Unleashing The Power of Tea Flavonoids - Two Decades of Research

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Tea Flavonoids are ubiquitous secondary metabolites with a multitude of biological properties. Flavones, flavonols, flavanols, anthocyanins and proanthocyanins are the main classes of flavonoids present in the tea plant, *Camellia sinensis. In both green and black tea flavanols (flavan-3-ols)*popularly known as catechins play a vital role in imparting taste, colour, astringency and a plethora of health-promoting effects. Considering the amount and variety of metabolites present in the tea plant could indeed be called a natural products laboratory.

Since the year 2000 our research has revealed the immense potential of flavonoids in the tea plant

Biochemical Marker for Identifying Resistance and/or Susceptibility of a Tea Plant to Blister Blight Leaf Disease Caused by the Fungus *Exobasidium vexans.*

Metabolites of the Tea leaves resistant and susceptible to the *Exobasidium vexans*, which causes blister blight leaf disease (Figure 1) were investigated with a metabolomics approach (Punyasiri et al, 2005).

Figure 1: Tea Leaves infected with *Exobasidium vexans* (Blister Blight leaf disease)

A study of the flavan-3-ol and caffeine content of resistant and susceptible tea cultivars showed that significantly higher levels of (j)-epicatechin (22.4 mg/g) were present in cultivars of tea resistant to blister blight compared with those in susceptible cultivars (11.3 mg/g) A significantly higher level of (j)-epigallocatechin gallate was present in susceptible cultivars compared with resistant cultivars. No significant differences were seen among cultivars in (+)-catechin, (j)-epigallocatechin, (j)-epicatechin gallate, or caffeine. Resistant cultivars with higher levels of epicatechin had lower levels of epigallocatechin gallate (Punyasiri et al, 2005). The resistance of apple cultivars (Treutter and Feucht, 1990) to *Venturia inaequalis*, and avocado (Prusky et al., 1996; Prusky, 1996) to anthracnose have been attributed to high levels of epicatechin, suggesting that epicatechin may be directly or indirectly involved in the resistance mechanism of tea against blister blight.

Enhancement of Levels of Secondary Metabolites with Fungal Infections

Histochemical staining of tea leaves with vanillin reagent (Broadhurst and Jones, 1978) and 4-dimethylaminocinnamaldehyde (DMACA) reagent (Li et al., 1996) gave evidence for the accumulation of proanthocyanidins in the infected areas of the leaf tissue (Figure 2 and 3) Vanillin reagent produced the deep red color characteristic for flavan-3-ols or proanthocyanidins, and the blue green color shown by DMACA (Figure 4) indicated that proanthocyanidins were formed on infection. DMACA has been shown to be more sensitive to soluble proanthocyanidins only with the terminal units of proanthocyanidins (Rohr, 1999).

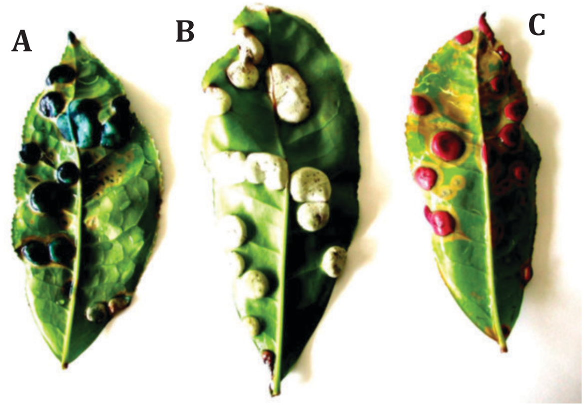

Figure 2: Histochemical staining of tea leaves with mature *E.vexans* blisters
A – DMACA staining, B – without staining,
C – Vanillin staining

