Genetic and environmental effects on polyphenols in *Plantago major*

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Summary

Leaves and seeds of *Plantago major* (common plantain or greater plantain) have been used for centuries to treat diseases relating to skin, digestive organs and blood circulation like wounds, inflammation and hypertension. Either whole or crushed leaves have been used to treat for example burns and all kinds of wounds to enhance the healing process, and to stop bleeding. To treat superficial wounds it is sufficient to apply the juice from the leaves. Both polysaccharides and polyphenols may have a synergistic effect on wound healing and other biological activities. Polyphenols extracted from leaves and seeds of *P. major* have been reported to have bioactive effects especially on wound healing, and to have antiulcerogenic, anti-inflammatory, antioxidant, anticarcinogenic and antiviral activity. Three subspecies have been described of *P. major*, two of which have been subjected to genetic and phytochemical analysis. *Plantago major* subsp. *major* is naturalized almost throughout the world and is mainly found as an agronomic weed. There has been little work emphasizing the utilization of the bioactive compounds from *P. major* in modern medicine. Similarly, the effects of genetic and environmental factors on the occurrence of these bioactive compounds have not been reported. The main emphasis of the introductor y paper is to highlight some factors that may be important for the utilization of *Plantago major* as a medicinal herb, providing the scope for the Ph.D. study. This paper also describes the taxonomy including morphological differences between the two subspecies, distribution, biology, genetics and DNA markers used in *P. major.*

Contents

Introduction

Plantago major belongs to the family Plantaginaceae and the highly diverse genus *Plantago* comprising approximately 256 species. *Plantago major* originated in Eurasia and is now naturalized almost throughout the world.

Taxonomy

Family

Plantago major belongs to the genus *Plantago* and family Plantaginaceae. The name comes from Latin 'planta', meaning 'sole of the foot' which refers to the broad leaves in the basal rosettes, often touching the ground in some species (Pilger, 1937).

Plantaginaceae can be treated as a cosmopolitan family consisting of three related genera, i.e. *Bougueria* Decne*, Littorella* Bergius and *Plantago* L. (Heywood, 1993; Mabberley, 1997). According to Rahn (1996) it is instead a monogeneric family containing only the genus *Plantago*.

Genus

There are about 256 species in genus *Plantago* distributed throughout the world. Pilger (1937) divided the genus into two subgenera: *Plantago* Harms (there are 18 sections in subgenus *Plantago*) and *Psyllium* (Miller) Harms including the branched species. Rahn (1978) instead subdivided the genus into three subgenera: subgenus *Plantago* L., *Coronopus* Lam. & D. C. and *Psyllium* Rahn (including subgenus *Psyllium* and 5 sections of subgenus *Plantago* in the sense of Pilger, 1937). Rahn (1996) proposed a new taxonomic treatment of the genus. He reclassified *Plantago* based on 90 morphological and anatomical characters, according to which genus

Plantago includes 6 subgenera: subgenus *Plantago*, *Coronopus* (Lam. & D. C.) Rahn, *Albicans* Rahn (includes different parts of subgenus *Plantago* sensu Pilger, 1937), subgenus *Psyllium* Harms (sensu Pilger, 1937, not in Rahn, 1978), *Littorella* Bergius (genus *Littorella* Bergius) and subgenus *Bougueria* Decne (genus *Bougueria* Decne). Sojak (1972), Holub (1973) and Dietrich (1982) accept the subgenus *Psyllium* Harms as a distinct genus. *Plantago major* belongs to subgenus *Plantago*.

