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Quantitative Estimation of Total Free Amino acids, Vitamins and Secondary Metabolites in Leaves of RC-1, RC-2, G-2 and G-4 Mulberry (Morus alba L.) Genotypes

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Abstract: ***Mulberry leaf exclusively assures the growth and development of the silkworm larvae, being considered a complete value nutrient, so that the knowledge of its nutritional status is of great interest. The experiment was made in order to study the total free amino acids, vitamins and secondary metabolites value in leaves of mulberry cultivars: RC-1, RC-2, G-2 and G-4. Among four cultivars G-4 is the best one containing highest total free amino acid in tender leaves (15.33µg/g), riboflavin in tender leaves (0.49 mg/g), niacin in coarse leaves (0.78mg/g), ascorbic acid in medium and coarse leaves (2.39mg/g) total phenols in medium leaves (4.96mg/g) and total flavonoids in medium leaves (5.68mg/g) compare to other three cultivars, so G-4 cultivar is highly recommendable feed for silkworm to increase their silk productivity.***
Keywords: Bombyx mori, free amino acid, riboflavin, ascorbic acid, phenols, flavonoids.

I. INTRODUCTION

Sericulture activities are agro-based and the industrial sector. The agro-based part involves mulberry cultivation and silkworm rearing. The economy of the sericulture industry depends not only on the race of the silk worm but also on the quality of the mulberry leaves. Mulberry (Morus spp.) is the exclusive source of feed for the silkworm – *Bombyx mori L.* in commercial sericulture (Ullal and Narasimhanna, 1981). Mulberry plays an important role in the quantity and quality of silk production, contribute to 38.20% for the success of cocoon crop (Miyashita, 1986). Silkworms feed on mulberry leaves during their entire larval period and utilize the leaf protein for the biosynthesis of silk. It is therefore clear that mulberry plays a dominant role in cocoon production as a source of nutrition to the silkworms.

The various compositional factors of mulberry leaves are liable for flourishing cocoon harvest and silk productivity, thus the mulberry leaf quality plays a predominant role in healthy growth of silkworm. Hence nutrition of silkworm is of primary importance as the cocoon production is directly influenced by the nutritive status of mulberry leaves. The quality of feed is determined by its major components such as water, carbohydrates, proteins, mineral, elements, fats, amino acids, secondary metabolites and vitamins (Thirumalaisamy et al., 2009). Silkworms obtain 72–86% of their amino acids from mulberry leaves and more than 60% of the absorbed amino acids are used for silk production. Increase in total proteins and free amino acid implies increased metabolic activities and activation of silk production.

Amino acids are building blocks for the synthesis of proteins, including antioxidant enzymes. Some amino acids and small peptides directly scavenge oxygen free radicals.

Vitamins are a group of unrelated organic compounds needed only in minute quantities in the diet that are essential for specific metabolic reactions within the cell and necessary for normal growth and maintenance of health of the plant. Ito (1978) determined that generally vitamins present in the mulberry leaves satisfy minimum needs of silkworm but the amount of vitamins present in mulberry leaves varies on the basis of environmental conditions, usage of fertilizers in field and mulberry varieties and other field practices. Riboflavin (vitamin-B2) is a water soluble vitamin, important in promoting the release of energy from carbohydrates, fats and proteins “i.e. in the metabolic pathway for ATP production” .Vitamin B3 (Niacin), also referred to as nicotinamide and nicotinic acid, is another water-soluble, B-vitamin involved with energy metabolism. The coenzymes of niacin (NAD/NADH/NADP/NADPH) are necessary for ATP synthesis (the body’s main energy source), synthesis of fatty acids and some hormones and the transport of hydrogen atoms. Ascorbic acid (vitamin C) is an important vitamin and is abundant in plant tissues:

green leaves have the same amount of ascorbate as chlorophyll (Foyer, 1993). Vitamin C also aids in detoxification of various metabolic or tissue toxins and acts as a strong antioxidant, increasing protein synthesis. Several reports are available where in ascorbic acid significantly increased the weight of silkworm larvae, silk filament length, weight and denier values (Babu et al., 1992), silk production (Sengupta et al., 1972**).**

Sengupta et al., (1972) have showed that B. mori requires specific essential sugars, amino acids, proteins and vitamins for its normal growth, survival and also for the growth of silk gland. Akhtar and Asghar, (1972**)** have found that vitamins and mineral salts played an important role in the nutrition of silkworm.

