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Review Paper

VALUE ADDITION TO SILKWORM RAISING BY EXPLORING OFF-STREAM AVENUES

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ABSTRACT

Sericulture, like any other agro based industry, faces tremendous challenges from escalating labour cost, conversion of sericulture land for real estate and industrial purposes, demographic changes, shortage of trained manpower, and economic volatility of world markets. In order to sustain the growth of any industry, profitability needs to be maintained at a healthy level. This is equally applicable to sericulture as well. Therefore, it is necessary not only to explore means and ways to curtail the production cost but also to add value through diversification of products and byproducts. Apart from the regular products, there are ample avenues to utilize the byproducts of sericulture which if used prudently and judiciously can help increase the value of sericulture products to maintain a healthy level of profitability. Owing to the recent advances in biomedical sciences, silk has increasingly been used as biomaterial to make scaffolds, sponges, films, gels, nano particles and nano fibrils. These high value products can definitely bring additional benefits to the sericulture industry. This review attempts to highlight these advances in the usage of silk and other products from silkworms with the objective of creating a changed perception on the economic returns from silkworms, to make sericulture more vibrant and sustainable.

Key words: Biomaterial, fibroin, sericin, silk, silkworm.

INTRODUCTION

Five major species of insects viz., the mulberry silkworm (*Bombyx mori*; Bombycidae), the tropical tasar silkworm (*Antheraea mylitta*; Saturniidae), the oak tasar silkworm (*Antheraea pernyi*; Saturniidae), the muga silkworm (*Antheraea assamensis*; Saturniidae) and the eri silkworm (*Samia ricini*; Saturniidae) produce silk fibres of textile value in a commercially viable scale and India is the only country where all the above silkworms are being utilized (Srivastava *et al.*, 2011). Morphologically, these insects distinctly differ from one another in all stages of their life cycle and also in the silk they produce (Figure 1) (Vijayan *et al.*, 2009). Among these silk producing insects, the mulberry silkworm *Bombyx mori* alone contributes about 87 % of the total raw silk production in India. *B. mori* being totally domesticated, is reared under controlled

conditions with much care and attention. Among the other four silkworms, the eri silkworm, *S. ricini* is also domesticated, though it is still included under the category of vanya (wild) silkworms (Vijayan *et al.*, 2006). The other three categories of silkworms viz., tropical tasar, oak tasar and muga still have to survive in the wilderness fighting against all odds. Traditionally, silk is used mainly by the textile industry to make exquisite fabrics because of its unique lustre, sensuousness, glamour and several other mechanical properties which claim it the title, queen of textile fibres. However, recently it has been observed that silk has a tremendous potential to be utilized in many fields, biomedical application being the prominent one among them. Additionally, a number of other products are also developed from silkworms. All these developments can add new dimensions to the value chain of sericulture.



Figure 1: Morphological variability among different commercially exploited silkworms available in India. A - Mulberry silkworm (*Bombyx mori*), B-Tropical tasar silkworm (*Antheraea mylitta*), C- Eri silkworm (*Samia ricini*); D-Muga silkworm (*Antheraea assamensis*). 1-eggs, 2-mature larvae, 3-cocoons, 4-moths and 5-silk fibers.

In view of the above, this article reviews the latest developments in the usage of silkworms and silks in brief with the objective of enhancing the awareness on the economic returns from silkworms to make sericulture more outreaching, vibrant and sustainable.

SILK AND ITS STRUCTURE

Silk, the major commercial product of silkworm, is a proteinaceous ectodermal secretion, synthesized and stored as hydrated jelly substance within specialized cells or cavities, which polymerises into water insoluble filament during the passage to the external environment (Sehna and Suderland, 2008). In the silkworm *Bombyx mori*, silk is produced in a pair of long, tubular organs called the silk glands which are divided into anatomically and functionally distinct regions such as anterior, posterior and middle silk glands (Figure 2).

The posterior region, 15 cm long with 500 secretory cells, synthesizes the fibroin part of the silk. The middle region, where the silk proteins are stored, is of 7 cm length and has about 300 secretory cells that produce the sericin protein. The anterior region is a 2 cm tube like structure composed of about 250 cells with no known secretory function (Mondal *et al.*, 2007).

