



REVIEW ARTICLE

Phytomelanin in Asteraceae: A Review

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Abstract

Phytomelanin is a dark brown to black, highly resistant amorphous layer, found in the pericarp of mature cypsela of Heliantheae Alliance (Asteraceae). It is a non-cellular organic mass, formed after fertilization as an extra cellular deposition in the schizogenous space between the hypodermal and fibre zones in the pericarp wall. It is an important taxonomic character which has been used in the tribal and sub tribal classification in Asteraceae. The phytomelanin is suggested to be a product deposited either from the hypodermis or epidermis which lie above the layer or from the cells of fibre zone lying inner to it. The different taxa of the Heliantheae alliance exhibit surface ornamentation due to the variation in the deposition pattern of phytomelanin. These variations are noticeable in the form of spines, pegs and striations.

Keywords: Cypsela, Phytomelanin, Heliantheae Alliance, Asteraceae.

Introduction

Phytomelanin is the dark amorphous and highly resistant substance present in the fruit walls (pericarp) of mature cypselae of some members of Asteraceae (Stuessy and Liu, 1983; Pandey *et al.*, 1989; Pandey and Dhakal, 2001; Pandey *et al.*, 2014). This highly resistant layer was reported to be present only in the members of the two tribes Heliantheae and Eupatorieae of Asteraceae in the early literature available (Misra 1972; Hegnauer 1977; Pandey and Singh 1982; Pandey and Singh 1983; Stuessy and Liu 1983; Pandey and Kumari 1987) against the recent understanding of it being present in the 11 tribes of the clade Heliantheae alliance within the family Asteraceae (Panero, 2007; Tadesse and Crawford, 2014; Pandey *et al.* 2014; Mathur and Pandey 2020). Apart from the

fruits of Asteraceae, it is also reported very recently to be present in the seeds of the monocot families of the order Asparagales like Agavaceae, Alocaceae, Amaryllidaceae, and Hyacinthaceae (Chase, 2004) as well as in the vegetative parts of different higher plants (Wichtl, 2004; Fritz and Saukel, 2011; Lusa *et al.*, 2018; Coutinho *et al.*, 2021).

Although Hanausek (1902) had recognized and reported the black carbonised layer, phytomelanin a century ago, attention was given to it only in the latter half of 20th century when its role in providing resistance against sunflower moth was detected in the cultivated sunflower, *Helianthus annuus* (Kiewnick, 1964; Carlson and Witt, 1974; Rogers and Kreitner, 1983). Hanausek (1913) reported its presence in the fruit walls of 98 species of Asteraceae belonging to the tribes Eupatorieae and Heliantheae. Many studies that followed also observed this chemically inert and tough, brownish black layer which occurs in the schizogenous space which develops between hypodermal and fibre zones after fertilization in Asteraceae fruits (Hanausek, 1902; Hanausek, 1907, 1911, 1912, 1913; Platechek, 1927; Putt, 1940; Politis, 1957; Pandey, 1976; Hegnauer, 1977; Pandey and Singh, 1982; Rogers *et al.*, 1982; Pandey and Singh, 1983; Rogers and Kreitner, 1983; Stuessy and Liu, 1983; Pandey and Kumari, 1987; Pandey *et al.*, 1989; Pandey and Jha, 1993a; Pandey and Singh, 1994; Pandey, 1999; Pandey and Dhakal, 2001; Pandey and Kumari, 2007; Tadesse and Crawford, 2014; Pandey *et al.*, 2014).

Robinson (1981) considered the presence or absence of the carbonisation or black layer formed by phytomelanin as the most significant feature of the cypselae of Heliantheae and closely related members. The significant modifications

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that had effected the changes in Asteraceae systematics has also resulted in the understanding of distribution of phytomelanin in Asteraceae in more tribes other than Heliantheae and Eupatorieae. Most of the past literature cites phytomelanin to be present in the fruit walls of the members of the tribes Eupatorieae and Heliantheae, excluding the subtribes Gaillardiiinae and Marshallinae (Misra, 1972; Pandey, 1976; Hegnauer, 1977; Pandey, 1977; Pandey and Singh, 1982; Pandey and Singh, 1983; Pandey and Kumari, 1987; Pandey and Jha, 1993a; Pandey and Singh, 1994; Pandey, 1999; Pandey and Dhakal, 2001; Pandey and Kumari, 2007).

Heliantheae alliance

The Heliantheae alliance (*sensu* Panero, 2007) or Helianthoid group (*sensu* Bremer, 1996) contains *ca.* 5500 species (Baldwin, 2009) within 13 tribes: Bahiaee, Chaenactideae, Coreopsideae, Eupatorieae, Helenieae, Heliantheae, Madieae, Millerieae, Neurolaeneae, Perityleae, Polymnieae, Tageteae and Feddeae (Funk *et al.*, 2009; Pandey *et al.*, 2014). Heliantheae alliance as one of the most highly derived clades formed of 13 tribes within the subfamily Asteroideae (Panero, 2007; Funk *et al.*, 2009). The only unique feature of most taxa of the Heliantheae alliance is the presence of a phytomelanin layer which imparts the fruit a black or dark brown colour in the cypselae (Panero, 2007). Phytomelanin is characteristic of all tribes of Heliantheae alliance, except the two the tribes Helenieae and Feddeae. Heliantheae alliance represent approximately half of the species of sunflower family in the Western Hemisphere and nearly one quarter of all species in the family. The members of the group are annual or perennial herbs with well represented shrub and tree species. Most species can be found in seasonally dry habitats (Panero, 2007).