Plant cells produce two types of metabolites. Metabolites are compounds synthesized by plants for both essential functions, such as growth and development (primary metabolites), and specific functions, such as pollinator attraction or defense against herbivores (secondary metabolites). Primary metabolites are involved directly in growth and metabolism. Flavonoids are a class of secondary metabolites with basic structure of two aromatic rings and an oxygen atom. They are water soluble phenolic glycosides (Deepika and Vidya, 2013). These secondary metabolites are also of interest because of their use as dyes, fibers, glues, oils, waxes, flavoring agents, drugs and perfumes, and they are viewed as potential sources of new natural drugs, antibiotics, insecticides and herbicides (Dewick, 2002; Croteau et al., 1987). Phenols are plant secondary metabolites, and they have an important role as defense compounds. The most important phenolic compounds implicated in defence mechanism of plants against pathogens are coumaric acid, phloretin, umbelliferous, caffeic acid, chlorogenic acid and ferulic acid (Agrios, 1969). Flavonoids are the largest group of plant phenols and the most studied (Dai and Mumper, 2010). Flavonoids have been reported to exert multiple biological property including antimicrobial, cytotoxicity, anti-inflammatory, antibacterial, antiviral (Cushnie and Lamb, 2005; Murray, 1998; Cook and Samman, 1996). The most important phenolic compounds implicated in the defense mechanism of plants against pathogens are coumaric acid, phloretin, umberlliferons, caffeic acid, chlorogenic acid and ferulic acid (Agrios, 1969). When such substances are ingested by the phytophagous insects along with the food, they get access to the natural defense mechanism. Therefore, the present investigation was conducted to evaluate the some biochemical composition of different maturity leaves of newly evolved mulberry (*Morus alba*) genotypes.

II. MATERIALS AND METHODS

The experiment was conducted in the Botanical garden of Botany Department, Sri Krishnadevaraya University. Mulberry (*Morus alba L*) varieties-RC-1, RC-2, G-2 and G-4 cuttings were collected from eight-months-old healthy plants from the Regional Sericultural Research Station (Central Silk Board-RSRS), Rapthadu, Anantapuramu, A.P, India. The cuttings made were of 5-6 inches long with a minimum of three to four active buds. The cuttings were brought to the laboratory and immediately planted in red soil containing FYM in 3:1 proportions. Each variety was divided into four sets and arrange in Randomized complete Experimental Block Design (REBD). The plants were kept under natural photoperiod of about 12-13hrs with a temperature of 28 ± 4^{o}C. The leaves were oven - dried and processed to analyze the free amino acids and secondary metabolites. The results were analyzed statistically by applying Duncans Multiple Range (DMR)-test

A. Estimation of total free amino acids:

Total free amino acid (Ninhydrin method) was estimated by the method of Moore and Stein (1948). 1ml of the sample was mixed with 1ml of Ninhydrin and kept in boiling water bath for 20minutes. Added 5ml of diluent (equal volume of water and n-propanol) and incubated at room temperature for 15minutes. The absorbance was read at 570nm against a reagent blank. The estimation was done in triplicates and the results were expressed as mg/g sample.

B. Riboflavin

The riboflavin extraction methodology was adapted from Esteve et al., (2001). 5g of the sample was extracted with 100ml of 50% ethanol solution and shaken for 1h. This was filtered into a 100ml flask; 10ml of the extract was pipette into 50ml volumetric flask. 10ml of 5% potassium permanganate and 10ml of 30% H_2O_2 were added and allowed to stand over a hot water bath for about 30min. 2ml of 40% sodium sulphate was added. This was made up to 50ml mark and the absorbance measured at 510nm in a spectrophotometer.

C. Niacin

Niacin was determined by the method of Scalar (2000). 5g of the sample was treated with 50ml of 1 N sulphuric acid and shaken for 30min. 3 drops of ammonia solution were added to the sample and filtered.10ml of the filtrate was pipette into a 50ml volumetric

flask and 5ml potassium cyanide was added. This was acidified with 5ml of 0.02 N H_2SO_4. The absorbance was measured in spectrophotometer at 470nm.