Fibroin: Silk comprises of two major fibers, inner fibroin (70-80 %) and sericin (20-30 %) and minute amount of other materials such as waxy matter (0.4-0.8 %), carbohydrates (1.2-1.5 %), inorganic matters (0.7 %) and pigments (0.2 %). Fibroin is a polymer of protein synthesized by the posterior silk gland (Kimura *et al.*, 1985). The fibroin is made up of a heavy chain (~390 kDa) and a light chain (~26 kDa) present in a 1:1 ratio and linked by a single disulfide linkage between the Cys-c20 of the heavy chain and Cys-172 of the light chain and a noncovalent bond by the P25, a 25 kDa glycoprotein (Tanaka *et al.*, 1999; Zhou *et al.*, 2000). Fibroin is mainly

made up of a recurrent amino acid sequence (Gly-Ser-Gly-Ala-Gly-Ala)_n, thus, has 43 % Glycine, 30 % Alanine, 12 % Serine and smaller amount of other amino acids (Kaplan *et al.*, 1998). Structurally, it has four components *i.e.*, (i) elastic β -spirals, (ii) crystalline β -sheets rich in alanine, (iii) tight amino acid repeats forming α -helices and (iv) spacer regions (Sirichaisit *et al.*, 2003). The elastic properties and other mechanical features of silk are due to the presence of α -helix and β -turns as these elastic domains alternate the hard β -sheets. The strong molecular cohesion occurring with amide-amide interactions in the β -sheet crystalline region containing hexapeptide repeat GAGAGS provides stiffness to silk fibers (Sponner *et al.*, 2007).

Sericin: It is produced and stored in the middle silk gland (Sutherland *et al.*, 2010). It is a nonfilamentous protein present as a protective coat over

the fibroin filaments and is generally removed during the processing of silk fibre by boiling the silk in an alkaline solution to improve the sheen colour, texture, and other properties (Freddi *et al.*, 2003). Recently, "Enzymatic degumming" using proteolytic enzymes has also been put into use as it is found giving a better dye uptake (More *et al.*, 2013; Suman *et al.*, 2013). Although sericin is insoluble in cold water, it can be removed from fibroin through alkaline hot water treatment (Gulrajani, 1988). In fact, based on the solubility, sericin is fractionated into three, the outermost hot water soluble layer, sericin-A, middle layer sericin-B and the innermost hot water insoluble layer, sericin-C. These three layers contain 17.2, 16.8 and 16.6 percentage nitrogen, respectively. Biochemically, sericin is composed of nearly 18 types of amino acids, of which serine, aspartic acid, glycine and threonine occupy a major share (Yamada, 1978; Mondal *et al.*, 2007; Patel and Modasiya, 2011). The amino acid composition of sericin is also found species specific as sericin isolated from wild silkworms contained more amino acids with non-polar side chains

than the same from domesticated species (Mondal *et al.*, 2007). Structurally, sericin is divided into two forms, α -sericin present in the outer layer of cocoon and β -sericin present in the inner layer of cocoon. The α -sericin contains lesser C and H, more N and O, and dissolves better in hot water than the β -sericin (Bose *et al.*, 1989).

USES OF SILK

Textile industry: It is believed that silk was discovered in China in 2852 BC by Shi Lin Chi, wife of the first Chinese Emperor Huang-Di, who also invented the first handloom. Silk was then reserved exclusively for the royal families; China took utmost care to guard the secret of the process of silk reeling and weaving until AD 300 (Reddy, 2009). Thus, it took a long time for silk to become a commercial product of the present stature. During the year 2011-2012, world raw silk production was 1,31,479 MT and China surged ahead of all the countries by producing 1,04,000 MT (79.10 %) silk, while India stood second with 23,060 MT (17.54 %) (Anonymous, 2011). Out of this, 18,272 MT was from the mulberry silkworm, 3072 MT from eri, 1590 MT from tasar and 126 MT from muga silkworm. Traditionally, silk has been used for making the most luxurious textile fabrics as silk fibres have outstanding natural properties which the most advanced synthetic polymers lack (Chen *et al.*, 2003). The mechanical properties of any woven fabric are features that provide basic texture, hand feel and dimensions to fabric. The mechanical properties are assessed to determine appearance, performance and serviceability of the fabric. The yarn count, cloth count, fabric mass, fabric thickness, dimensional stability, crease recovery, stiffness *etc.* are some of the mechanical properties. Similarly, tensile strength, tear strength, abrasion, drapability, piling *etc.* are some of the functional properties that decide the durability and serviceability of the fabric. Silk fabrics are superior in all these properties. Further, silk contains natural cellular albumen, which helps speed up metabolism of skin cells helping to reduce signs of aging. Silk is a natural heat regulator which helps in maintaining body temperature as silk does not conduct heat or static electricity like other fibres. Therefore, heat is retained during cold



Figure 2: Silk glands of the silkworm, *Bombyx mori*

temperatures and the redundant heat is shed during summer, keeping the skin cool in the summer and warm in the winter. Thus, silk fabrics are equally suitable in all climates.

