International Journal of Medicinal Plants Research ISSN 2169-303X Vol. 11 (3), pp. 001-005, March, 2022.
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Review

Advances in the study of flavonoids in *Ginkgo biloba* leaves

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Accepted 16 December, 2021

***Ginkgo biloba* flavonoids are one of the most popular herbal supplements, taken for their perceived "memory enhancing" properties. In this paper, the current studies of *Ginkgo biloba* flavonoids from leaves is been reviewed. Flavonoids are derived from the products of the shikimate and acetate-malonate pathways. Flavonoid concentration in *G. biloba* leaves is not only regulated by the involvement of genes, but also by other factors, such as photosynthesis, tree age, season, extent of leaf maturity, plant seed- load, the reproductive ways of nursery stock and the variety, time, quantity of fertilizer application and so on. Ecological factors influence flavonoid concentration primarily during the young stage of ginkgo development. Among all ecological factors, light and temperature are the most important. Great progress in producing flavonoids by means of biological technology and chemical synthesis has been made. Comments and analysis on the methods of increasing flavonoid content in *G. biloba* leaves were made in this review.**

Key words: *Ginkgo biloba*, flavonoid, mechanism of control, external synthesis.

INTRODUCTION

In the present time, *Ginkgo biloba* is one of the most popular functional plants, especially as medicinal plants. Extracts of *G. biloba* leaves contain active compounds such as flavonoids and terpene lactones (ginkgolides and bilobalide) and can therefore be use to increase peripheral and cerebral blood flow (Smith and Luo, 2004; van Beek, 2002). So far, about 38 kinds of flavonoids have been isolated from *G. biloba* and among them were 28, 6 and 4 kinds of mono-, di- and tri- glycosides of flavonols, respectively (Hasler et al., 1992; Dubber and Kanfer, 2004; van Beek and Montoro, 2009). In recent years, there have been some documents on the changes of flavonoids content (FC) and their affecting factors, the mechanism of flavonoids formation (FF), the extranal chemical synthesis of flavonoids and tissue culture producing flavonoids among others (Lobstein et al., 1991; Shi et al., 1998; Sun et al., 1998; Kim et al., 1998; Cheng et al., 2001a, 2002, 2004a; Zhu et al., 2007; Hu et al., 2009). This review tries to reassess our present knowledge in order to synthesize the findings into a clear scheme of action, to identify the connections between the many still isolated findings, to understand the present contradiction and to identify future issues of research. The idea behind this is that improved cultural practices for high FC require improved theoretical foundation.

FF IN *G. BILOBA* LEAVES

Though many studies on FF have been carried out for a long time and the great progresses have been made, reports about FF based on *G. biloba* have not been found. However, the basic process of FF is clear with the help of studies on other plants. Modified graph of FF according to other documents has been found in Forkmann (1991), Lancaster (1992), Ju et al. (1995) and Holton and Cornish (1995). Flavonoids came from the

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Abbreviations: ABA, abscisic acid; **BV,** blue-violet light; **CHI,** chalcone isomerase; **CHS,** chalcone synthase; **FB,** flavonoids biosynthesis; **FC,** flavonoids content; **FF,** flavonoids formation; **FR,** far-red light; **GA,** gibberellic acid; **PAL-L**, phenylalanine ammonia-lyase; **R,** red light; UFGT-UDP-3-0-glucosyltransferase; **UV,** ultraviolet light.

derivatives of the products of shikimate pathway and acetate-mdonate pathway. The central step in flavonoids biosynthesis (FB) is the condensation of the molecules of malonyl-COA with a molecule of *p*-coumaroyl-COA to give the C15 chalcone intermediate (naringenin chalcone), a reaction catalysed by CHS and it is further transformed into various flavonoids. In general, flavornoids refer to a series of chemical compounds in which two aromatic rings are connected by tri-carbon chain, including flavones, flavanones, flavonols and cyanidind. Because of their specific property of light-absortion, cyanidins are classified as an individual type (van Beek, 2002). The flavonoids discussed in this paper do not include cyanidins.