D. Ascorbic acid

Ascorbic acid of leaf samples were estimated by titration method using DCPIP (2, 6-dichlorophenolindophenol) dye. Pipette out 5ml of the working standard solution into a 100ml conical flask. Added 10ml of 4% oxalic acid and titrate against the dye (V1ml). End point is the appearance of pink color which persists few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid. Extract the leaf sample (500mg) in 4% oxalic acid and make up to a known volume (100ml) and centrifuge. Pipette out 5ml of this supernatant, add 10ml of 4% oxalic acid and titrate against the dye (V2 ml).

Amount of Ascorbic acid mg/g of sample was calculated by the following formula,

Ascorbic acid (mg/g) = (0.5mg/V1ml) × (V2/5ml) × (100ml/wt of Sample taken) ×100

E. Estimation of total phenols

Total phenolic content (Folin - Ciocalteau method) was estimated by the method of Bray and Thorpe (1954). Added 1ml of sample with 0.5ml of Folin phenol reagent and incubated at room temperature for 3minutes. Added 2ml of 20% Na_2CO_3 after 3minutes, mixed well and incubated in boiling water bath for 1minute. Rapidly cooled and the absorbance was read at 650nm against reagent blank. The estimation was done in triplicates and the results were expressed as mg/g sample.

F. Total flavonoids

Flavanoids was estimated by the method of Jia et al., (1999). 1ml of the extract was mixed with 0.075ml of 5% Sodium nitrite solution and incubated at room temperature for 10minutes. 10% aluminum chloride was then added and incubated at room temperature for 6minutes. Then 1N sodium hydroxide was added. The absorbance was read at 510nm against a reagent blank. The estimation was done in triplicates and the results were expressed as mg catechin equivalent/g sample.

III. RESULTS

The results that are obtained are given in tables as follows under each of its respective topics. All the experiments performed were under standard laboratory conditions with standard protocols.

A. Determination of total free amino acids

The amino acid content from mulberry leaf changes in accordance with the age of the leaf and provides the nutritional needs of the silkworm larva. Quantitative determination of free amino acids in tender, medium and coarse leaves of RC-1, RC-2, G-2 and G-4 mulberry cultivars were presented in Table I. Showed that significantly high (15.33µg/g) content of free amino acid was observed in tender leaves of G-4 cultivar followed by tender leaves of RC-2, G-2 and RC-1 cultivars. Whereas, significantly lower (9.28µg/g) levels was observed in coarse leaves of RC-1 mulberry cultivar.

Table I: Analysis of total free amino acid content in tender, medium and coarse leaves of RC-1, RC-2, G-2 and G-4 mulberry cultivars

Mulberry cultivars	Total free amino acid (µg/g)		
	Tender	Medium	Coarse
RC-1	9.28 ±1.21	9.31 ±1.26	8.12 ±1.15
RC-2	13.28 ±2.35	13.57 ±1.42	12.45 ±1.47
G-2	10.54 ±1.52	10.74 ±1.29	10.29 ±1.34
G-4	15.33 ±1.23	14.36 ±1.22	13.62 ±1.25

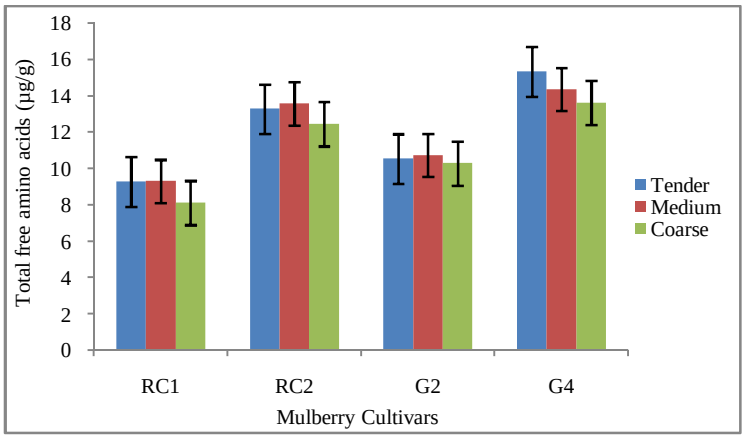

Fig: 1. Comparison of total free amino acid content in leaves of mulberry cultivars