Biomaterial/Biomedical applications of silk:

Biomaterials are materials which can be interfaced with biological systems to evaluate, treat, augment or replace

any tissue, organ or function of the body (Williams *et al.*, 1999). Out of the many biomaterials, polymers, ceramics, metals, and biocomposites are the most commonly used ones (Table 1). Although silk is yet to receive the deserving attention, there is a growing awareness among the biomedical fraternity about its biomaterial qualities (Table 2). In fact, silk is the

Table 1: Commonly used biomaterials

Sl. No.	Material	Use
1	Stainless steel	Bone fracture fixation, dental implants, joint replacements.
2	Titanium	Bone fracture fixation, dental implants, joint replacements, electrodes, stents, pacemaker encapsulation.
3	Cobalt chrome alloys	Bone fracture fixation, dental implants, joint replacements, dental implants, heart valves.
4	Gold	Dental fillings, crowns and bridges.
5	Silver	Antibacterial agents, pace maker wires, sutures, dental amalgams.
6	Platinum	Electrodes
7	Alumina	Joint replacements, dental implants.
8	Zirconia	Joint replacements
9	Calcium phosphate	Bone repair, surface coating of joint replacements, cell scaffolds.
10	Calcium sulphate	Bone graft substitutes
11	Procelain	Dental restoration
12	Carbon	Heart valves, dental implants, percutaneous devices, orthopaedic implants.
13	Glass	Bone graft substitutes, fillers for dental materials.
14	Nylon	Surgical sutures, gastrointestinal segments, tracheal tubes.
15	Polyethylene	Joint replacement, artificial tendons and ligaments, synthetic vascular grafts, dentures, facial implants.
16	Polypropylene	Sutures
17	Polyesters	Vascular prostheses, drug delivery systems, resorbable sutures, cell scaffolds, skin wound covering.
18	Polyamides	Sutures
19	Polymethylmethacrylate (PMMA)	Bone cement, dental restorations, intraocular lenses.
20	Poly vinyl chloride (PVC)	Tubing facial prostheses
21	Silicon rubber	Finger joining, artificial skin, breast implants, intraocular lenses, catheters.
22	Hydrogels	Ophthalmology, drug delivery systems.
23	Collagen and gelatine	Cosmetic surgery, wound dressings, tissue engineering, cell scaffold.
24	Cellulose	Drug delivery
25	Chitin	Wound dressing, cell scaffolds, drug delivery.
26	Ceramics	Bone graft substitute
27	Alginate	Drug delivery, cell encapsulation.
28	Hyaluronic Acid	Post-operative adhesion prevention, ophthalmic and orthopaedic lubricant.
29	Silk	Tissue engineering, drug delivery.
30	Dextran	Tightening ligaments
31	Heparin	Anti-coagulant for blood
32	Glycosaminoglycan	Tissue engineering, alone or in combination with collagen.

(Adopted from Coburn and Pandit, 2007)

Table 2: Some of the recent publications expounding the importance of silk biomaterials in biomedical applications