Species

Three subspecies of *Plantago major* have been recognized; *P. major* subsp. *major, P. major* subsp. *intermedia* and *P. major* subsp. *winteri.* The first two subspecies are often acknowledged. Although morphologically similar, they are still distinct entities with different habitat requirements (Zhukova *et al.*, 1996). The third subspecies has been reported in the literature but there is not much research on this subspecies. The first two subspecies have distinct cytotypes. The difference in cytotypes and in number of seeds per capsule is used as an indication of taxonomic identity. Evolution of the *P. major* groups (subspecies) may be in part due to chromosomal rearrangement. Most *P. major* karyotypes are more symmetrical than those of *P. major* subsp. *intermedia*, which may indicate that *P. major* subsp. *intermedia* is the derived type (El-Bakatoushi and Richards, 2005). Morphological characters and habitat differentiate between the two subspecies. *Plantago m.* subsp. *major* is winter hardy and is more abundant on footpaths and rough surfaces, and in cultivated areas and grassy places, whereas *P. m* subsp. *intermedia* is less winter hardy and is more abundant near the sea (Molgaard, 1976; Stace, 1997). According to Molgaard (1976) *P. m* subsp. *major* has wider leaves and produces only a few larger seeds per capsule (4–15) while *P. m* subsp. *intermedia* has narrower leaves and usually produces a large number of smaller seeds in each capsule (12–25). Morgan-Richards and Wolff (1999) made a study on the genetic structure of the two subspecies and based on the results of this study, they proposed that the two taxa should be treated as different species, *P. major* and *P. intermedia.*

Distribution

Genus

Species of the genus *Plantago* grow in almost every type of habitat including deserts, sea cliffs, woodlands, disturbed areas and tropical mountains. Species vary greatly in distribution with many species restricted to a specific area while others are more widespread (Primack, 1978).

Species

Plantago major is a temperate-zone plant with extreme ranges to the north and south, almost from pole to pole although very rare in lowland tropics. In its wild form, it grows from sea level to 3500 m altitude (Sagar & Harper, 1964). The species grows best in moist areas such as river beds, seepage areas on hillsides, drains, places subjected to water runoff from buildings, along road sides and in costal areas (Webb *et al*., 1988).

Plantago major originated in Eurasia but is now naturalized almost throughout the world. Research on pollen has shown that this species was introduced to the Nordic countries 4000 years ago (Jonsson, 1983). It is known to have been present in England in 1672 and is found in Canada since 1821. The Indians named it 'white man's footprint' because it is found everywhere the Europeans have been (Samuelsen, 2000). Early Eurasian settlers introduced *P. major* to North America, and now both native populations and those naturalized from Eurasia can be found. The species is a common weed in most of the agricultural areas of the world including also places where tropical crops are grown (Anderson, 1999)

Biology

Species of genus *Plantago* vary from spring annuals to summer annuals, biennials, and perennials. There are repeated evolutionary shifts in both directions between annual and perennial habit (Primack, 1976). *Plantago major* occurs both as a perennial and as an annual.

Stem and leaves

Plantago major has a short, stout and erect herbaceous stem. Leaves form a basal rosette and grow up to 30 cm long (Sagar & Harper, 1964). The leaves are ovate to elliptic in shape with parallel venation (5–9 veins). Leaf blade is entire or irregularly toothed, and narrows into a petiole. Leaf petiole is of almost equal length as blade. Leaves are glabrous or hairy, normally green in color, sometimes with purple shading (Samuelsen, 2000). Total number of leaves and amount of biomass is affected by growth habit of the plant. Warwick (1980) reported that prostrate individuals of *P. major* produce a significantly lower number of leaves compared to the erect plants. Generally, prostrate plants of *P. major* are less damaged than erect individuals by simulated trampling.

Roots

Plantago major produces many adventitious roots of whitish color. The roots grow up to 1 m in length (Sagar & Harper, 1964). Prostrate individuals of *P. major* produce a significantly lower number of roots than those with erect growth habit (Warwick, 1980).

Flowers and inflorescences

Flowering time for *P. major* is from May to September in the temperate zone but it can vary depending on where the plants are grown (Long, 1938). Normal age of plant for first flowering is approximately 13 weeks (Warwick & Briggs, 1980) but plants may flower and start setting seeds just 6 weeks after germination (Sagar & Harper, 1964).