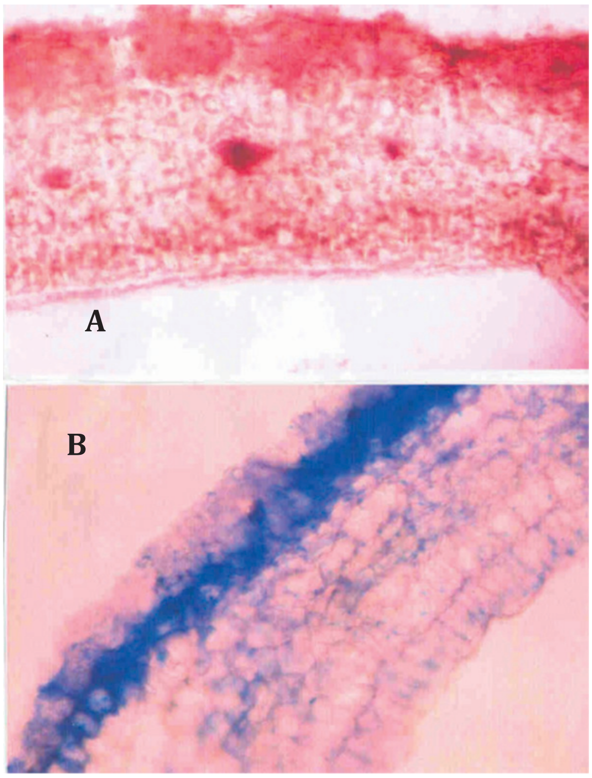

Figure 3: Histochemical staining of t.s sections of *E.vexans* infected tea leaves
A- Vanillin staining; B- DMACA staining

Enhancement of Levels of Secondary Metabolites with Fungal Infections

Acid-catalyzed oxidative depolymerization of proanthocyanidins to the anthocyanidins, cyanidin, and delphinidin has been used to detect proanthocyanidin levels in plants (Porter et al., 1986). Depolymerization of infected tissue gave higher levels of cyanidin and delphinidin compared with healthy tissue. The observed reduction in catechin levels and increase in proanthocyanidin levels on infection suggest a possible role for proanthocyanidins in the defense mechanism. Of the cultivars studied here, TRI 2043 is the most resistant cultivar to blister blight. Substantially higher amounts of cyanidin and delphinidin were formed during the acid hydrolysis of the extract of the purple green leaf of this cultivar when compared with other cultivars. The identities of the anthocyanidins were confirmed by comparing their ultraviolet (UV) visible spectra, paper chromatography, TLC, and HPLC values (Harborne, 1958, 1967) with those of standards. The color of the leaf was, therefore, attributed to the presence of high levels of anthocyanins.

The higher tolerance of this cultivar may be explained as being due to the enhanced levels of catechins, which are proanthocyanidin precursors formed from the anthocyanins. The increase in epicatechin gallate at the translucent stage could be due to the conversion of epicatechin into its gallic acid ester upon infection, since the translucent stage is accompanied by a significant decrease in epicatechin content. Similar observations were seen with epigallocatechin gallate and epigallocatechin content, although the increase in the former at the translucent stage was not significant Epicatechin appears to play an important role in the resistance mechanism of tea against blister blight disease, while methylxanthines may play a role during initial attack by the fungus, E. vexans. The high resistance of the cultivar TRI 2043 is attributed to the high level of anthocyanins present (Punyasiri et al 2004a, Punyasiri et al, 2005).

Elucidation of the Flavonoid Biosynthesis Pathway in Tea and Reporting of the Enzyme, Anthocyanidin Reductase for the First Time

The high polyphenol contents in tea leaves, particularly the flavan 3-ols and derivatives, hamper the isolation of active enzymes. Applying an enzyme preparation method developed for other plant tissues with high concentrations of polyphenols, enzymes of flavonoid biosynthesis were successfully characterized in combination with 14C-labeled flavonoids as substrates. For anthocyanidin reductase (ANR) a non-radioactive enzyme test was developed and used for