Sl. No.	Biomaterial	Utilization	Reference
1	Fibroin matrix	Corneal reconstruction	Bray <i>et al.</i> (2013)
2	Fibroin scaffolds	Nerve conduit scaffolds for growth factor delivery	Catrina <i>et al.</i> (2013)
3	Silk fibroin nanofiber	Testing the effect of Spirulina containing silk fibroin nano fiber for tissue engineering with high hemocompatibility	Cha <i>et al.</i> (2013)
4	Silk-gelatin scaffolds	Chondrocyte culture	Das <i>et al.</i> (2013)
5	Fibroin-Chitosan-silk scaffolds	Bone tissue engineering	Deng <i>et al.</i> (2013)
6	Polypropylene (PP) grafted muga (<i>Antheraea assama</i>) silk fibroin polymer	Suture biomaterial	Gogoi <i>et al.</i> (2013)
7	Transparent and flexible resistive memory devices with a very high ON/OFF ratio incorporating gold nano particles into the silk protein fibroin biopolymer	To make building blocks for the next generation of printable bio-electronic devices	Gogurla <i>et al.</i> (2013)
8	Silk fibroin scaffolds	Human mesenchymal stem cells (hMSC) culture for bone regeneration.	Hofman <i>et al.</i> (2013)
9	Silk fibroin membranes	Keratinocytes cultured	Levin <i>et al.</i> (2013)
10	Chitosan-silk fibroin (CHI/SF) composite porous 3D-scaffolds	Bone tissue engineering	Lima <i>et al.</i> (2013)
11	Silk fibroin scaffolds	Vascular cell growth	Liu <i>et al.</i> (2013a)
12	Bilayered vascular graft containing inner layer composed of silk fiber tube containing heparin and a highly porous silk fibroin external layer	Development of small diameter vascular grafts	Liu <i>et al.</i> (2013b)
13	Metallic implant titanium with silk protein sericin	Osteoblast cell culture	Nayak <i>et al.</i> (2013)
14	Eri silk fibroin matrix	Fibroblasts and osteoblast culture	Pal <i>et al.</i> (2013)
15	Silk based micro-needle	Drug delivery	Raja <i>et al.</i> (2013)
16	Silk hydrogels from non-mulberry and mulberry silkworm fibroins	Stem cell culture	Silva <i>et al.</i> (2013)
17	Silk scaffold	Tissue regeneration in a porcine model	Tu <i>et al.</i> (2013)
18	Sericin film	Resistance switching device	Wang <i>et al.</i> (2013 b)
19	Electrospun silk fibroin matrix	Urethra reconstruction in dogs	Xie <i>et al.</i> (2013)
20	Small-diameter silk vascular grafts	Coronary arteries	Aytemiz <i>et al.</i> (2012)
21	Silk fibroin-chitosan composite nano fibrils	Osteoblastic culture	Chen <i>et al.</i> (2012)
22	Nanocomposite gold-silk nano fibers	Human mesenchymal stem cell culture	Cohen-Karni <i>et al.</i> (2012)
23	Silk protein films	Human corneal-limbal epithelial cell culture	Lawrence <i>et al.</i> (2012)
24	Collagen-reinforced electrospun silk fibroin tubular	Biomimetic small-calibre blood vessels (SCBV) grafts	Marelli <i>et al.</i> (2012)
25	Silk protein biofilm	Wound healing	Padol <i>et al.</i> (2012)
26	Biopolymeric matrix fabricated by chemically cross-linking poly (vinyl alcohol) with silk sericin protein	Biocompatible and biopolymeric material for tissue-engineering	Mandal <i>et al.</i> (2011)
27	Silk-fibroin nano particles	Drug delivery	Lammel <i>et al.</i> (2010)

Cont'd

Sl. No.	Biomaterial	Utilization	Reference
28	Silk-Collagen sponge	Tendon regeneration	Shen <i>et al.</i> (2010)
29	Silk-tropoelastin films	Mesenchymal stem cell culture	Hu <i>et al.</i> (2010)
30	Silk fibroin 3-D matrix	Cartilage tissue regeneration	Wang <i>et al.</i> (2010)
31	Silk-Collagen-hyaluronan scaffolds	Anterior cruciate ligament cell and T-lymphocyte culture	Seo <i>et al.</i> (2009)
32	Silk-gelatin scaffolds	Ligament tissue engineering	Fan <i>et al.</i> (2008)
33	Mineralized silk protein composite polyamine scaffolds	Human bone marrow stem cell culture	Kim <i>et al.</i> (2008)
34	Silk fibroin nano fibrous tubular scaffolds	Bioengineering of small diameter vascular grafts	Soffer <i>et al.</i> (2008)
35	Fibroin scaffolds	Chondrogenic differentiation of human stem cells	Uebachs <i>et al.</i> (2008)
36	Silk fibroin scaffolds	Osteopromotive implants	Kirker-Head <i>et al.</i> (2007)
37	Silk-lactose scaffolds	Hepatocyte culture	Gotoh <i>et al.</i> (2004)

strongest and toughest natural biopolymer with numerous prospects for processing, functionalization, and biological integration (Lawrence *et al.*, 2008; Gogurla *et al.*, 2013).

In order to make the silk suitable for biomedical applications it needs to be processed and converted into different forms such as fibroin solution, films, sponges, porous scaffolds, foams, electrospun fibers, yarns *etc.* (Gupta *et al.*, 2007; Li *et al.*, 2001; Nazrov *et al.*, 2004; Bini *et al.*, 2006; Chen *et al.*, 2006; Horan *et al.*, 2006; Aytemiz *et al.*, 2012; Cha *et al.*, 2013; Levin *et al.*, 2013; Tu *et al.*, 2013). It may also be used alone or in combination with other materials, *e.g.*, with gelatine to form hydrogels (Gil *et al.*, 2007; Das *et al.*, 2013), with chitosan to develop scaffolds (Chen *et al.*, 2012; Deng *et al.*, 2013) with Spirulina to develop scaffolds with higher hemocompatibility (Cha *et al.*, 2013). These processes are often used for incorporating physical, chemical, and biological cues into the protein matrix to guide the growing cells to perform the desired functions such as cell migration, adhesion, and differentiation. Further, the biomaterial matrix needs to degrade at a rate commensurate with new tissue formation to allow cells to be deposited into new extracellular matrix (ECM) and regenerate functional tissue and also it should elicit a little host immune response. Silk fibroin is well known for its biocompatibility, the reason for it being in use since 19th century for sutures (Kearns *et*