Traditionally the tribe Heliantheae was considered primitive and close to the ancestors of the family (Bentham, 1873; Bessey, 1915; Hutchinson, 1916; Cronquist, 1955; Turner, 1977). It has been now considered highly nested in the phylogenetic ladder (Funk *et al.*, 2009). Further phylogenetic investigations have been especially helpful in revealing relationships within the Heliantheae alliance (Panero *et al.*, 1999; Baldwin *et al.*, 2002; Panero and Funk, 2002; Kimball and Crawford, 2004; Crawford and Mort, 2005; Mort *et al.*, 2008; Panero and Funk, 2008; Funk *et al.*, 2009).

ITS data equally has played an important role in delimiting the tribes of the Heliantheae alliance (Baldwin *et al.*, 2002). Molecular phylogenetic analyses have shown Eupatorieae to be nested within the Heliantheae alliance (Baldwin *et al.*, 2002; Panero and Funk, 2002) and also revealed Helenieae to be the basal sister clade (Jansen and Palmer, 1987a, Jansen and Palmer, 1987b; Jansen and Palmer, 1988; Jansen *et al.*, 1991; Jansen and Kim, 1996; Panero and Funk, 2002). The alliance evolved as a well-supported monophyletic group in the further studies (Jansen *et al.*, 1990, 1991; Kim *et al.*, 1992; Bayer and Starr, 1998). Further *ndhF* sequence data revealed

strong sister group relationship between Athroismeae-Feddeae as sister groups of Heliantheae alliance (Kim and Jansen, 1995; Eldenäs *et al.*, 1999; Funk *et al.*, 2005; Cariaga *et al.*, 2008).

Panero (2008) recognized a clade to include the tribes with phytomelanin in their fruit walls which included 11 tribes from the alliance and the tribe Athroismeae and recognized a clade- The Phytomelanin Cypselae Clade (PCC), encompassing the tribes Coreopsideae, Bahiaee, Chaenactideae, Tageteae, Neurolaeneae, Polymnieae, Heliantheae, Millerieae, Madieae, Perityleae and Eupatorieae, and part of Athroismeae to include 5400 species in 460 genera mainly distributed in the Neotropics and are endemic to the New World (Panero, 2007; Panero, 2008; Panero and Funk, 2008).

Phytomelanin outside Heliantheae alliance

Besides its occurrence in the members of Heliantheae alliance, the phytomelanin has also been reported to occur in some other members of the Asteraceae. Phytomelanin is also reported in very few members of the tribes Athroismeae (Athroisminae), Vernonieae and Carduae (Fritz and Saukel, 2011; Freitas *et al.*, 2015; Coutinho *et al.*, 2021) which are not part of the alliance. The cypselae of four genera in the *Sipolisia* group of Vernonieae show spotty phytomelanin in the pericarp (Robinson, 1996; Robinson, 2007; Robinson, 2009; Loeuille, 2011). It is secondarily absent in some members of Heliantheae alliance whilst occur as a parallelism in a few Vernonieae (Panero, 2007). Katinas and Crisci (2008) reported the presence of phytomelanin in the cypselae of some species of *Panphalea* and *Holochelium* of subtribe Nasauviinae of tribe Mutisieae. The report of phytomelanin deposition around the fibre tissue of the mesocarp and vascular bundles in *Wunderlichia* spp. takes the count of phytomelanin presence to a new subfamily Wunderlichioideae (Bonifácio *et al.*, 2019).

Phytomelanin in Monocots

The term phytomelanin is also used for the black-coloured deposits in different tissues of certain plants outside the family Asteraceae (Tremolieres, 1982; Dahlgren *et al.*, 1985; Metche and Manbenot, 1970; Wittich and Graven, 1995 and 1998; Dahlgren and Clifford 1982). In monocots it occurs in the outer epidermis of the seed testa (Huber, 1969; Dahlgren and Clifford, 1982; Dahlgren *et al.*, 1985). Pigments in the epidermis when present are intracellular in Asparagales, more specifically on the inner walls of the seed testa (Wittich, 1998). Wittich and Graven (1995, 1998) studied in detail the deposition of phytomelanin in the seeds of *Gasteria verrucosa* (Mill.) Duval (synonym of *Gasteria carinata* var. *verrucosa* (Mill.) van Jaarsv., family Xanthorrhoeaceae). In their study a callose deposition is formed internally on the cell wall which is degraded by the phytomelanin precursors. Pigments in the epidermis when present are intracellular

in Asparagales, more specifically on the inner walls of the seed testa (Wittich, 1998). The presence of dark-coloured integuments in the seed coat of members of Orchidaceae like *Pogoniopsis schenckii* and *Vanilla planifolia*, and *V. palmarum* is suggestive of phytomelanin like substances (Alves *et al.*, 2019).

Morphological Nature of Phytomelanin

In the earlier studies on phytomelanin, it has been classified as an exclusive inert dark substance present in the walls of mature fruits of some selected Asteraceae members. It was described as “the coal-like black substance” (Hanausek, 1913), “the rigid part of the seed coat” (Putt, 1940), “armour layer” (Arnoldova 1926; Platechek 1927), “brittle, coal-like substance, rich in carbon” (Dahlgren and Clifford (1982), “the black pigment stored in epidermal cells forming a continuous melanin cork” (Takhtajan, 1985; Takahama, 2004), “phytomelano-plaster” (Politis, 1957), “carbon layer” (Robinson, 1981) or as black pigment layer (Roth, 1977).