B. Estimation of Vitamins

Quantitative determination of riboflavin, niacin and ascorbic acid in tender, medium and coarse leaves of RC-1, RC-2, G-2 and G-4 mulberry cultivars were presented in Table II. Showed that significantly higher levels (0.52 mg/g) of riboflavin was observed in medium leaves of G-4 mulberry cultivars and lower levels (0.33mg/g) were presented in tender leaves of RC-1 cultivar. Significantly maximum (0.78 mg/g) content of niacin was observed in coarse leaves of G-4 variety and minimum (0.65mg/g) content was presented in both tender and medium leaves of RC-1 mulberry cultivars. The content of ascorbic acid was significantly higher (2.39 mg/g) in both medium and coarse leaves of G-4 cultivar and lower (2.21 mg/g) in tender leaves of RC-1 mulberry cultivars.

Table II: Analysis of riboflavin, niacin and ascorbic acid contents in tender, medium and coarse leaves of RC-1, RC-2, G-2 and G-4 mulberry cultivars

Mulberry cultivars	Riboflavin (mg/g)			Niacin (mg/g)			Ascorbic acid (mg/g)		
	Tender	Medium	Coarse	Tender	Medium	Coarse	Tender	Medium	Coarse
RC-1	0.33 ±0.05	0.35 ±0.06	0.36 ±0.04	0.65 ±0.08	0.65 ±0.04	0.67 ±0.05	2.21 ±0.08	2.25 ±0.07	2.24 ±0.09
RC-2	0.47 ±0.06	0.46 ±0.04	0.48 ±0.07	0.72 ±0.05	0.70 ±0.06	0.71 ±0.07	2.39 ±0.04	2.34 ±0.06	2.34 ±0.11
G-2	0.38 ±0.04	0.39 ±0.08	0.41 ±0.08	0.73 ±0.03	0.70 ±0.08	0.69 ±0.06	2.25 ±0.02	2.23 ±0.05	2.24 ±0.06
G-4	0.49 ±0.05	0.52 ±0.02	0.48 ±0.08	0.74 ±0.05	0.72 ±0.09	0.78 ±0.05	2.38 ±0.06	2.39 ±0.05	2.39 ±0.08