Inflorescence of *P. major* is a spike, which grows 1–30 cm in length, usually simple but very rarely branched. The spike is not usually consumed by grazing animals because it is hard as compared to the succulent and soft leaves. Spikes bear yellowish white flowers of 2–4 mm diameter. Flowers are protogynous (stigmas are exserted 1–3 days before anthesis) (Sharma *et al.*, 1992).

Fruits and seeds

Fruit of *P. major* is a capsule, which is 5 mm long. Large numbers of capsules are produced on a spike. Number of capsules per cm of spike is 23–26. Seeds are produced in capsules and the number of seeds per capsule is 4–15 (Samuelsen, 2000; Warwick & Briggs, 1980; Sagar & Harper, 1964). Prostrate individuals of *P. major* produce significantly less seeds than the erect plants. There were no significant differences between the two growth forms (prostrate and erect) in case of spike dry weight (Warwick, 1980).

Seeds set rapidly within three weeks after flowering. *Plantago major* plants produce a large amount of seeds, up to 20 000 per plant. The seeds are quite small

(0.4–0.8 × 0.8–1.5 mm) with an ovate to elliptic shape, which varies according to number of seeds in capsule. The large endosperm forms the major part of the seed and surrounds the embryo completely. Seeds become thick when moistened because of polysaccharides present in the seed coat and can become attached to animals and humans and thus be spread over large distances (Samuelsen, 2000; Kuiper & Bos, 1992; Sagar & Harper, 1964).

Genetics

Genetics is the science of heredity and variation in living organisms. All the living organisms inherit traits from their parents and this fact has been used since prehistoric times to improve plants and animals through breeding (Weiling, 1991). Genetic variation is the tendency of genetic characters to vary and is a prerequisite for breeding. Mutation, recombination and hybridization are the factors responsible for genetic variation, while recombination is the main source of variation in most sexually reproducing species. Breeding and meiotic systems together constitute the “genetic system” and determine the nature and rate of recombination (Darlington, 1939; Stebbins, 1950).

Breeding system and genetic variation

Species in the genus *Plantago* have a wide range of mating systems, from inbreeders to obligate outcrossers. *Plantago major* is wind pollinated, self-compatible and highly inbreeding (Kuiper & Bos, 1992). Outcrossing rate in *P. major* subsp. *major* (10–14%) is slightly higher than in *P. major* subsp. *intermedia* (3–6%). Both species exhibit lower variation within populations and higher proportion of variation among populations (Wolff, 1991; Squirrell & Wolff, 2001). Other investigated

Plantago species are, e.g., *P. coronopus* which is selfcompatible and has a variable level of outcrossing; from 34 to 100% (Wolff *et al.*, 1988), and *P. lanceolata* which generally exhibits a higher genetic variation within populations because it is self-incompatible and thus obligatory outcrossing (Hale & Wolff, 2003).

From comparative studies on inbreeding and outbreeding species, it has become clear that generally outbreeding species have higher genetic variability within populations and lower genetic variability among populations, whereas inbreeding species possess lower genetic variability within populations and higher genetic variability between populations (Solbrig, 1972; Brown, 1979; Schoen, 1982; Layton and Ganders, 1984; Van Dijk *et al.*, 1988). Populations of inbreeding species sometimes lack genetic variability altogether and are then considered to be pure lines (Jain, 1976).

A similar pattern occurs in the case of morphological variation; in general, outbreeders have higher morphological variability within population, and inbreeders in contrast have a higher variability between populations (Carey, 1983). In some studies no differences between outbreeding and inbreeding species were observed (Brown & Jain 1979) and even a reversed result with higher intra-population variation in inbreeding species and higher inter-population differences in outbreeding species has been found (Hillel *et al.*, 1973).

According to Wolff (1990), both the inbreeding *P. major* and the outbreeding *P. lanceolata* showed a high degree of morphological differentiation between populations. It appeared that besides the influence of the mating system, selection might diminish morphological variability in the case of strong directional selection, especially in *P. major* and *P. lanceolata.*

Chemistry in *Plantago*

There is an increasing interest in phytochemicals, because of their potential use in functional food products and medicines. *Plantago major* has numerous phytochemicals in its leaves, seeds and roots, which apparently have medicinal properties and also can be used as taxonomic markers (Samuelsen, 2000).