Developmental Pattern

De Vries (1948a) suggested that in the genera *Helianthus* and *Tagetes*, the phytomelanin deposition starts with the appearance in intercellular spaces of colorless, oily substances which gradually solidifies and becomes brown to black. With no physical structure of its own, phytomelanin forms characteristic ornamentations on the surface of the mature fruits following the contour of the walls of hypodermis (Stuessy and Liu, 1983; Pandey *et al.*, 1989). Though always found in a solid state, phytomelanin has an original fluid or plastic consistency when it is freshly synthesised as inferred from the different shapes in different species (Pandey and Dhakal 2001; Pandey *et al.*, 2014).

The developmental pattern of phytomelanin in the walls of the cypselae has been observed and documented in detail using both light microscope and electron microscope (Stuessy and Liu, 1983; Pandey *et al.*, 1989). Putt (1940) had stated that in sunflower (*Helianthus annuus*), the brown black pigment starts appearing 4 to 6 days after anthesis, a view also shared by Arnoldova (1926). However, Rogers and Kreitner (1981); Rogers *et al.* (1982); Pandey and Dhakal (2001) and De-Paula *et al.* (2013) suggested it to initiate its deposition on the 3rd day after fertilization which continues rapidly till 13th day after which the process slows down. Based on ultrastructural analysis, phytomelanin deposition in the stem tissues of *Piptocarpha axillaris* of tribe Vernoniaeae Coutinho *et al.* (2021) reported initiation of polymerization of phytomelanin starts at the second stem node, in the pith region, and proposed that the plastids actively participate in the phenolic material synthesis, following the “tannosome” and the “pearl necklace” models, which is then polymerized in the intercellular spaces during the sclerification process as phytomelanin.

Source of phytomelanin

Features of seeds and dry indehiscent fruits have been the subject of numerous studies among vascular plants which have yielded valuable taxonomic data (Brisson and Peterson, 1977). Cypselar and seed-coat anatomy have been shown on numerous occasions to be useful in inferring taxonomic and evolutionary relationships in Asteraceae (Dittrich, 1977; Heywood and Humphries, 1977; Grau, 1980; Stuessy and Liu, 1983; Singh and Pandey, 1984). The phytomelanin is suggested to be a product deposited either from the hypodermis or epidermis which lie above the layer or from the cells of fiber zone lying inner to it. Hanausek (1910) and Roth (1977) had proposed three possible processes responsible for the synthesis of the “carbon or phytomelan layer”- intracellular deposition by the accumulation of a black pigments within the epidermis cells or intercellular deposition, that is formed in the lysigenous intercellular spaces; or degradation of entire cells or cell layers, with subsequent carbonization of their walls.

Sárkány (1947) hypothesized that the hydration of carbohydrates by high metabolic changes in the hypodermal cells result in the formation of phytomelanin. De Vries (1948a) suspected hypodermis as the source of origin of phytomelanin based on his observation of granules which show cellulosic reaction, in the cytoplasm of the hypodermal cells of *Tagetes* fruits. It has also been envisaged as the by-product of disintegration of the fibre cells (Knowles, 1978).

Most of the researchers, however, believe that phytomelanin is a non-cellular substance that is an exudate of the hypodermis. Many authors provided ultra-structural data to support this theory based on light microscopy, that the hypodermis is the source of phytomelanin (Sárkány, 1947; De Vries, 1948a; De Vries, 1948b; Misra, 1964; Misra, 1972; Pandey and Singh, 1982; Pandey and Singh, 1983; Pandey *et al.*, 1989; Pandey and Singh, 1994; Pandey, 1999; Pandey *et al.*, 2014).

Phytomelanin is suggested to be the lytic product of inner cell layers of the hypodermis (Rogers *et al.*, 1982). It has also been suggested to be ametabolic precipitate from the catabolism of hypodermal cells (Rogers and Kreitner, 1983). Both hypodermal and fibre cells synergistically is involved in the hardening of phytomelanin (Rogers and Kreitner, 1983; Pandey and Singh, 1983; Stuessy and Liu, 1983). Phytomelanin precursors are hypothesized to be produced in the hypodermis and the polymerization is speculated in the extracellular space of the schizogenous cavity (Pandey *et al.*, 1989). Marzinek and Oliveira (2010) also agree with Pandey *et al.* (1989) in suggesting that, the outer mesocarp (hypodermis) is of secretory in nature which is corroborated by the presence of dense cytoplasm and nuclei. De-Paula *et al.* (2013) suggested the role of only fibre cells in the secretion of phytomelanin.