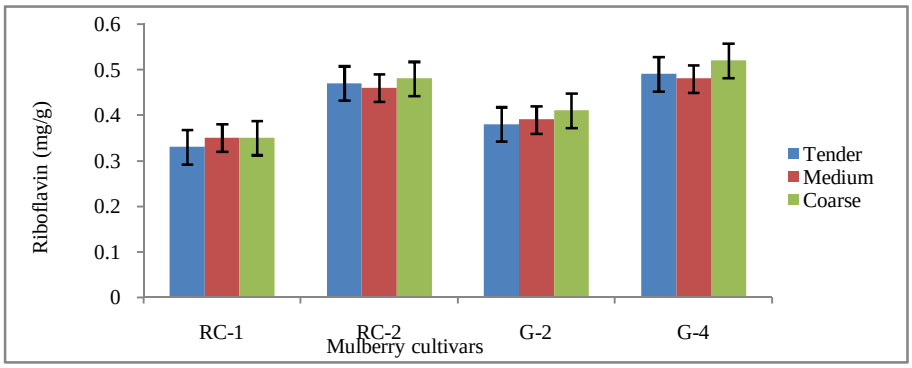

Fig: 2. Comparison of riboflavin content in leaves of various mulberry cultivars

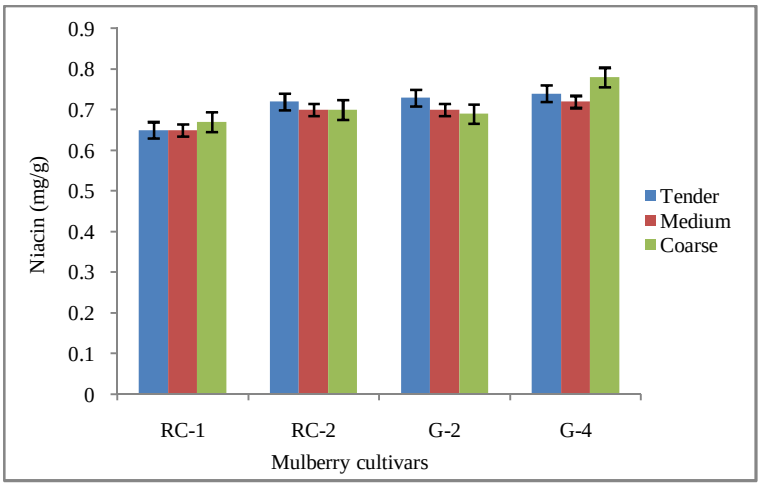

Fig: 3. Comparison of niacin content in leaves of various mulberry cultivars

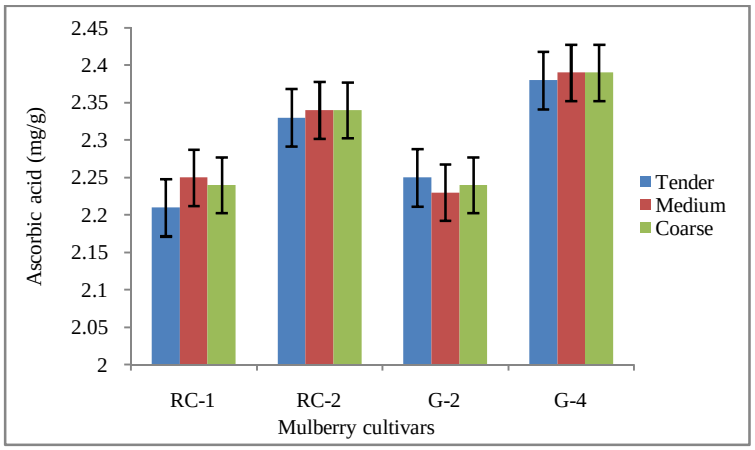

Fig: 4. Comparison of ascorbic acid content in leaves of various mulberry cultivars

C. Quantitative estimation of Secondary metabolites

Secondary metabolites are organic molecules that are not involved in the normal growth and development. Quantitative analysis of secondary metabolites in tender, medium and coarse leaves of RC-1, RC-2, G-2 and G-4 mulberry cultivars were presented in Table III. Shows that total phenols and flavonoids contents were found significantly high (4.96mg/g, 5.68mg/g) in medium leaves of G-4 cultivar and low (2.46mg/g, 4.42mg/g) in tender leaves of RC-1 mulberry cultivar respectively.

Table III: Analysis of total phenols and flavonoids content in tender, medium and coarse leaves of RC-1, RC-2, G-2 and G-4 mulberry cultivars

Mulberry cultivars	Total phenols (mg/g)			Total flavonoids (mg/g)		
	Tender	Medium	Coarse	Tender	Medium	Coarse
RC-1	2.46 ±0.21	2.78 ±0.26	2.68 ±0.15	4.42 ±0.38	4.35 ±0.25	4.32 ±0.29
RC-2	3.22 ±0.35	3.56 ±0.42	3.35 ±0.41	4.55 ±0.34	4.72 ±0.34	4.68 ±0.27
G-2	3.98 ±0.52	4.34 ±0.29	4.12 ±0.34	4.59 ±0.29	4.98 ±0.37	4.84 ±0.32
G-4	4.27 ±0.23	4.96 ±0.22	4.58 ±0.25	5.42 ±0.31	5.68 ±0.33	5.43 ±0.28