EXTERNAL CONTROL OF FF

FF is affected by internal and external factors. The effects of them on FF are different with the exact factors.

THE COURSE OF GROWTH AND DEVELOPMENT

FC of leaves in ginkgo is influenced by season, year, tree age, leaf maturity, seed-load of plant and so on. There are four kinds of viewpoints about seasonal changes of FC (Shi et al., 1998; Hasler et al., 1992; Yuan et al., 1997; Li et al., 2006). Firstly, FC is the highest in fall, next in spring and the lowest in summer. Secondly, FC increases continuously from May to October. Thirdly, FC is the highest during sprouting (April), next in fall (August) and fourthly, there is no conclusive rule for the changes of FC. Though many contradictions can be discovered from the above, it is popular that ginkgo leaves are harvested in fall, sometimes together with ginkgo seeds. FC also varied with different years (Jiang and Tuo, 2006). There was some consistency between season and leaf maturity. However, this consistent relationship did not exist under special circumstances when leaves were collected more than one each year or when there was environmental stress. It was reported that the rate of FB was faster while FB ability was lower during leaf division. There was again a higher peak of FB while FC was dramatically negatively related to moisture content of leaves when ginkgo leaves stopped growing and approached old period (Lister et al., 1994). It therefore indicated that there could be more than one peak of FB during the whole leaf life. Considering that flavonoids are secondary metabolites and their formations are based on photosynthesis, it could be believed that FC in fall is the highest throughout a year. In addition, FC can be maintained at a stable level provided that the leaves still remained on trees in late fall or that post-harvest leaves were stored with sealed chamber (Xie, 1995). Lobstein et al (1991) and Yuan et al (1997) observed that flavonoid varieties and their proportions were regulated by seasonal change.

There is a trend that the reduction of FC is accompanied by the increase of tree age. FC was higher in leaves of young trees or shoots. The highest level was reached by 3 - 5 years old seedling plant. When tree age surpassed 20 years, FC remained stable at a lower level (Yang et al., 1997) . This result suggested that the influence of tree age on FC mainly took place at young stage of ginkgo tree. There were two oppositions about the effect of seed-load of plant on FC (Chen et al., 1997a) . One viewpoint is that FC of leaves in seed-load tree is lower than that in non-seed-load tree, while the other viewpoint is that FC is higher in the leaves of seed –load tree, and the difference of FC between them increases after July every year. It is related to the growing of vigor of trees.

ECOLOGICAL ENVIRONMENT

As a whole, the influences of ecological environment on FC are synthetically effective. The same cultivar can be tamed into different ecological types and FC of the same cultivar could also be different under different ecological environment. However, there were some inconsistent reports that FC and its changes did not show obvious regularity among different planting regions (Wu et al., 1995). For example, Yang et al. (1997) demonstrated that FC of the leaves in old ginkgo trees was not statistically different although they grew in different places. This indicated that the influence of ecological environment on FC was dependent upon tree age. These effects were only evident for the plants at young stage.

Light and temperature are the most important factors among all the ecological factors. FB requires light or is enhanced by light (Xie and Wang, 2006) . FF is absolutely light dependent and its biosynthetic rate is related to light intensity and density. Light quality also affected FC, with BV or UV being the most effective than white light even at low intensity. The duration of light treatment had no direct relationship with FF. An appropriate low temperature and a great temperature difference between daylight and night were beneficial to FF. It is one of the most important reasons that made FC increased gradually from South to North to a certain degree within given regions. Several researchers found that FF could be induced by the shift of high and low temperature with proper frequency. Low temperature was not the direct reason for the promotion of FF. Besides, a proper drought is also beneficial to FB (Leng et al., 2001). Recent progresses showed that the principal environmental factors influencing FC were latitudes, annual mean precipitation, annual mean sunshine percentage and annual mean temperature. A valuable viewpoint was therefore put forward that ginkgo leave-cultivation for higher content of flavonoids should be settled in circumstances with certain stress (Sun et al., 1998).