Anatomy of Cypsela

Detection of the phytomelanin layer in most of the studies was mainly done anatomically by microscopic examination of fruit cross sections (Putt, 1940; Kiewnick, 1964; Johnson and Beard, 1977; Rogers and Kreitner, 1981; Pandey and Singh, 1983; Rogers and Kreitner, 1983; Stuessy and Liu, 1983; Pandey *et al.*, 1989; Pandey *et al.*, 2014) or by chemical tests (potassium dichromate-sulfuric acid) (Carlson *et al.*, 1972; Rogers *et al.*, 1982). Initial reports of phytomelanin from the anatomical studies have been mainly on *Helianthus annuus* as more attention was given to economically and agronomically important crops (Putt, 1940; Sárkány, 1947; Kiewnick, 1964; Carlson and Witt, 1974; Knowles, 1978; Rogers and Kreitner, 1983). Developmental anatomy of cypselae of the members of tribe Heliantheae has been studied by several workers (Kapil and Sethi, 1962; Chopra, 1968; Misra, 1972; Rajan, 1972; Chopra and Singh, 1976; Maheshwari Devi and Ranjalkar, 1979; Pandey and Singh, 1982; Stuessy and Liu, 1983; Maheshwari Devi and Padma, 1985; Pandey *et al.*, 1986a; Pandey and Kumari, 1987). All the studies invariably report the existence of a resinous or tanniferous substance in the schizogenous space of the pericarp. In all the early studies on Asteraceae members of Heliantheae alliance, the presence of phytomelanin was specifically reported as an intercellular material exclusively developing as a post fertilization deposition in the schizogenous cavity (Stuessy and Liu, 1983; Pandey and Dhakal, 2001; Pandey *et al.* 2014).

Pigments in the epidermis and hypodermis are intracellular whereas the phytomelanin deposition is extracellular (Putt, 1940; Misra, 1972). The tannin deposition has been found to be a common feature within Heliantheae fruits *viz.*, *Verbesina encelioides* (Misra, 1972), *Blainvillea rhomboidea*, *Spilanthes acmella* (Rajan, 1972), *Xanthium strumarium* (Chopra, 1968), *Guizotia abyssinica* (Chopra and Singh, 1976), *Eclipta erecta* (Pandey, 1976). Knowles (1978) observed that the phytomelanin gets deposited between the hypodermis and outer cell layer of the middle layer (fibre zone) as 'pointed cones and hooklets', initially from the tangential walls and later on from the radial walls also. He also observed that initially the layer appears dark and later becomes black. The discontinuous nature of the fibrous tissue in the cypsela has been correlated with the discontinuous phytomelanin layer in *Guizotia* by Chopra and Singh (1976) and species of *Coreopsis* and *Cosmos* (Pandey and Jha 1992; Pandey *et al.*, 1986 b). The same is corroborated by Mathur and Pandey (2020) based on studies on 12 taxa of the tribe Coreopsideae. Similar pattern of deposition of phytomelanin between the hypodermal and fibre layers has been reported in Eupatorieae (Robinson and King, 1977; Pandey and Singh, 1983; Pandey and Singh, 1994) and in Helenieae *s.l.* (Griebel, 1913; Pandey *et al.*, 1986a; Pandey *et al.*, 1986b; Karis and Ryding, 1994). Freitas *et al.* (2015) studied cypsela anatomy of *Heterocoma* (Vernonieae) and proposed

a different pattern of deposition of phytomelanin- between the inner parenchyma and outer fibrous mesocarp. Different patterns of deposition of phytomelanin has been reported in literature- in the schizogenous space between the outer parenchymatic and inner lignified mesocarp (Pandey and Singh 1983, 1994; Marzinek and Oliveira, 2010; De-Paula *et al.*, 2013; Freitas *et al.*, 2015); between the inner parenchymatous layer and inner lignified cells (Misra 1964, 1972; Pandey and Singh 1982, 1994; Stuessy and Liu 1983; Pandey, 1998, Julio and Oliveira, 2009; Frangiote-Pallone and Souza, 2014; Pandey *et al.*, 2014; Freitas *et al.*, 2015; Mathur and Pandey, 2020) and between the outer (lignified) and inner (parenchymatous) mesocarp (Freitas *et al.*, 2015). Each of the groups where phytomelanin presence in the fruit wall also exhibit a unique pattern of deposition (Freitas *et al.*, 2015).

Phytomelanin in Vegetative parts

Presence of phytomelanin in parts other than the cypsela has been reported in the cells of secondary phloem in the cortex, and in pith of roots in *Centaurea jacea* of tribe Cardueae (Fritz and Saukel, 2011). The presence of similar phytomelanin-coated cells has also been reported in the underground vegetative structures of *Parthenium integrifolium* (Upton *et al.*, 2011) and *Echinacea angustifolia* (Wichtl, 2004; Upton *et al.*, 2011) of tribe Heliantheae. Recently, Coutinho *et al.* (2021) reported the presence of phytomelanin more frequently in the cortex, pericycle, primary phloem, secondary phloem, and pith regions of the stem of *Piptocarpha axillaris* (Less.) Baker, tribe Vernonieae (Asteraceae). Lusa *et al.* (2018) had reported the occurrence of phytomelanin in the aerial vegetative organs (stems and leaves) of Asteraceae for the first time. Presence of phytomelanin in the intercellular space of the outer mesocarpic sclerenchyma of pericarp has been reported in *Lychnophora salicifolia* of Vernonieae (Marques *et al.*, 2021).

Phytomelanin and Crystals- Antagonistic or not?

Mukherjee and Nordenstam (2010) have reported that the formation of calcium oxalate crystals may be antagonistic to the formation of phytomelanin pigment, since all taxa with a phytomelanin layer do not possess these crystals. Helenieae (Bremer, 1996) comprises taxa that have raphides or druses rather than phytomelanin in the cypselae walls unlike the other ingroup and outgroup lineages of the Heliantheae alliance (Baldwin, 2009). Similar type of statement was advocated by Robinson and King (1977) on the cypsela wall of the tribe Eupatorieae. Although *Gaillardia* with crystalliferous tissues in the pericarp (earlier placed in Heliantheae, presently placed within subtribe Gaillardiiinae, Helenieae) has no phytomelanin layer (Mukherjee and Nordenstam, 2010). Freitas *et al.* (2015) reported phytomelanin in *Heterocoma* spp. (Vernonieae) and observed distinct absence of crystals in the fruit walls in their study. Marques *et al.* (2021) for the first time reported

the occurrence of the phytomelanin along with the calcium oxalate crystals in the mesocarp of *Lychnophora salicifolia* (Vernonieae) Asteraceae.