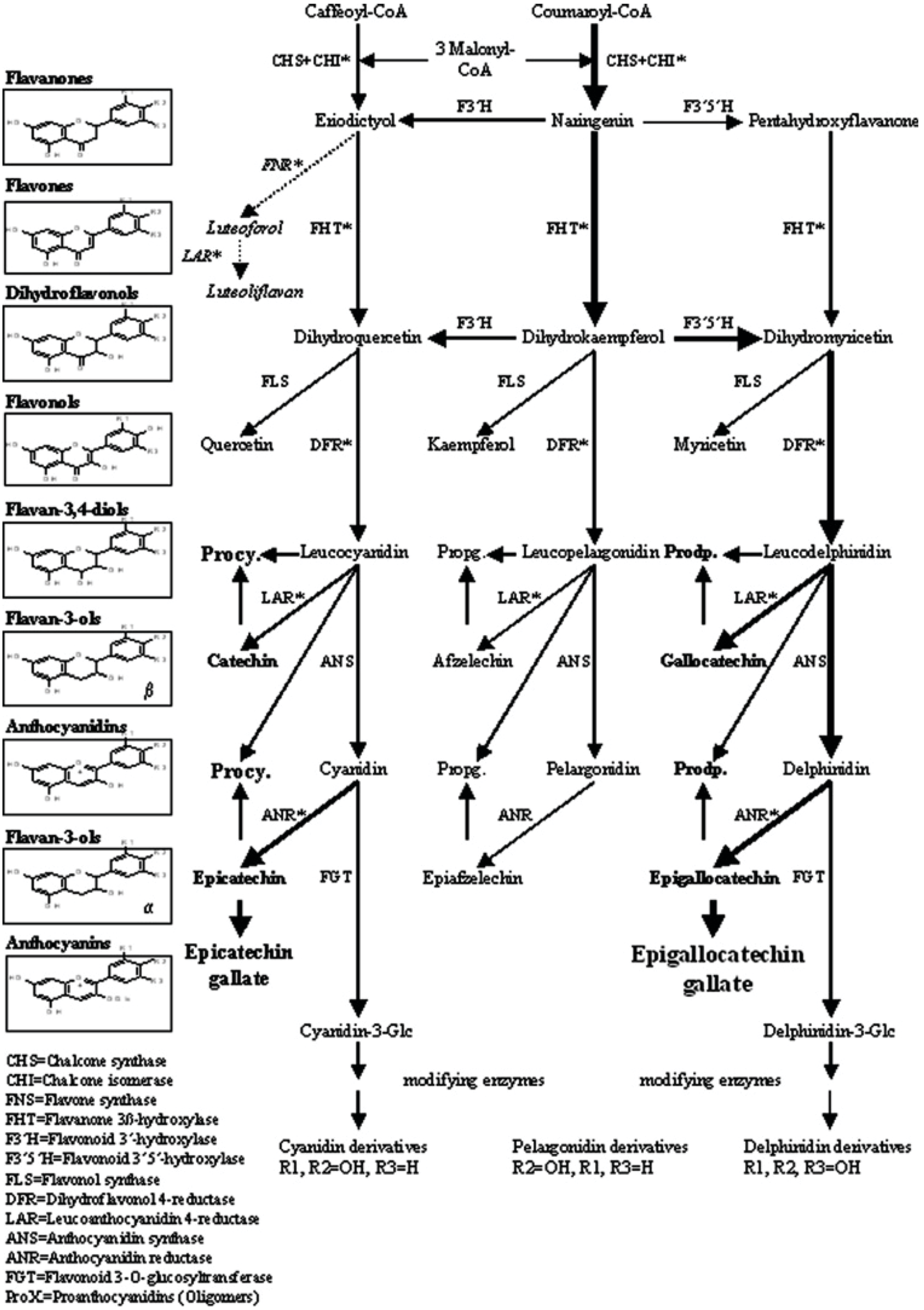

Figure 4: (Adapted from Punyasiri, 2006)

characterization of tea ANR activity (Punyasiri et al, 2004b).