al., 2008) and recently for developing matrices, films, scaffolds and sponges for growth, proliferation and differentiation of cells into tissues and organs (Minoura *et al.*, 1995; Chiarini *et al.*, 2003; Gotoh *et al.*, 2004; Min *et al.*, 2004; Unger *et al.*, 2004; Cha *et al.*, 2013; Gogi *et al.*, 2013; Levin *et al.*, 2013; Tu *et al.*, 2013). Further, it is observed that silk from the wild silkworm, *A. pernyi*, contains the Arg-Gly-Asp (RGD) sequence which has the property of supporting cell attachment and growth to a greater extent than *B. mori* silk (Minoura *et al.*, 1995). There are various techniques by which silk fibroin is adapted to suit to the objectives and applications. Tissue engineering is one such technique wherein knowledge of organic chemistry, cell biology, genetics, mechanics and transport processes are integrated to create artificial organs for transplantation, basic research, or drug development. It is an interdisciplinary field of biological sciences and engineering to develop tissues that restore, maintain, or enhance tissue function (Langer and Vacanti, 1993). It uses biomaterials and cells to produce new tissues and has several advantages over other therapies due to the ability of providing a permanent solution to the problem of organ failure. Over the years, three distinct approaches such as (i) direct implantation of freshly isolated or cultured cells; (ii) *in situ* tissue regeneration and (iii) implantation of tissues assembled *in vitro* from cells and scaffolds, have been evolved within discipline

of tissue engineering (Anderson and Van den Berg, 2004; Khademhosseini *et al.*, 2006; Shen *et al.*, 2010; Catrina *et al.*, 2013; Cha *et al.*, 2013; Hofmann *et al.*, 2013; Levin *et al.*, 2013; Liu *et al.*, 2013 a,b; Tu *et al.*, 2013).

Direct cell implantation entails isolation of individual cells or small cellular aggregates from the donor to grow and inject it directly into the damaged tissue. *In situ* tissue regeneration involves the use of bioactive and/or bioresorbable natural or synthetic scaffolds to exploit the body's natural ability to regenerate. Despite the early success of tissue engineering, workers in the field have faced challenges in repairing or replacing tissues that serve a predominantly mechanical function. Two potential obstacles to the creation of functional tissues to integrate with host tissue are inadequate mechanical properties (*e.g.* ability to withstand haemodynamic stresses) and adverse host-tissue reactions due to immunogenicity of the cellular components or the presence of residual non-degraded polymer scaffold. Recent research has shown that silk fibroin has such desired properties, hence, is suitable for the tissue engineering (Ma *et al.*, 2006). For instance, sulphonated and heparinised silk fibroin films have the desirable mechanical properties to be used as artificial blood vessels (Ma *et al.*, 2005, 2006). In addition to this, silk fibroin has good biocompatibility and biodegradability which are determined by their special molecular structures (Huang *et al.*, 2007; Cao and Wang 2009). Because of the biocompatibility, silk fibroin has been used for making collagen gel-silk filament composites for vascular tissue engineering (Couet *et al.*, 2007; Marelli *et al.*, 2012), patterned silk films for supporting cell proliferation, films and sponges for wound healing (Roh *et al.*, 2006; Lawrence *et al.*, 2009, 2012). It is also understood that the wound healing property of silk film is due to increased collagen synthesis *via* activation of fibroblast (Min *et al.*, 2004) and the ability of the silk film to keep the wound moist is by retaining water, proteins and electrolytes (Sugihara *et al.*, 2000). Silk films prepared from aqueous silk fibroin solution had oxygen and water vapour permeability, which depends to a great extent to the

content of silk I and silk II structures (Minoura *et al.*, 1990; Vepari and Kaplan, 2009). Nanoscale silk fibroin films can also be formed from aqueous solution using a layer-by-layer technique (Wang *et al.*, 2005).