Developmental study

Understanding of the origin and synthesis of phytomelanin has improved considerably after the availability of ultra-structural data. Detailed study of phytomelanin as a taxonomic marker using electron microscopic studies has been worked out only in a few genera- *Tagetes patula* (Pandey, 1999), *Ageratum conyzoides* (Pandey *et al.*, 1989), *Clibadium*, *Desmanthodium* and *Ichthyothere* (Stuessy and Liu, 1983), *Bidens biternata* (Kothari and Sharma, 2009), 6 members of the tribe Eupatorieae (Marzinek and Oliveira, 2010), *Praxelis diffusa* (De-Paula *et al.*, 2013), *Coreopsis* and *Bidens* species (Tadesse and Crawford, 2014). Other works include Stuessy and Liu, 1983; Pandey *et al.*, 1989; Mukherjee and Sarkar, 1998; Pandey, 1999; Julio and Oliveira, 2009; De-Paula *et al.*, 2013; Jana and Mukherjee, 2012; Jana and Mukherjee, 2013; Tadesse and Crawford, 2014; Pandey *et al.*, 2014). Stuessy and Liu (1983) had given a substantial indication of phytomelanin as a valuable taxonomic marker in their study on *Clibadium*, *Desmanthodium* and *Ichthyothere*. The pioneering work which revealed the developmental pattern was attempted by Pandey *et al.* (1989) on *Ageratum conyzoides* that redefined the studies on phytomelanin. The present understanding of phytomelanin development and deposition is based on this study.

Electron microscopic studies (Pandey *et al.*, 1989; Pandey, 1999) have shown that abundant smooth and apparently tubular endoplasmic reticulum in the hypodermal cells is similar to the reticulum characteristic of and often found in secretory cells of other plants (Luttge and Schnepf, 1976; Robards and Stark, 1988). During post-fertilization stages, the outer zone of pericarp (epidermis and hypodermis) detaches from the inner zone (fibre layers and parenchyma layers) following which there is the development of a schizogenous gap between the fibres and the hypodermis where phytomelanin gets deposited. Simultaneous to the development of this space, there also develops small 'cone-shaped extensions' (Stuessy and Liu, 1983) or 'papillae' (Pandey *et al.*, 1989; Pandey, 1999; Pandey and Dhakal, 2001) which are outgrowths that connect the fibre to the inner hypodermal cells, from the outer tangential walls of the fibres and later from the radial walls. Marzinek and Oliveira (2010) reported formation of numerous anticlinal projections from the external cells of inner mesocarp (fibrous zone) towards the outer mesocarp (hypodermis) which produces the schizogenous space between the two mesocarpic regions. They also have reported that the width of phytomelanin layer varies in different species and that sclerification of peripheral layers of inner mesocarp happens in later stages in all the Eupatorieae species they have studied. According to Marzinek and Oliveira (2010) the schizogenous space

between the two layers is contiguous with the intercellular spaces of the outer mesocarp (hypodermal), resulting in an irregular (discontinuous) deposition of phytomelanin in some members.

According to Pandey *et al.* (1989) it is along the tips of the papillae that the phytomelanin layer is first visually expressed followed by the inner hypodermal walls where the papillae are in contact. The deposition of phytomelanin which is observed by the 3rd day after fertilization, increases till the 13th day, after which it wanes off (Rogers *et al.*, 1982). Fibre cell walls are covered with phytomelanin earlier to the hypodermal cells. The lignification of the sclerenchymatous cells of pericarp of cypselae with phytomelanin also were reported to start much earlier and appear thicker (Rogers *et al.*, 1982; Rogers and Kreitner, 1983). Pandey *et al.* (1989) described the hypodermal cells to have large central vacuole and high metabolic activity with active mitotic phase. The genera lacking phytomelanin show thin-walled hypodermis which are compactly arranged whereas the hypodermal layers become disarranged and impacted (Pandey and Dhakal, 2001). With regard to fibre cells, the sclerenchyma lignifies earlier than in those genera lacking phytomelanin (Rogers *et al.*, 1982; Rogers and Kreitner, 1983).

The ultra-microscopic studies done by Pandey *et al.* (1989) have revealed that the hypodermal cells to possess ample smooth endoplasmic reticulum/ tubular ER similar to the secretory cells (Luttge and Schnepf, 1976; Robards and Stark, 1988). There is no secretory ER in fibres encountered before phytomelanin deposition (Pandey, 1999). Fresh deposits of phytomelanin flows from the hypodermal walls (facing the schizogenous space) that are not covered by phytomelanin (Pandey and Dhakal, 2001). They have also discussed an option that the older or earlier deposited phytomelanin might be retaining a degree of porosity to allow the migration of fresh phytomelanin.

Pandey *et al.*, (1989) proposed that phytomelanin polymerization takes place outside the walls of fibre cells due to enzymatic activity in the apoplast (Pandey *et al.*, 1989). Based on their studies on selected species of tribe Eupatorieae, Marzinek and Oliveira (2010) have reported that the differences among the thicknesses of the phytomelanin layers among the various species may be related to the varying numbers of secretory layers in the outer mesocarp (hypodermis) of these species. The ones with more secretory cells in their hypodermis according to them have thicker phytomelanin layers.