The enormous content of flavan 3-ols is the most striking feature of tea leaf chemistry, it is up to 20% (w/w, dry) and depends mostly on tea variety. These flavan 3-ols are presented by or derived from catechin (3b-hydroxy group), epicatechin (3a-hydroxy group) or the respective trihydroxyflavan 3-ols, gallocatechin and epigallocatechin. Demonstration of flavonoid enzymes with their respective substrate specificities and substrate conversions of the cytosolic enzymes allows us to outline the most important branches of pathways in tea flavonoid biosynthesis (Punyasiri et al, 2004b)

The ANR reaction is of great importance in tea since the flavan 3a-ols, epicatechin and epigallocatechin, and their derivates are more abundant in tea leaves than the flavan 3b-ols catechin and gallocatechin.

Despite the small, stereochemical difference of both groups with respect to C-3, their biosyntheses are conspicuously different. As shown and discussed below, catechin and gallocatechin are synthesized from the respective leucoanthocyanidin, leucocyanidin, and leucodelphinidin, respectively, by the action of LAR. For the biosynthesis of epicatechin and epigallocatechin in contrast, the leucoanthocyanidins are not the direct precursors.

First anthocyanidin synthase has to convert the leucoanthocyanidins to anthocyanidins, which may be glucosylated to anthocyanins. Alternatively, as has been demonstrated here, anthocyanidins are converted to flavan 3a-ols (epicatechins). Recently it was reported (Xie et al, 2003) that the banyuls gene of *A. thaliana* as well as a homolog from *Medicago truncatula* code for anthocyanidin reductases (anr), which reduce anthocyanidins such as cyanidin and delphinidin to their corresponding flavan 3a-ols using NADPH as a cosubstrate. Sequences of putative anr cDNAs are also reported from Phaseolus, Gossypium, and Vitis (Tanner et al, 2003). This remarkable report elucidated the biosynthetic pathway of flavan 3a-ol biosynthesis and, on the other hand, showed, that anthocyanidins are not only the precursors for anthocyanin biosynthesis. It was now shown that tea leaves contain a strong ANR activity producing epicatechin and epigallocatechin. Even at the suboptimal pH 7.5, the ANR activity measured by epicatechin production is about seven times higher than the combined activity of DFR and LAR leading to catechin. The high ANR activity seems to be essential with respect to the dominance of epicatechin and epigallocatechin and also their galloyl esters as the major flavonoid components in tea leaf. Epicatechin and epigallocatechin, together with catechins, are also the building blocks of proanthocyanidins reported from tea (Kiehne et al,.1997) Thus, ANR together with LAR, may be of great importance for the biosynthesis of proanthocyanidins.

The substrate specificities of the cytosolic flavonoid enzymes provide an explanation for the flavonoid contents in the tea leaf. The flavan 3,4-diols delivered by CHS, CHI, FHT, and DFR are either converted by ANS and the high ANR activity to epicatechin and epigallocatechin or by LAR activity to catechin and gallocatechin. This explains why epicatechin-type and catechin-type flavan 3-ols are the dominating flavonoids of the tea leaf.

Role of flavan-3-ols in Establishing High Quality in Tea Cultivars Suitable for both Green Tea and Black Tea Manufacture.

Tea (*Camellia sinensis* L.) is well known for its biochemical constituents that define the product quality and confer pest and disease resistance. Seven major metabolites – epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), epigallocatechin gallate (EGCg), caffeine, theobromine, and gallic acid – of 87 beverage type and six non-beverage type tea accessions from Sri Lankan tea germplasm were profiled using HPLC and LC-MS/MS. All seven metabolites varied widely in the beverage type accessions. The non-beverage types only contained gallic acid and epicatechin. Results prove the presence of high EC and ECg contents in green leaves to be a reliable marker for identifying high-quality black tea producing accessions. High EC and low EGCg contents in green tea leaves appear to enhance traits of resistance to blister blight disease. Significant variations detected in theobromine, caffeine, and total polyphenol content define the affinity of germplasm to the main three tea taxa, and we conclude that C. sinensis ssp. lasiocalyx predominates the Sri Lankan germplasm collection (Punyasiri et al, 2017).

