLUSIONS

The cytological events and histological analysis that were observed during pollen development in *G. quadriradiata* Ruiz & Pav in our experiment could be treated as factors that have an influence on its high generative reproducibility and can support the invasive character of this species. The regular and proper course of meiosis with the correct callose formation and degradation, chromosome bouquets and the appearance of cytoplasmic channels, the asynchronous development of pollen and the high viability of pollen grains make *G. quadriradiata* a good reproductive species, which is correlated with their invasive character and can support this feature and thus lead to the formation of aggressive weedy races that have the ability to colonise highly disturbed ecological habitats. It appears that *G. quadriradiata*

from Polish habitats has a great generative potential that is similar to that described by Jursik et al. (2003) from Czech flora and slightly smaller than the one from Indian flora (Gopinatha and Babu, 1982).

AUTHORS' CONTRIBUTIONS

All of the authors contributed to the conception and design, acquisition of data, analysis and interpretation of the data, and drafting or critical revision of the paper. The authors declare that they have no conflicts of interest.

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