Micromorphological study

The surface ornamentation in different taxa of the Heliantheae alliance is due to the variation in the deposition pattern of phytomelanin. These variations are noticeable in the form of spines, pegs, striations etc. (Pandey *et al.*, 1989; Pandey and Dhakal, 2001; Pandey *et al.*, 2014). The contour of the hypodermal inner walls and outer walls of

fibre cells decides the morphology of the phytomelanin layer (Rogers and Kreitner, 1983; Stuessy and Liu, 1983). The ornamentation on this hardened layer is the reflection of the contour of the cell-walls lining the schizogenous space (Pandey *et al.*, 1989; Pandey and Dhakal, 2001). Tadesse and Crawford (2014) based on light microscopic and SEM studies have used the pattern of development of phytomelanin to segregate the messed up complex involving the genera of *Bidens* and *Coreopsis*. They concluded all those taxa which lacked striations in the phytomelanin layer to be *Coreopsis* and those with striated cypselae as belonging to the genus *Bidens*.

Pandey *et al.*, (2014) studied the micro-morphology of the cypselae of various tribes and analysed the inter-tribal relationships within the family. Biswas and Mukherjee (2007) classified the cypselar wall structure of selected genera under five different groups based on distribution pattern on the pericarp-phytomelanin net-like in appearance, phytomelanin braces vertically oriented, phytomelanin braces irregularly lobed with variable orientation, phytomelanin layers oriented indifferent horizontal lines and phytomelanin layers oriented in different horizontal lines but inter-connected. Different types of deposition of phytomelanin have been demonstrated by Mukherjee and Sarkar (1998) in other genera of this tribe. Micromorphological and anatomical studies on fruits of *Heterocoma* (Vernonieae-Lychnophorinae) established the presence of phytomelanin outside Heliantheae alliance of Asteroideae (Freitas *et al.*, 2015).

Taxonomic Significance of Phytomelanin

Chloroplast DNA studies (Jansen and Palmer, 1987b) and morphological cladistic studies have shown that Mutisieae is the most basal tribe in the Asteraceae and Heliantheae as the evolved one. All tribes basal to Heliantheae alliance lack phytomelanin. Phytomelanin is present only in some of the highly evolved tribes of Asteraceae. According to Robinson (1981), the absence of phytomelanin in Gaillardiiinae and in the subfamily Cichorioideae represents independent losses.

Helianthoid relationship of the Eupatorieae was clearly established from molecular studies (Jansen *et al.*, 1990; Bremer *et al.*, 1992) and supported by later morphological studies (Karis, 1993). Absence of calcium oxalate crystals in the mature cypselae of non-phytomelanin containing taxa is probably the primitive condition. Presence of calcium oxalate crystals without presence of phytomelanin may be one step evolved condition, and absence of calcium oxalate crystals in phytomelanin bearing taxa may be highest evolved state, since these taxa have been considered as belonging to the most advanced lines in the family. Based on the phylogenetic results, presence of those epidermal-cell crystals is probably a derived condition in Helenieae, although lack of cypselar phytomelanin may be ancestral; Panero (Funk *et al.*, 2005; Panero, 2007) noted that two

of three subtribes (*i.e.*, Anisopappinae and Centipedinae) of the most basally divergent tribe of the Helianthoid group, Athroismeae, also lack fruit blackening, as in tribe Inuleae, which may be most parsimoniously interpreted as indicating independent expressions of phytomelanin in subtribe Athroisminae and in the bulk of the Heliantheae alliance (*i.e.*, in the sister group to Helenieae). Cariaga *et al.*'s (2008) finding that the Cuban genus *Feddea* (Feddeae), with no-phytomelanin fruits (and lacking epidermal-cell crystals), is sister to all other members of the Heliantheae alliance except Athroismeae strengthens the hypothesis of two origins of cypselar phytomelanin expression in the Helianthoid clade and ancestral absence of blackened fruits in Helenieae.

According to Robinson and King (1977), the Heliantheae and Eupatorieae share close relationship than any other tribes which is supported by the presence of phytomelanin in both the tribes. The nature of changes in phytomelanin structure suggests that the tribe Heliantheae evolutionarily preceded the Eupatorieae (Pandey and Dhakal, 2001). Though presence of phytomelanin layer is the characteristic feature of the tribe Heliantheae, it is reported to be absent in *Helianthus annuus* by Basak and Mukherjee (2001). They believed *H. annuus* being the most primitive of the seven species studied by them, was the only species lacking phytomelanin. It is interesting to note that it was in *H. annuus* that phytomelanin was first reported and described by Hanausek (1902).

Pericarp anatomy in different members of Asteraceae has thrown light on the distribution of phytomelanin in various members within Heliantheae alliance and across the non-Heliantheae members of the family. Taxonomic assessment of delimiting various taxa at different levels has been suggested based on pericarp anatomy Pandey (1986, 1999); Pandey and Kumari (2007); Pandey and Jha (1993b); Pandey and Singh (1983, 1994). Pandey and Kumari (1987) reported that phytomelanin presses the hypodermal cells which gives it an undulated appearance. Pandey *et al.* (1989) has reported that cypselae of certain taxa of Heliantheae show striations/ interruptions in the phytomelanin layer while such striations are totally absent in Eupatorieae. Based on their studies on selected species of tribe Eupatorieae, Marzinek and Oliveira (2010) have reported that the differences among the thicknesses of the phytomelanin layers among the various species may be related to the varying numbers of secretory layers in the outer mesocarp (hypodermal) layers of these species.