Flavonoids

Flavones are the main flavonoids in *P. major* (Kawashty *et al*., 1994; Nishibe *et al*., 1995). Flavones tend to replace flavonols in *Plantago* (Harborne & Williams, 1971). Subgenera *Plantago* and *Coronopus* have a tendency to produce flavones, luteolin and 6-hydroxy luteolin. Attempts have been made to use flavonoids as taxonomic markers in *Plantago* (Kawashty *et al.,* 1994).

Caffeoyl phenylethanoid glucosides

Verbascoside is usually present in *Plantago,* sometimes together with plantamajoside. A number of other caffeoyl phenylethanoid glucosides have been reported in *Plantago*. Attempts have been made to use caffeoyl phenylethanoid glucosides also as taxonomic markers (Ronsted *et al*., 2000). The concentration of verbascoside is higher in seeds and flowering stalks of *P. major,* whereas the concentration of plantamajoside is higher in leaves (Zubair *et al*., 2008b).

Iridoid glucosides

Iridoid glucosides have been found to be valuable taxonomic markers of subgenus *Plantago* and the sections within this subgenus (Andrzejewska-Golec & Swiatek, 1984). Bartsioside and plantarenaloside are associated with subgenus *Psylliun*

(Andrzejewska-Golec, 1997). Distribution pattern of the iridoids in *Plantago* showed good correlation with the classification made by Rahn (1996) (Ronsted *et al.*, 2000).

Ronsted *et al.* (2003) concluded from their study (Chemotaxonomy and evolution of *Plantago*) "compounds of majoroside type may be of taxonomic value within subgenus *Plantago*, and the common presence of 5-hydroxylated iridoids and caffeoyl phenylethanoid glucoside (β-hydroxyacteoside) support a relation between subgenera *Coronopus* and *Plantago*".

Other chemical compounds, which have been reported in *Plantago*, are aucubin, melittoside, 10-acetylaucubin (Andrzejewska-Golec & Swiatek, 1984; Ronsted *et al.*, 2003), 10-O-acetylgeniposidic acid (Ronsted *et al*., 2003), asperuloside (Bianco *et al*., 1984), melampyroside, plantarenaloside, ixoroside (Afifi *et al*., 1990), majoroside (Handjieva *et al*., 1991), 10-hydroxymajoroside, 10-acetoxymajoroside (Taskova *et al*., 1999), geniposidic acid, hellicoside, acteoside, plantaginin, 6-hydroxyluteolin 7-glocoside, β-hydroxyacteoside, orobanchoside (Nishibe, 1994) and gardoside (Murai *et al*., 1996).

Polyphenolic compounds in *Plantago major*

Both polysaccharides and polyphenols have been proposed to act as bioactive compounds in this species. The antiviral activity of *P. major* is derived mainly from its phenolic compounds (Chiang *et al.,* 2002). Phenols constitute a group of structurally related compounds containing a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group, and are present in many natural products. The phenols in natural products range from simple molecules such as phenolic acid to highly polymerized, large polyphenolic compounds such as tannins (Jurisic Grubesic *et al*., 2005).

There seems to be an increasing interest especially in natural polyphenols due to their potentially positive effect in controlling certain diseases. The polyphenols have free radical scavenging ability by naturalizing dangerous reactive oxidants, as well as metal ion chelators. Therefore, polyphenols are antioxidants in nature. Polyphenols are considered responsible for wound healing and have antimicrobial and anti-inflammatory activity (Brantner *et al*., 1994).

Plantamajoside is the major known phenolic compound in *P. major*. Well-documented biological effects of this compound include anti-inflammatory activity (an inhibitory effect on arachidonic acid-induced mouse ear oedema; Murai *et al*., 1995), free radical scavenging activity (Skari *et al*., 1999) and some antibacterial activity (Ravn & Brimer, 1988). Verbascoside is the second major phenolic compound present in seeds and flowering stalks of *P. major*. Verbascoside has shown pronounced anti-hepatotoxic activity (Xiong *et al*., 1998), activity against several kinds of cancer cells (Pettit *et al*., 1990; Saracoglu *et al*., 1997) and antiviral activity against vesicular stomatitis virus (Bermejo *et al*., 2002). These compounds in plants also function as protectants and repellents against herbivores (Ravn & Brimer, 1988).