CULTURAL PRACTICES

Cultural practices contained many respects and depended on research angle and depth. The reproducible ways

on nursery stock brought about great influence on FC of the leaves in ginkgo. For example, FC of seedling was higher than that of grafted seedling (Chen et al., 1997a). The difference of FC was due to growing vigour of tree, namely, the vigorous trees yielded higher FC (Jang and Tuo, 2006).

Current studies on the influence of other cultural practices on FC were concentrated on the relationship between the yield of leaves and the factors such as the application of fertilizers and growth regulators, the density of planted nursery stock and the set-up of orchard with grafted seedlings. These cultural practices were to improve the quality of leaf—refer to formal index, that is, leaf area, leaf color and leaf thickness to raise leaf yield per unit area in order to accomplish the increase of total quantity of flavonoids (Chao et al., 1996; Chen et al., 1997b; Zhou and Xu, 1997). Excessive nitrogeneous (N) fertilizer interfered with FF, especially when applied to foliage in late stage of growth development, but it could increase leaf yield (Wang et al., 1995), Hu (1998) stated that N^+ ion implantation in *G. biloba* could lead to changes of shape and characters of leaves, including FC. Effect of potassium (K) fertilizer on FF was opposite to N. Wang (1995) pointed out that the leaf yield was almost increased to 200% by collecting leaves twice a year, but FC may have decreased because of the change of leaf color. Nevertheless, direct relationship between leaf yield and FC remains unclear.

MECHANISMS OF CONTROL

From the proceeding section, it became obvious that various external factors may have some effects on FF in *G. biloba*. However, little is known about how they exert their influence on the mechanisms of endogenous control with which they interfere. So far, insufficient FC has generally been attributed to a lack of the prerequisites for FF promotion, either inside or outside the leaf.

HEREDITY

Leaf FC of *G. biloba* was controlled by genes to a great extent. Chen et al. (1997a) reported that FC of Taian-4 was the highest (2.64%) and Jiandiyanlin the lowest (0.95%) among 44 experimental *G. biloba* cultivars, but FC of 68.4% *G. biloba* cultivars varied from 1.4 to 1.9%. This result implied a dual character that showed a tremendous difference of FC among the minority of cultivars and nearness of FC among the majority of cultivars. There was also a very high hereditary capacity in FC. It indicated that genotype was the most important factor to FC. Even FC of the same cultivar varied with plant sexual difference, but an essential distinction between male and female plant still remained a dispute. The ripening date of cultivar has effect on FC as the FC of early-maturing variety is usually lower than that of late variety (Shi et al., 1998; Fan et al., 1998).

ENZYMES AND PHOTOSYNTHESIS

The process of FB contains more than ten reactions and more than ten kinds of enzymes are involved. With the help of radioisotope technique, the biosynthesis and transformation of flavonoids have been known. The first source of flavonoids comes from photosynthetic products. Their precursors are simple phenols. The reaction catalysed by PAL is the deamination of $_L$-phenylalanine to yield trans -cinnamic acid and ammonia, which is the first committed step for the biosynthesis of the phenylpropanoid skeleton (Xu et al., 2008a). Activity of this key enzyme is closely related to the physiological or developmental status of a plant. Concomitant increase in the levels of PAL and phenolic compounds has been demonstrated in many tissues. In many cases, the increase in PAL is also coordinated with the appearance of other enzymes associated with the FB pathway (Cheng et al., 2005) . However, many other products such as anthocyanin, cutin and lignin are biosynthesized in the course of phenolic compounds being transformed into flavonoids (Graham, 1998). Therefore, sometimes FF is not dependent upon PAL activity.

Chalcone synthase (CHS), the first enzyme in the FB pathway, is responsible for the establishment of the C15 skeleton of flavonoid compounds. Its activity is related to light intensity, and it is considered a key enzyme in regulating FB (Xu et al., 2007). 4-Coumanate: CoA ligase, having many isoenzymes, which lies at a branch point from which many different types of products are derived, is also regarded as an important enzyme (Kumar and Ellis, 2003). Besides, UDP-3-o-glucosyltransforase (UFGT), catalyzing the formation of stable anthocyanin from unstable anthocyanidin and the formation of quercetin glycosides from quercentin, is closely correlated with the biosynthesis and transformation of flavonoids and anthocyanin (Xu et al., 2008b). Isomerization and transformation of flavonoids often take place automatically without chalcone isomerase (CHI), but it can be sped up by CHI (Muir et al., 2001).