There is a wide variation in the contents of TPP, major catechins, caffeine, theobromine, and gallic acid in the Sri Lankan tea germplasm. The amounts of dihyroxylated, trihydroxylated, gallated, and non-gallated catechins and their ratios present in tender tea leaves (two leaves and a bud) determine the ultimate quality of black tea. The accessions used to produce high-quality black tea had high amounts of dihydroxylated catechins, EC and ECg, high ratios of dihydroxylated to trihydroxylated catechins, and low ratios of gallated to non-gallated catechins. Metabolic profiles can be effectively used in choosing accessions of desired tea quality for propagation and in tea breeding programmes to generate progenies with wide variation.

Establishing the Involvement of the Major Flavonols in Tea Accessions and Correlating them with Exotic Tea Accessions.

Flavonol glycosides in tea leaves have been quantified as aglycones, quercetin, myricetin, and kaempferol. Occurrence of the said compounds was reported in fruits and vegetable for a long time in association with the antioxidant potential. However, data on flavonols in tea were scanty and, hence, this study aims to envisage the flavonol content in a representative pool of accessions present in the Sri Lankan tea germplasm. Significant amounts of myricetin, quercetin, and kaempferol have been detected in the beverage type tea accessions of the Sri Lankan tea germplasm. This study also revealed that tea is a good source of flavonol glycosides. The *Camellia sinensis* var. *sinensis* showed higher content of myricetin, quercetin, and total flavonols than var. *assamica* and ssp. *lasiocalyx*. Therefore flavonols and their glycosides can potentially be used in chemotaxonomic studies of tea germplasm. The nonbeverage type cultivars, especially *Camellia rosaflora* and *Camellia japonica* Red along with the exotic accessions resembling China type, could be useful in future germplasm studies because they are rich sources of flavonols, namely, quercetin and kaempferol, which are potent antioxidants. The flavonol profiles can be effectively used in choosing parents in tea breeding programmes to generate progenies with a wide range of flavonol glycosides (Punyasiri et al, 2016).

Significant amounts of myricetin, quercetin, and kaempferol have been quantified in the beverage type tea accessions of the Sri Lankan tea germplasm. The var. *sinensis* showed higher content of myricetin, quercetin, and total flavonol than var. *assamica* and ssp. *Lasiocalyx*; therefore flavonols and their glycosides can be useful to chemotaxonomic studies of tea germplasm.The nonbeverage type cultivars, especially *Camellia rosaflora* and *Camellia japonica* Red along with the exotic accessions resembling China type, are worthwhile in future germplasm studies because they are rich sources of flavonols, namely, quercetin and kaempferol, which are potent antioxidants and health-promoting aspects. Additionally, the flavonol profiles can be effectively used in choosing accessions in tea breeding programmes to generate progenies with wide variations (Punyasiri et al, 2016).

The above findings are presently applied in the plant breeding programme of the Tea Research Institute, Sri Lanka.

References

- Brasathe Jeganathan, P.A Nimal Punyasiri, J. Dananjaya. Kottawa-Arachchi Mahasen.. Ranatunga I.Sarath.B.Abeysinghe , M. T. Kumudini Gunasekare , and B.M.Ratnayake Bandara (2016). Genetic Variation of Flavonols : Quercetin, Myricetin and Kaempferol in the Sri Lankan Tea (*Camellia sinensis* L.) and Their Health-Promoting Aspects. International Journal of Food Science. Article ID 6057434, 9 pages, http://dx.doi.org/10.1155/2016/6057434
- Broadhurst, R.B and Jones, W.T (1978),. Analysis of condensed tannins using acidified vanillin. J. Sci. Food Agric. 29:788Y794.
- De-Yu. Xie, S.B. Sharma, N.L. Pavia, D. Ferreira, R. Dixon (2003), Role of Anthocyanidin Reductase, Encoded by BANYULS in Plant Flavonoid Biosynthesis, Science 299 (2003) 396–399.
- Harborne, J. B. (1958). Spectral methods in characterizing anthocyanins. Biochem. J. 70:22Y28.
- Harborne, J. B (1967.) Anthocyanins, in Comparative Biochemistry of Flavonoids. Academic Press, London.
- Kiehne, A, Lakenbrink, C, Engelhardt, U.H (1997). Analysis of Proanthocyanidins in Tea

samples -LSMS Results, Lebensm. Unters. Forsch. 205.153–157.