Hydrogels, the three-dimensional polymer networks, are also made for encapsulation and delivery of biologically relevant agents (Vepari and Kaplan, 2009). Hydrogels are structurally similar to the extracellular matrix of many tissues and are mostly delivered in a minimally invasive manner. Consequently, hydrogels have been utilized as scaffold materials for drug and growth factor delivery, engineering tissue replacements, and a variety of other applications (Drury and Mooney, 2003). Three-dimensional silk hydrogels can be formed by dissolving degummed silk in a suitable solution and transforming the aqueous fibroin solutions into gels in the presence of acids, dehydrating agents and ions (Kim *et al.*, 2004; Mandal *et al.*, 2009). However, a number of factors may affect the gel formation. Important among them are concentration, temperature and pH of the solution, concentration of Ca^{++} *etc.* (Kim *et al.*, 2004). For instance, a 3 % solution can gel within two days at pH 3-4 whereas, it may take 8 days at pH 5-12 (Ayub *et al.*, 1993). The pore size also depends on silk fibroin concentration and temperature (Kim *et al.*, 2004). Thus, it is important to keep these facts in mind while making the hydrogel.

Electrospun scaffolds are also widely used in tissue engineering. Wang *et al.* (2013 a) developed a technique to develop scaffolds with macropores and high porosity. Scaffolds developed from silk fibroin were used recently for tissue regeneration in a porcine model (Tu *et al.*, 2013) and the study demonstrated that silk scaffolds support regeneration of innervated vascularised smooth muscle and urothelial tissue within three months. Polybutylene succinate (PBS) is used to develop weft knitted silk fibrous porous architectures to have superior control over scaffold design (Almeida *et al.*, 2013). Likewise, silk fibroin-chitosan composite scaffolds developed through freeze drying were seen to promote better cell growth (Lima *et al.*, 2013). To make silk fibroin scaffolds more growth promoting, Shi *et al.* (2013) developed a low crystallinity hydroxyapatite and

modified the scaffolds used for repairing bone/ligament defects. This scaffolds promoted the osteogenesis in a faster rate in animal trial as 60 % of the original bone volume and 80 % of the mechanical strength could be recovered after four months. In another effort, Levin *et al.* (2013) developed silk fibroin scaffolds for tympanic membrane keratinocyte growth to aid myringoplasty surgery. Silk hydrogel is much useful for sustained release of monoclonal antibodies (Guziewicz *et al.*, 2011).

Another very important application of silk protein is the preparation of nano particles for sustained and long-term release of drugs (Kundu *et al.*, 2010; Mathur and Gupta 2010; Numata and Kaplan, 2010). Nano particles, the minute particles, of fibroins can be prepared by dissolving fibroin in 9.3 M Lithium bromide solution at 60 °C for 5 h, dialyzed in double distilled water for 4 days to remove the Lithium bromide from the solution and subsequently mixing the aqueous solution containing dissolved fibroin with organic solvents such as acetone and isopropanol and centrifuging at appropriate rpm (Tudora *et al.*, 2003). Gholami *et al.* (2011) used electrospraying of dilute fibroin solution in formic acid to produce nano particles with average particle size as low as 80 nm. Recently, Zhao *et al.* (2012) developed a solution-enhanced dispersion by supercritical CO₂ (SEDS) method to develop nanoparticles having good spherical shape, smooth surface, and narrow particle size distribution with a mean particle diameter of about 50 nm. Silk nano particles are used for therapeutic purpose because they are stable, spherical, negatively charged, possess large surface area for displaying a large number of functional groups, the size and surface area of the nano particle can be tailored and controlled and they have rapid absorption and release capabilities (Kundu *et al.*, 2010; Nitta and Numata, 2013). Yan *et al.* (2009) observed that newly synthesized nano particles are capable of sustained release of loaded growth factors, conjugation with drugs and peptides of importance to deliver them into the targeted sites. Composite nano particles (<100 nm) of silk fibroin and chitosan were used for local and sustained therapeutic delivery of curcumin to cancer

cells (Gupta *et al.*, 2009). Likewise, insulin, and L-asparaginase conjugated into nano particles showed better storage stability and efficiency in dispensation (Yan *et al.*, 2009; Zhang *et al.*, 2011). A dual-drug release system has also been developed to control the release rate of the loaded bioactive molecules on silk nanoparticles (Numata *et al.*, 2012). Also, nano particles composed of DNA and recombinant silks containing cell-penetrating peptide, tumour-homing peptide, Arg-Gly-Asp (RGD) motifs and/or cationic sequences, have been designed for gene therapy (Numata *et al.*, 2012; Nitta and Numata, 2013). In general, it is noted that protein nano particles are degraded into harmless peptides by proteolytic enzymes (Elzoghby *et al.*, 2012), besides having several other qualities including electrostatic attractions, hydrophobic interactions and covalent bonding (Nitta and Numata, 2013). Attempts have also been made to develop gold-fibroin composite nanofibrils to enhance the rate of cell growth (Cohen-Kami *et al.*, 2013). All these studies demonstrated unequivocally the divergent applications of silk fibroin in biomedical fields evincing the future of silk in a new dimension.