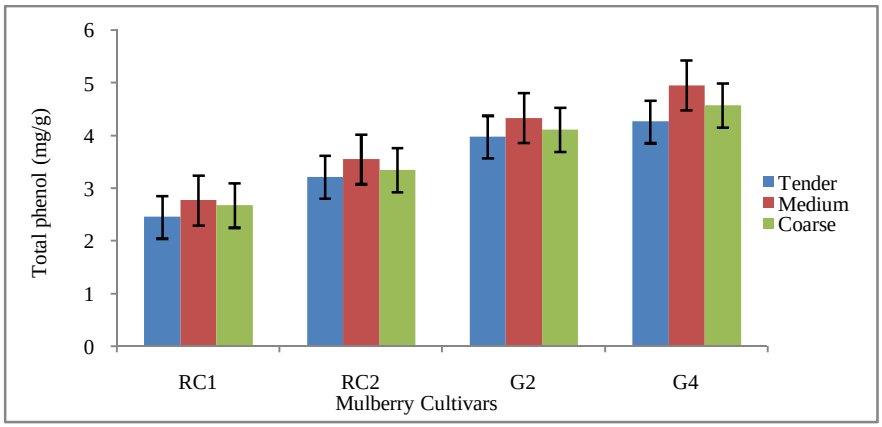

Fig: 5. Comparison of total phenol content in leaves of mulberry cultivars

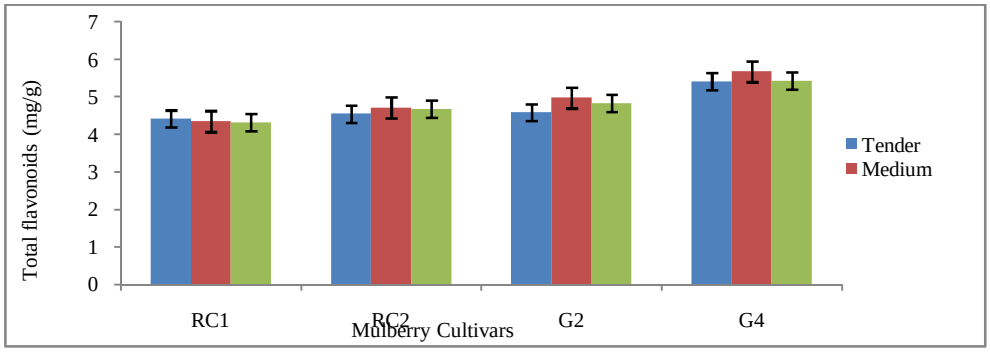

Fig: 6. Comparison of total flavonoids content in leaves of mulberry cultivars

IV. DISCUSSION

The current study provides the estimated amounts of the primary metabolites (total free amino acids) and secondary metabolites (phenols and flavonoids) present in the leaves of RC-1, RC-2, G-2 and GRC-1, RC-2, G-2 and -4 mulberry cultivars. This may provide knowledge on the nutritional quality and also its biological activities.

Mulberry varieties possessing high nitrogen and amino acids contents in leaves are nutritively superior and promote growth and development of silkworm (Machii and Katagiri, 1991; Suryanarayanan and Shivashankar Murthy, 2002). The silkworm, *Bombyx mori* requires 10 essential amino acids which can be synthesised by silkworm only when their precursors are present in its diet i.e., mulberry leaves (Bajpeyi *et al*., 1991). Higher amino acids and protein content are of particular importance to the silkworm larvae because of their active utilization for the synthesis of silk protein (Sujathamma and Dandin, 2000). Horie (1978) who studied the utilization of food stuff by silkworm reported that the optimum dietary protein (amino acid) level of 20-21 per cent is required for better growth of silkworm larvae. The actual silk fibre fibroin is derived from four kind's amino acids viz., Alanine, Serine, Glycine and Tyrosin, come from the food material of silkworm. The amino acid Alanine plays an important role in metabolism of glucose, tryptophan and organic acid. Riboflavin enhances the silk production and reduced the uric acid excretion and the choline and its derivatives sprayed on mulberry leaf and feeding to silkworm enhanced the fiber yield (Ito, 1978). Niacin and ascorbic acid is reported to enhance the larval survival rate (Ito and Niminura, 1966 a and b). El-Karaksy and Idris (2009) observed that ascorbic acid significantly increased the weight of B. mori larvae and pupae. Gomaa et al. (1977) also reported that ascorbic acid significantly increased the weight of B. mori larvae. Several authors suggested that the enhancement in larval weight was related to phagostimulation of ascorbic acid (Singh and Reddy, 1981). Plants produce many compounds as secondary metabolites that have no apparent metabolic, physiologic and structural role in the producer, but often have effects on other organisms (Samatha *et al*., 2014). Good quality plants (on the basis of rearing performance) showed higher total phenol content, which ranged from 1.98% in least preferred leaves to 6.26% in most preferred genotypes of P. bombycina (Hazarika *et al*., 1995). Total phenol content was found to be significantly more in tender leaves of P. bombycina (1.946%) compared to medium (1.182%) or mature leaves (0.712%). Phenols are said to offer resistance to diseases and pests in plants. Age dependent variations in the distribution of chemical defenses within and among plants have bearing on herbivore behaviour and fitness (Shelton, 2004). Higher content of phenols in tender leaves also

supports the optimal defence theory, where young and semi-mature leaves having high fitness and high probability of attack tend to have higher concentration of defence metabolites. Phenolic compounds are important in several respects during the development of Bombyx mori (Neog et al., 2011).