Regulating mechanism of PAL in FF include (1) Relief of PAL inactive system, speeds up FF; (2) Activity and content of PAL have a direct effect on FF; (3) PAL regulates FF with the help of phytochrome. Red light (R) advances FF, whereas far-red light (FR) can offset this influence. Nevertheless, another viewpoint is that, long wave light increases activity of PAL and CHS and enhances flavonoids accumulation (Liu and Cheng, 2003; Cheng et al., 2005). In addition, PAL activity is inhibited by the products of the reaction. For example, cinnamic acid. Compared with PAL, little is known about the mechanisms of the other enzymes.

Though previous studies on the relationship between photosynthesis and FC have been carried out, most of the results remained contradictory. As for this, there were three different kinds of viewpoints: (1) There is a notable positive relationship between them (Xie and Wang, 2006); (2) There is a positive but indirect relationship un-

der given conditions. For example, the enhancement of sugar resulted in an increase of FC in plant tissue culture and glucose was the best treatment (Kim et al., 1998); (3) There is no relationship, not even a negative one. It was proved that the increase of sugar content in leaf and dry weight of leaf was not accompanied by the increase of FC and FB speed (Liu et al., 2001). On the contrary, flavonoids accumulation was observed when sugar content was lower. Reduction of chlorophyll content in fall is usually followed by an increase in FC (Fan et al., 1998). Considering the complexity of the process of FB and flavonoids metabolism, it is difficult to find out definite linear relationship between flavonoids and their precursors.

PLANT HORMONES

Plant hormones play roles in FF. Many reports indicated that ethylene and abscisic acid (ABA) enhanced FB, while the effect of gibberrllic acid (GA) was opposite with the growth promoting hormones giving inconsistent results (e.g. Cheng et al., 2004a, 2004b). The mechanisms by which plant hormone is connected with the regulation of enzyme activities has been reported. For example, ethylene improved FB by means of improving activities of enzymes. However, some researchers are of the view that ethylene increased FC by way of regulating the course of growth and development and accelerating the ripening, as was demonstrated in apple (Saure, 1990). As long as fruit is at the ripening stage, the accumulation of flavonoids in its peel can be examined even though there is no visible peak of release of enthylene. This may imply that maturity is a more important factor than ethylene to FB. GA speeds growth and reduces CHS activity so that FB is exhibited (Carrier et al., 1990). ABA increases FB by repressing GA activity (Carrier and Laurain, 2000). However, there was no report on the mechanism of FB control based on ginkgo until now. Thus, it is really necessary to study FB in the leaves of ginkgo plant.

EXTERNAL SYNTHESIS OF FLAVONOIDS

Owing to the difficulty of regeneration, changeable browning and others in tissue culture of ginkgo, it is very hard to produce flavonoids practically by taking advantage of biological technology. However, some progresses have been made (e.g. Kim et al., 1998, 1999; Hao et al., 2007; Zhu et al., 2008). Most studies not only induced callus and found the accumulation of flavonoids in callus, but also obtained some useful data on tissue culture conditions. However, the FC from ginkgo callus was very low. Tang et al. (2004) succeeded in the chemical synthesis of flavonoids, but the procedure was very complex and was also very expensive. In addition, chemical synthetic flavonoids were poorer in medicinal effects than natural biosynthetic ones due to the different varieties and proportions of flavonoids in the former.

Above all, either tissue culture or chemical synthesis is far from practical application. However, with the development of molecular biology, for example, the successful cloning of genes encoding some key enzymes, the prospect for external synthesis of flavonoids is optimistic.

ACKNOWLEDGEMENTS

The work was supported by the Natural Science Foundation of China (30971974), and Natural Science Fund of Hubei Province (2008CDA061) and University-Industry Cooperation Fund of Hubei Educational Office (CXY2009B009).

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