Medicinal uses of *Plantago major*

For the past few decades, a growing number of people have been turning to alternative forms of medicine in response to disillusionment with the modern medical system. Many botanical, especially herbal, products have gained popularity for the treatment of ailments and diseases such as the common cold, wounds, hypertension, inflammation, viral infections, depression, insomnia, and even cancer (Blumenthal *et al*., 2006).

Plantago major has been used for different purposes in folk medicine all over the world. The biological activities of *P. major* leaves and seeds are wound healing, anti-inflammatory, analgesic, antioxidant, weakly antibiotic, immuno-modulating, antiulcerogenic, antihypertensive (Samuelsen, 2000; Nyunt *et al*., 2007), antileukemia, anticarcinogenic, antiviral, cell-mediated immunity modulating (Chiang *et al*., 2003), anticandidal (Holetz *et al*., 2002), antitumor (Yaremenko, 1990), antinociceptive (reducing sensitivity to painful stimuli) (Atta & El-Sooud, 2004) and reduction of immunodepressive effects of anticancer drugs (Shepeleva & Nezhinskaya, 2008). This plant has traditionally been used in e.g. China for numerous diseases varying from cold to hepatitis (Chiang *et al*., 2002). *Plantago major* has also been used to neutralize poisons internally and externally (Lithander, 1992).

Antiulcerogenic activities

Plantago major leaves produce an antiulcerogenic effect against alcohol- and aspirin-induced gastric ulcer (Atta *et al*., 2005; Than *et al*., 1996). The leaves have been used as an antiulcerogenic in Turkey (Yesilada *et al.,* 1993). A combined methanol and water extract inhibited ulcer formation by 40% relative to the control group, while a water extract inhibited ulcer formation by 37% and a methanol extract by 29%. However, when compared to other Turkish plants with antiulcerogenic properties, *P. major* leaves did not constitute one of the most active remedies against ulcer (Yesilada *et al*., 1993).

Anti-inflammatory and immuno-modulating activities

Extracts of *P. major* enhance the production of nitric oxide and tumor necrosis factor-alpha (TNF-∝), which protect the host against the development of infection and tumors (Nathan & Hibbs, 1991). The main effect of nitric oxide is to inhibit the synthesis of DNA and ATP. Tumor necrosis factor-alpha (TNF-$\propto$) is one of the essential mediators of host inflammatory responses in natural immunity. The regulation of immunity parameters induced by *P. major* may be clinically relevant in numerous disease processes including tuberculosis, AIDS and cancer (Flores *et al*., 2000).

Antioxidant activities

Oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases, including atherosclerosis, cancer and Parkinson's disease, and is also involved in aging (Halliwell, 2000; Young & Woodside, 2001). Antioxidants are substances that possess the ability to protect the body from damages caused by free radical-induced oxidative stress (Souri *et al*., 2008). Antioxidants, whether synthetic or natural, can be effective in prevention of the free radical formation by scavenging and suppression of such disorders (Halliwell, 2000; Young & Woodside, 2001). Some medicinal plants are promising sources of potential antioxidants (Souri *et al*., 2008). Tea made from green leaves of *P. major* has antioxidant properties but the antioxidant capacity is higher in fresh green leaves (Campos & Lissi, 1995). Environmental factors such as altitude affect the antioxidant activitity differently in roots and leaves of *P. major*; antioxidant activitity of roots increases with an increase in altitude whereas antioxidant activitity of leaves decreases with an increase in altitude (Argueta *et al*., 1994; Ren *et al*., 1999).