Genetics

Most of the work on genetics of phytomelanin has been undertaken in *Helianthus annuus*, as it is the cash crop greatly affected due to pest infestation where presence or absence or variation in pattern of phytomelanin layer clearly affects the yield. The value of phytomelanin as a potential

pest deterrent based on sunflower seed studies prompted studies on the inheritance of the phytomelanin layer in sunflower (Bird and Allen, 1936). The pericarp of sunflower is either with or without phytomelanin with striated patterns in phytomelanin (Rogers *et al.*, 1982).

Studies on inheritance of phytomelanin (Arnoldova, 1926; Platechek, 1927; Pustovoit, 1961; Kiewnick, 1964; Metche and Manbenot, 1970; Johnson and Beard, 1977; Rogers and Kreitner, 1983) present clear evidence that phytomelanin is controlled genetically by a single gene locus with dominant allele (Pml) and recessive allele (pml). Inheritance of the phytomelanin layer in sunflower cypselae was studied in the light of its deterring effect on the sunflower moth (Johnson and Beard, 1977). The involvement of polygenes was speculated by Mosjidis (1982) and Rogers and Kreitner (1983) as the cypselae exhibited wide variations. No positive correlation was established between the presence of phytomelanin in the pericarp and percentage of oil or sclerenchyma thickness in the pericarp (Rogers *et al.*, 1982; Rogers and Kreitner, 1983). The hull colour in sunflower seeds is determined by the pigments of the exoderm and phytomelanin (Tang *et al.*, 2006). The genes controlling phytomelanin pigment (P) and hypodermal pigment (*Hyp*) were mapped on linkage groups 16 and 17, respectively. The hull colour differences distinguishing "stripped" confectionery from "black" oilseed cultivars were caused by P and *Hyp* mutations.

Lately, molecular genetic studies have opened new dimensions regarding patterns of expression of genes in controlling expression of both anthocyanin as well as phytomelanin pigments. Park *et al.* (2007) have found that a bHLH regulatory gene in the common morning glory, *Ipomoea purpurea*, controls anthocyanin biosynthesis in flowers, proanthocyanidin and phytomelanin pigmentation in seeds, and seed trichome formation. Such examples indicate that there may be different sets of genes causing variable expressions of phytomelanin and anthocyanin.

Resistance to insect-predation

Phytomelanin is insoluble in water and organic solvents and shows exceptional resistance against concentrated acids and alkalis, and is highly resistant to microbes and pests (Platechek, 1927; Arnoldova, 1926; Pandey and Dhakal, 2001). Experimental studies support the concept of defensive role of phytomelanin in cypselae against predatory insects (Johnson and Beard, 1977). Pandey and Dhakal (2001) reviewed the available literature concerning phytomelanin in the Asteraceae, and pointed out that this substance has a deterrent effect on insect predators, which was confirmed in experiments with sunflower seeds (*Helianthus* spp.). Phytomelanin as an effective tool against larvae of sunflower moth (*H. nebulella* and *H. electellum*) is well established by many studies (Sárkány, 1947; Kiewnick, 1964; Carlson *et al.*, 1972; Carlson and Witt, 1974; Shapiro,

1975). Sárkány (1947) and Kiewnick (1964) reported that phytomelanin in the pericarp gives mechanical protection against penetration of larvae of the European moth, *H. nebulella*, which lays its eggs at the time of pollination of the heads (Stafford *et al.*, 1984). The resistant layer hinders the insect from penetrating the pericarp, affording mechanical protection against larval damage. Rogers and Kreitner (1983) also suggest antixenosis and antibiosis towards the larvae of *Homoeosoma electellum*. Prasifka *et al.* (2014) discussed the pericarp strength of sunflower and its value for plant defence against the sunflower moth, *Homoeosoma electellum*.

Carlson *et al.* (1972) and Carlson and Witt (1974) reported that in cultivated sunflower (*H. annuus*), cypselae having the phytomelanin layer were damaged less by larvae of *H. electellum* than cypselae that lacked the layer. Studies conducted on pericarp resistance to mechanical puncture in sunflower cypselae by Stafford *et al.* (1984) have indicated that phytomelanin containing cypselae showed a significantly greater resistance to mechanical puncture than those lacking phytomelanin. The adaptive nature of phytomelanin in sunflowers has been studied by Seiler *et al.* (1984) suggesting the protective nature of the substance in fruits against predators.

Susceptibility to the sunflower moth under natural infestation shows variation (Teetes *et al.*, 1971). Resistance was directly correlated with presence of phytomelanin in the cypselae (Rogers *et al.*, 1982). European researchers believe phytomelanin as physical barrier whereas the American studies mostly agrees upon the fact that it provides a physiological resistance due to toxic effect (Pandey and Dhakal, 2001). Phytomelanin exhibited both anti-xenosis and antibiosis towards larvae of *H. electellum* (Rogers and Kreitner, 1983). Pandey and Dhakal (2001) concluded that phytomelanin has both mechanical as well as physiological resistance against the sunflower larvae. However, Marzinek and Oliveira (unpublished data quoted in Marzinek and Oliveira, 2010) analyzed the cypselae of *Mikania micrantha* and *Symphopappus reticulatus* and found insect larvae in 10% and 22% of the fruits, respectively. As these were the species with the thickest recorded phytomelanin layers within their study material, the deterrent effect of this substance could not be confirmed, and the role of phytomelanin in the cypselae still needs to be elucidated (Marzinek and Oliveira, 2010).