- Li, Y. G., Tanner, G., and Larkin, P. (1996). The DMACA / HCl protocol and threshold proanthocyanidin content for bloatVsafety in forage legumes. J. Sci. Food Agric. 70:89Y91.
- Porter, L. J., Hrstich, L. N., and Chan, B. G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25:223Y230
- Punyasiri, P.A.N, Fischer, T.C Maretns, S., Forkmann, G., and Fischer, T.C. (2004). Flavonoid Biosynthesis in the Tea plant *Camellia sinensis*: Properties of prominent Epicatechin and Catechin Pathways. Archives of Biochemistry and Biophysics 431 (1), 22-30.
- Punyasiri, P.A.N., Tanner.G, Abeysinghe, I.S.B., Kumar, V., Campbell, P.M and Pradeepa, N.H.L (2004). Exobasidium vexans infection of leaves of tea (*Camellia sinensis*) increases 2,3-cis isomers and gallate esterification of proanthocyanidins Phytochemistry 65, 2987–2994.
- Punyasiri, P.A.N., Abeysinghe, I.S.B. and Kumar., V. (2005). Preformed and Induced Chemical Resistance of tea plant against Exobasidium vexans infection. Journal of Chemical Ecology 31,1315-1323.
- Punyasiri, P.A.N, Brasathe Jeganathan, J. Dananjaya Kottawa-Arachchi, Mahasen Ranatunga, I. Sarath B. Abeysinghe, M. T. Kumudini Gunasekare, and B. M. Ratnayake Bandara (2017). Genotypic variation in biochemical compounds of the Sri Lankan Tea (*Camellia sinensis* L.) accessions and their relationships to quality and biotic stresses. J. Horticultural Science and Biotechnology, Volume 92 (5)
- Punyasiri, P.A.N, Brasathe Jeganathan, J. Dananjaya. Kottawa-Arachchi Mahasen. Ranatunga I.Sarath.B.Abeysinghe , M. T. Kumudini Gunasekare , and B.M.Ratnayake Bandara (2015). Optimization of Sampling Methodology for Metabolite Profiling of Sri Lankan Tea. J. Analytical Methods in Chemistry, ID 964341, 6 pages. http://dx.doi.org/10.1155/2015/964341
- Punyasiri P.A.N (2006), PhD Thesis - Preformed and Induced Chemical Resistance of tea plant against Exobasidium vexans infection, Postgraduate Institute of Science, University of Peradeniya Sri Lanka.\
- Prusky, DPRUSKY, D. (1996). Pathogen quiescence in post harvest diseases. Annu. Rev. Phytopathol. 34:413Y434.
- Prusky, D., Hamadan, H., Arfi, R., and Keen, N. T. 1996. Induction of biosynthesis of epicatechin in avocado suspension cells treated with an enriched CO_2 atmosphere. Physiol. Mol. Plant Pathol. 48:171Y178.
- Rohr, G. E. 1999. Analytical investigation on and isolation of procyanidin from Crataegus leaves and flowers. PhD dissertation. ETH, Zurich.
- Tanner,G.J, Francki,K.T, Abrahams, S, Watson, J.M, Larkin, P.J and Ashton A.R (2003). Proanthocyanidin biosynthesis in plants. Purification of legume leucoanthocyanidin reductase and molecular cloning of its cDNA, J. Biol. Chem. 278 (34),31647– 31656.
- Treutter, D and Feucht, W. (1990). The pattern of flavan-3-ols in relation to scab resistance of apple cultivars. J. Hortic. Sci. 65:511Y517.