Antiviral activities

Certain pure compounds of *P. major* possess antiviral activity. Chemical compounds found in extracts of *P. major* (mainly phenolic compounds) exhibit potent anti-herpes virus and anti-adeno virus activities (Chiang *et al*., 2002). Extracts of *P. major* also showed antimicrobial activity against yeasts (Stanisavljevic *et al*., 2008). *Plantag major* leaves extract exhibited weak antibacterial activity in vitro, but the extract has an effect on infected wounds in vivo. While the application of antibiotics on infected wounds had no effect, treatment with a *P. major* extract removed the infections and healed the wounds (Samuelsen, 2000). Leaves have also traditionally been used for the treatment of skin infections and for bacterial infections (Holetz *et al*., 2002).

Anticarcinogenic activities

Leaves of *P. major* have been utilized for treatment of skin cancer (Samuelsen, 2000). Yaremenko (1990) found that *P. major* was effective in a screening system for prophylactic oncology. An aqueous extract of *P. major* was shown to have a prophylactic effect on mammary cancer in mice (Lithander, 1992). A leaf-derived extract was injected subcutaneously in mice that had developed cancer. After 60 weeks, only 18.2% of the treated mice had tumors as compared to 93.3% of the untreated.

Wound healing

Wounds

Wounds can be defined simply as the disruption of the normal cellular and anatomic continuity of a tissue as a result of injury (Bennet, 1988). Wounds may be produced intentionally such as a surgical incision or accidentally by physical, chemical, thermal, microbial or immunological insult to the tissue. Wound healing is the body's natural process of regenerating dermal and epidermal tissue. The process of wound healing consists of integrated cellular and biochemical events leading to reestablishment of structural and functional integrity and regain of strength of the injured tissue (Stadelmann *et al.* 1998). Herbal medicines are often used for the treatment of wounds, especially in developing countries (Azaizeh *et al.*, 2003).

Plantago major and wound healing

Leaves of the common weed *P. major* have been used, and are still being used as a wound healing remedy in almost all parts of the world in folk medicine. Greek physicians described the traditional use of *P. major* in wound healing already in the first century A.D. (Samuelsen *et al.*, 1999). Either whole or crushed leaves are used to treat for example burns and other kinds of wounds to enhance the healing process, and to stop bleeding. The leaves of *P. major* have thus been prescribed for the treatment of wounds caused by for example dog bites (Roca-Garcia, 1972). Normally, it is sufficient to apply only the juice from leaves to heal superficial wounds (Brondegaard, 1987). In Scandinavian countries, *P. major* is well-known for its wound healing properties. The Norwegian and Swedish people call this plant 'groblad' which can be translated as 'healing leaves' (Samuelsen, 2000).

The extract of *P. major* contains a mixture of antioxidants; those antioxidants may constitute one of the mechanisms that contribute to its wound healing properties (Yokozawa *et al.*, 1997).

Greenhouse cultivation of *Plantago major*

Conditions

Plantago major has been used as a model species for genetic, environmental, photochemical and medicinal studies. Cultivation conditions for *P. major* plant material have varied with the purpose of the study. *Plantago major* plants have thus been grown in growth chambers or greenhouses in many studies whereas other studies have been based on field-collected material (Molgaard 1976; Van Dijk 1984; Wolff 1991a, 1991b). In the wild, seeds germinate at or very near to the soil surface. Growth place and soil moisture content affect seed germination; seedlings emerge earlier on paths than on riverbanks (Lotz, 1990). Freshly shed seed germinate in the following spring (Sagar & Harper, 1964). Germination occurs throughout the growing season, seedlings start to emerge in April and maximum numbers of seedlings emerge during the months of May and June.

In the greenhouse, seeds start to germinate when soil moisture is adequate and soil temperature reaches 10°C. However, germination is more rapid as temperature increases, and the ideal temperature for germination is around 25°C. Seeds can be germinated in seed trays, germination tray or pots filled with soil, sand, soil mixed with sand, peat, soil mixed with peat, sand mixed with peat, vermiculite or perlite (Murr & Stebbins, 1971; Blom, 1978; Maddox and Antonovics, 1983; Reekie, 1998; Smekens & Tienderen, 2001; Rosenhauer, 2007). Imbibition treatment before sowing increases germination percentage (Gorski *et al.*, 1977). Other pretreatments also increase seed germination; a 3-months period of moist storage at 5°C increases germination from 31 to 100%, and pre-chilling of seeds at 5°C for 7–14 days is also very useful in increasing germination percentage (Sagar & Harper, 1960; Grime *et al.*, 1981).