Chemical factor of host resistance in sunflower

Hegnauer (1977) suggested that the polymerization of highly unsaturated acetylenes like phytomelanin are effective against nematodes and bacteria. If polyacetylenic nature of phytomelanin is considered, its presence in cypselae has an adaptive significance (Hegnauer, 1977). Young larvae of sunflower moth showed high mortality when fed with synthetic diet containing extract from the florets of resistant variety of sunflower. Waiss *et al.* (1977), based on feeding

experiments where the larvae of *Homoeosoma electellum* were fed with a synthetic mixture containing phytomelanin, interpreted the casualty in them due to the larvicidal nature of phytomelanin. This is well supported by the studies of Carlson and Witt (1974); Beard *et al.* (1977) and Rogers and Kreitner (1983). Phytomelanin has been proposed to have antimicrobial activity to resist the fungal infestation (Swain, 1965).

Embryo protection and germination

In mature cypselae, phytomelanin forms a protective covering around the embryo. It is this layer that protects the developing embryo of the seed during its production on the plant and after dispersal or harvesting (Hanausek, 1910; Hanausek, 1912; Roth, 1977; Pandey *et al.*, 1989; Pandey and Dhakal, 2001; Tadesse *et al.*, 2001). It is this layer that protects the developing embryo of the seed during its production on the plant and after dispersal or harvesting.

Adaptive significance

Dark colour of reproductive disseminules in plants and skin of animals is mainly due to melanins (Nicolaus, 1968). Melanins have protective function against UV radiation, ionizing radiation, high and low temperatures in various developmental stages of the organisms (Kerestes *et al.*, 2003). According to Hanausek (1910, 1912) and Roth (1977), phytomelanin may serve as a protective screen against excessive insolation or in mechanical protection of the pericarp. Studies conducted by Bidzilya (1972) and Zherebin and Litvina (1991) have shown that melanin pigments from plants, or phytomelanins, exhibit radioprotective properties. Pollination, breeding system, defense, dispersal and germination could be the major factors that could have related to phytomelanin origin and variation (Stuessy and Spooner, 1988). Phytomelanin has the major role of embryo protection as implied by its development after fertilization. The phytomelanin is quite hard and highly resistant to alkali and acids (Kiewnick, 1964) and with properties which have been shown to decrease predation of cypselae of the Asteraceae (Carlson and Witt, 1974; Rogers and Kreitner, 1983). Protection of the embryo occurs during development of the cypselae and after the dispersal. Dahlgren and Rasmussen (1983) and Netolitzky (1926) suggest its role in seed protection. The recent reports on the presence of phytomelanin in the vegetative tissues apart from the tissues of seeds and fruits suggest a role beyond protection of embryo and from predators. Marzinek and Oliveira (2010) concluded that the species they selected from the *cerrado* biome (hot, dry biome in Brazil) are with thicker layer of phytomelanin which clearly indicates the role of phytomelanin in protection against excessive insolation.

In cypselae where these parenchymatous areas are absent and the germinating seedlings emerge by breaking through the margins at the lower half of the cypselae (Pandey and Dhakal, 2001). The striations on the cypselae is proposed

to help in entry of water and emergence of seedling during germination (Tadesse *et al.*, 2001). Katinas and Crisci (2008) offered a contrary interpretation that the phytomelanin coating might be a water-resistant layer in the cypselae of taxa such as *Panphalea heterophylla* (Nassauviinae, Mutiseae) that inhabit marshy areas it could serve to keep water out.

Conclusions

An appraisal of the literature indicates the taxonomic and evolutionary significance of phytomelanin in different groups. Reports of presence of phytomelanin in parts other than the fruits and seeds of selected members of Asteraceae and Asparagales suggest that it has evolved independently in different groups in response to different stress conditions. The presence of phytomelanin in shoots and leaves of Lychnophorinae species is considered as a homoplasy within Asteraceae (Marques *et al.*, 2021) contrary to the earlier reports of it being a synapomorphy based on phylogenetic studies (Panero and Funk 2008). It is interesting to observe that in members with phytomelanin in cypselae, it is conspicuously absent from the vegetative parts and vice versa. The chemical nature and developmental patterns of all these black substances referred to as are, however, not clear yet. The report on coexistence of calcium oxalate crystals and phytomelanin in *Lychnophora salicifolia* (Vernonieae), a member outside the Heliantheae alliance affirms the monophyly of the alliance clade where calcium oxalate crystals are absent. Reports of phytomelanin from subfamilies of the basal grade- in the fruit walls of Wunderlichioideae and vegetative parts of Carduoideae (Fritz and Saukel, 2011) is clearly contrary to the earlier understanding of the inert substance being restricted to the advanced members of the family. It has opened the probability of the evolution of phytomelanin more than once independently. Reports of phytomelanin from outside the Heliantheae alliance based on anatomical studies from lower nested groups have opened the need for more data from other related clades to have clinching decision on the significance of presence of it in different members (Freitas *et al.*, 2015; Lusa *et al.*, 2018; Marques *et al.*, 2021). The chemical nature of the melanin from all these related groups needs to be assessed to verify the taxonomic and evolutionary significance of the recent studies.

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