Sericin is known to cause hypersensitivity and inflammatory responses when attached with fibroin (Altman *et al.*, 2003). Hence, it is removed from fibroin and once it is detached, it loses the above properties and can, thus, be used for several cosmetic and therapeutic purposes. Sericin has several useful properties including resistance to oxidation, UV rays and bacterial attacks to un-degummed silk (Panilaitis *et al.*, 2003). It also absorbs and releases moisture easily. Owing to these properties, sericin films are used for cell culture (Minoura *et al.*, 1995; Tsubouchi *et al.*, 2005), constructing nonvolatile memory applications (Wang *et al.*, 2013 b) *etc.* Nayak *et al.* (2013) used sericin from *A. mylitta* to immobilize on titanium surface to culture osteoblast cells, which resulted in enhanced cell adhesion, proliferation, and differentiation. Mandal *et al.* (2011) developed biopolymeric matrix by chemically cross-linking poly (vinyl alcohol) with silk sericin protein obtained from cocoons of the tropical tasar silkworm, *A. mylitta* with high cytocompatibility.

Sericin has been used to cap the silver nano particles (Bhat *et al.*, 2011), to make polyurethane forms with high moisture absorption / desorption ability (Minoura *et al.*, 1995), to prepare synthetic polymers with Acrylonitrile to separate water from organic solutions (Yamada *et al.*, 1993), to make antifrost film for refrigerators (Tanaka and Mizuno, 2001), and to coat surfaces of various durable materials to enhance functionality (Li, 1996). The antioxidant property of sericin was experimentally proved by suppressing the *in vitro* lipid peroxidations (Kato *et al.*, 1998). Since sericin has a strong resemblance with the natural moisturizing factor (NMF), it is being used as a valuable ingredient of cosmetics as it can inhibit tyrosinase activity responsible for the biosynthesis of skin melanin. The gel prepared from sericin solution with pluronic and carbopol as a stabilizer is used as a moisturizing, semi-occlusive and protective film on the skin surface to provide an immediate, long lasting, smooth, silky feeling (Padamwar *et al.*, 2005). The other uses of sericin includes, as a soil conditioner, coagulant for purification of waste waters, hygroscopic moisture-releasing polyurethane foams and their manufacture for furniture and interior materials, as additives for health foods to prevent colon cancers, medical composites of sericin, additives to rice cooking, fabric care compositions, light and sunscreen compositions, foam-forming aerosol shaving gels, sericin-coated powders for cosmetics, as dermatitis inhibitor, as wound protection film, nail cosmetics, and chewing gums (Gulrajani, 2005). The silk protein, sericin due to its saturation, revitalization and UV rays absorption properties has got enormous potentials to be used as skin moisturizer, anti-irritant, antiwrinkle and sun protector in addition to shaping the hair by making it soft and flexible (Kumaresan *et al.*, 2007). Sericin M, a 400 kDa protein, supports cell attachment of skin fibroblasts to collagen (Tsubouchi *et al.*, 2005). Sericin-S (5-100 kDa) has been used to increase proliferation of mammalian cells like T-lymphocytes and hybridomas (Terada *et al.*, 2005). Fibroblast cells cultured on sericin matrices showed non-elongated morphology, unlike the normal spindle shaped morphology observed on silk fibroin and collagen matrices (Minoura *et al.*, 1995). Biomaterials

like tricalcium phosphate have also been coated with sericin to improve biocompatibility (Takeuchi *et al.*, 2005).

OTHER BYPRODUCTS OF SILKWORM RAISING

The silkworms rejected for not spinning cocoons and the pupae of the reeled cocoons are excellent sources for value addition. Equally important are silkworm excreta, rejected silkworm and egg shell. Thus, other than silk fibres, silkworms, silkworm excreta, pupae, moth and egg shell can also be considered as sources of economically useful products. If properly used, it can contribute handsomely to the value chain of sericulture. Table 3 gives a fair idea about the source, products and their use (Nair *et al.*, 1999):

Some of the important such products / byproducts are briefed hereunder.