The seedling stage lasts for 8 to 15 weeks, depending on temperature and cultivation conditions (Blom, 1978). The seedlings (5–16 days old) can be transferred to bigger pots and grown in a greenhouse at 18°C to 27°C temperature (12–16 hr) during the day and 15°C to 20°C (8 to 12 hr) during the night.

Effects and uses

Cultivation conditions affect not only the plant growth but also the morphology of leaves and stem (Warwick & Briggs, 1980). Greenhouse conditions thus have important consequences for the synthesis of various chemical compounds (Murr and Stebbins, 1971; Molgaard 1976; Van Dijk 1984; Wolff 1991 a). Carefully controlled greenhouse conditions, i.e. temperature, ventilation, humidity, day length, light intensity, irrigation schedule, and fertilizers are therefore necessary for obtaining repeatable results. Change in a single condition can greatly affect total biomass production and the concentration of polyphenols. A series of experiments carried out at Balsgård have shown that *P. major* plants grown in a greenhouse without any fertilization produced less biomass as compared with plants grown with additional fertilizers (Rosenhauer, 2007; Zubair *et al*., 2008b). Plants subjected to continuous removal of flowering stalks produced more biomass as compared with plants grown without any removal of flowering stalks. Application of fertilizers also affected the concentration of polyphenols in *P. major*; plants grown without fertilization produced a higher dry weight concentration of total phenols compared to plants grown with additional fertilizers.

Harvesting and post harvest handling

Polyphenols are not evenly distributed between different plant organs. Variation in concentration of polyphenols in finished products can be due to genetic variation in the plant species, lack of organ specificity, stage of growth, cultivation parameters (soil, light, water, temperature and nutrients), contamination by microbial and chemical agents, drying method, extraction strategy and finished product storage. To obtain reproducible results for the extraction of polyphenols, all of the above-described operations need to be conducted according to a specific protocol. In order to obtain the maximum concentration, careful optimization of these operations and conditions is necessary (Kabganian *et al.,* 2002; Zubair *et al.,* 2008b)

Harvesting of different pant organs

Contents of a specific phenolic compound often vary greatly with the plant organ used, and growth stage of plant when harvested (Gray *et al.*, 2003). Zubair *et al.* (2008b) reported that concentrations of plantamajoside and verbascoside showed large variation in different aerial organs of *P. major*. Concentration of plantamajoside reached its maximum in samples of flowering stalks and its minimum in old leaves, whereas concentration of verbascoside reached its maximum in samples of flowering stalks and its minimum in seeds. Concentration of plantamajoside in flowering stalks was 77 times higher than in seeds, and concentration of verbascoside in flowering stalks was 360 times higher than in old leaves.

The concentration of aucubin in *P. lanceolata* reached a maximum 98 days after germination, and the concentration of acteoside 126 days after germination, while the level of catalpol remained essentially constant over the course of an experiment conducted for 126 days (Tamura & Nishibe 2002).

Drying method

Freshly harvested *P. major* plants occupy large volumes and thus can pose difficulties in transportation and storage. Dried plant material is easier to handle and less prone to microbial degradation. There are two different methods for drying the plant material based on heat source or energy utilization (Cai *et al*., 2004). In natural drying, the plant material is exposed to the sun and/or air; the sun energy and the desiccating air currents promote the removal of water from the plant material. Natural air-drying and sun drying is easy to control and seldom damages the crop (Downs & Compton, 1955). Natural drying is useful if the phytochemicals are not photo-sensitive.