V. CONCLUSION

We concluded, after the quantitative determination of total free amino acids, vitamins and secondary metabolites in RC1, RC2, G2 and G4 mulberry cultivars, the most efficient mulberry variety estimated on the bases of their quantitative production is G-4 mulberry cultivar and recommended as best genotypic feed for the production of quality silk to the silk worm.

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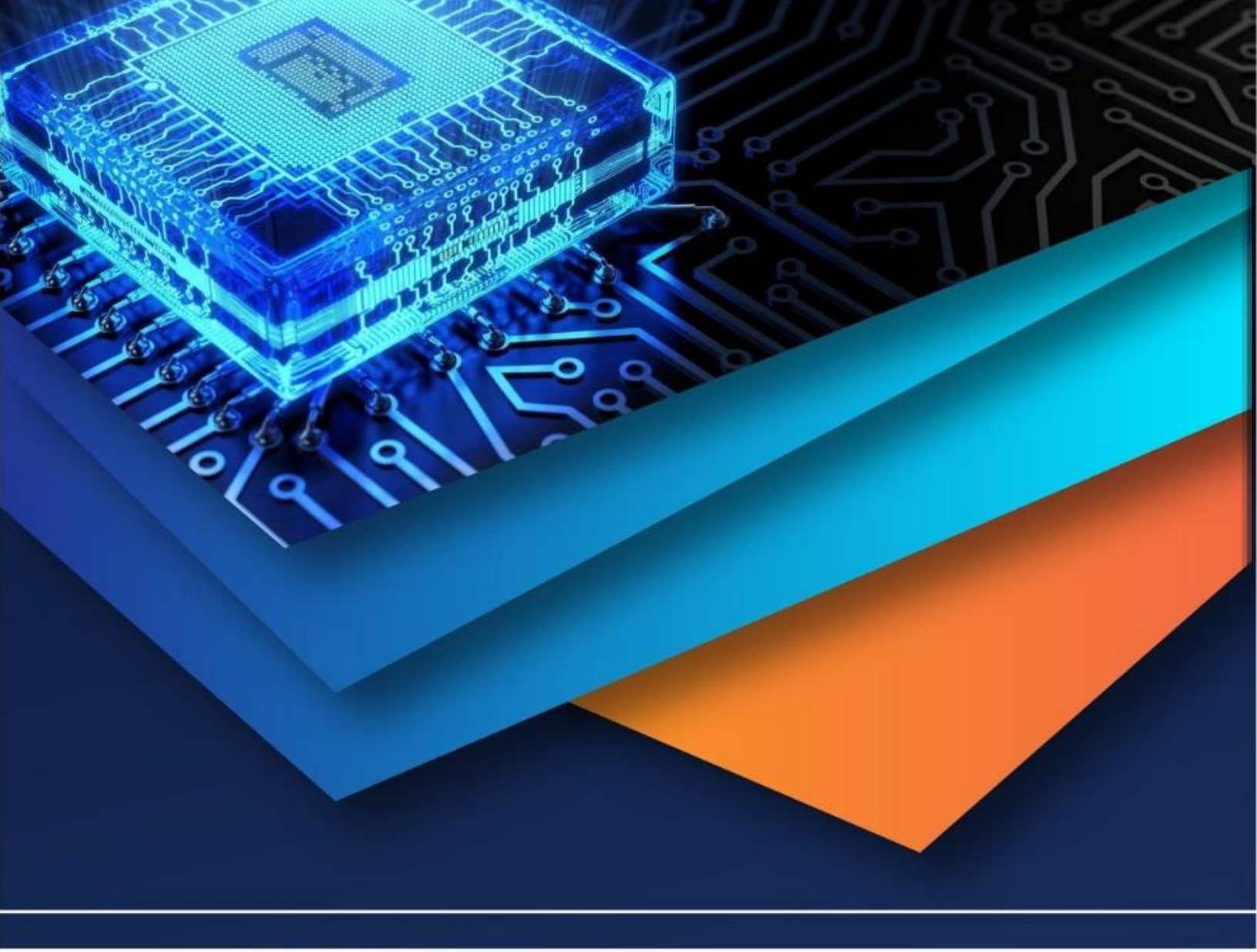

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