Mechanical drying includes freeze-drying, artificial drying, microwave drying, vacuum drying and spray drying. Freeze-drying is an ideal method for drying plant material containing heat- and photo-sensitive compounds. Unfortunately, freeze-drying is a very expensive method and it is used only for drying high-value products. Tamura and Nishibe (2002) reported that phytochemicals in *P. lanceolata* are sensitive to drying treatments. As compared to fresh biomass, plantain phytochemicals like catapol decreased by 50%, aucubin by 25% and acteoside decreased by 29%, when dried for 8 h at 60°C. Zubair *et al.* (2008a) reported that the concentration of plantamajoside was 68% higher in freeze-dried samples than in the samples dried at 50°C, and the concentration of verbascoside was 52% higher in freeze-dried samples than in the samples dried at 50°C.

Extraction method

Extraction is the main operation for botanical preparations (Shi *et al.*, 2002). The concentration of the phenolic compounds varies greatly with solvent used for extraction. Total amount of phenolic substances extracted with ethylacetate was somewhat smaller as compared to the amount obtained with ethanol (Bazykina *et al.* 2002). Yilmaz and Toledo (2006) carried out extractions at 60°C for 5 hours, using pure ethanol and different ethanol-containing volumes of water (10, 20, 30, 40, 50 and

60%). A mixture of ethanol and water was revealed to be more efficient than water or ethanol separately. They also found that the phenol content of ethanol extracts from grape seeds increased with increasing water in the mixture from 0% to 30%, stayed constant for 30, 40 and 50%, and decreased for higher percentages of water.

Molecular markers in *Plantago major*

Populations of a species can become genetically isolated in various ways due to e.g. their reproduction system or geographical distances and can then diverge from each other through drift or differential selection. If the populations have diverged sufficiently, they may be called different ecotypes, forms or even different subspecies. It is generally accepted that morphological characters and ecological niche are a good guideline to distinguish two forms or subspecies within a species (Molgaard, 1976). The study of morphological characters, allozymes and PCR-based DNA polymorphism not only helps in the classification of closely related taxonomical units like ecotypes, forms and subspecies, but also provides information about the evolution of characters and molecules.. The variability of molecular markers also indicates aspects of the breeding system and help to identify the mating system of a species.

Plantago major is a highly inbreeding species with very low outcrossing rate. Therefore, each population can be regarded as an inbred line, which is highly adapted to its specific habitat (Wolff, 1991b). Although general appearance of the two subspecies of *P. major* is very similar, several morphological characters such as the number of seeds per capsule, number of veins in leaf, number of inflorescences and leaf length discriminate these subspecies (Molgaard 1976; Van Dijk 1984; Wolff, 1991a). Allozyme studies have been performed on both subspecies of *P. major* collected from nine locations in the Netherlands. The two subspecies shared 27 invariable allozyme loci, and showed similar allele frequencies also in three out of the nine polymorphic loci. These results suggest that the morphological differences between the two subspecies are maintained mainly by selection since they occupy different ecological niches (Van Dijk & Van Delden 1981).

Different molecular marker systems show different levels of genetic variability. Studies using random amplification of polymorphic DNA (RAPD) have thus shown more genetic variation than studies of the same material examined for allozyme variation (Hidayat *et al.*, 1996; Haig *et al.*, 1994).. Morgan-Richards and Wolff (1999) studied the two subspecies of *P. major* using RAPD and ISSR (inter simple sequence repeats) procedures and found two well-differentiated groups of plants. One group was identified as *P. m.* subsp. *intermedia.* Within this group plants clustered first with other plants collected from the same locality. The second group was identified as *P. m.* subsp. *major.* In this group plants clustered but with much less structure than in *P. m.* subsp. *intermedia.* Five Swedish populations of *P. major* collected from southern (Skåne), southeastern (Blekinge), eastern (Stockholm) and western (Västergötland) parts of the country were studied at SLU Balsgard, Sweden. Two well-separated groups of plants were found; one with the populations from Skåne and the other with the other three populations. Within each group, plants clustered first with other plants collected from the same locality (Zubair *et al*. 2010).

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