- a) Chitin, one of the components of pupal skin, is a linear homopolymer of β -1-4-linked N-acetylglucosamine and has unique structures and multidimensional properties (Pillai *et al.*, 2009). Extracts from different races of mulberry silkworm showed that multivoltine pure races contain more chitin (3.152 %) than the bivoltine (3.010 %). Likewise, chitin was more in male pupae (3.225 %) as compared to that in female pupae

Table 3: Economically important compounds which can be isolated from silkworm byproducts

Source	Product	Use
Silkworm excreta	Chlorophyll	Pharmaceutics, food products and cosmetics
	Pectin	Pharmaceutics and food products
	Carotene	Synthesis of vitamin A and medicines
	Phytol	Synthesis of vitamin E and K
	Triaccontanol	Plant growth regulator
Silkworm pupa	Lysine	Pharmaceutics-Fortification of proteins
	Protease	Food, beverages and leather industries
Pupa cuticle, silk-moth and egg shell	Chitin	Pharmaceutics, food and paper adhesive industry, as chilling agent in waste management and for treatment power plant



Figure 3: Value adding products from silkworms. A- whole pupae, B- Extracted and purified amino acids, C- Pupal oil.

(3.078 %) (Suresh *et al.*, 2012). Since chitin has several biomedical properties such as excellent biocompatibility and biodegradability, low immunogenicity, low toxicity and good anti-microbial activity, it is being considered as one of the potential biomaterials for the future (Jayakumar *et al.*, 2007; Pillai *et al.*, 2009). Chitin is now being used in post operational treatments such as conchotomy, deviatomy, polypectomy because of

its easy usability, less hemophase, greater pain relief and ability to fasten healing of wounds (Katti *et al.*, 1996), as an anti microbial and immune-adjuvant (Katti *et al.*, 1996), as a filling agent for defective tissues and also as a wound dressing agent (Okamoto *et al.*, 1993).

Table 4: Particulars of essential amino acids in pupae, cocoon pelade and silk fiber

Sl. No	Amino acid	Quantity of amino acids		
		Pupa (mg/g)	Pelade (g/100 g)	Silk fiber (g/100 g)
1	Glycine	14	37	14
2	Aspartic acid	21	03	17
3	Glutamic acid	05	03	04
4	Serine	05	13	34
5	Threonine	07	02	10
6	Alanine	94	30	06
7	Lysine	05	01	03
8	Arginine	19	01	03
9	Valine & Methionine	199	04	03
10	Leucine & Isoleucine	500	02	02
11	Tyrosine	nil	nil	03
12	Histidine & Tryptophan	nil	nil	01
13	Phenyl alanine	nil	nil	01
14	Proline & Cystine	nil	nil	01

(Adopted from Ramakanth and Raman, 1997; Kumaresan *et al.*, 2007; Roychoudhury and Joshi, 1995)

- b) Chitosan is another important product from silkworm skin, which is a GAG-like linear polysaccharide composed of glucosamine and N-acetyl glucosamine linked in a β (1-4) manner and is deacetylated from chitin (Martino *et al.*, 2005). Analyses showed that chitosan is higher (2.451 %) in male pupae than in female pupae (2.291 %) (Suresh *et al.*, 2012). Like chitin, chitosan also has a variety of properties such as antibacterial, anti-fungal, anti-viral, anti-acid, anti-ulcer, non-toxic, non-allergenic, totally biocompatible and biodegradable *etc.* (Pillai and Sharma, 2009; Pillai *et al.*, 2009). Since chitosan has good biocompatibility and high water absorbent capacity, film made of chitosan is used for treating burns (Muzerelli *et al.*, 1988) and experiments in rat and dog models showed that chitosan accelerates wound healing (Nagai *et al.*, 1984). Chitosan-silk fibroin composite nano fibres developed through electrospinning were found suitable for growth and osteogenic differentiation of human foetal osteoblastic [hFOB] cells for bone tissue engineering (Chen *et al.*, 2012). It is also found

- that chitosan can be used to remove arsenic from potable water (Saha and Sarkar, 2012).
- c) The whole pupae (Figure 3A) is an excellent source of food that lowers body fat, blood pressure and blood sugar and used to treat liver hepatitis, pancreatitis, leukocytopenia, neurological, ophthalmic, anti-histaminic and gastric ailments (Velayudhan *et al.*, 2008). The chemical composition showed that pupae contain crude protein (55~60 %), total lipids (26 %), free amino acids (5~8 %) and several essential amino acids. Extracted and purified amino acids are now available commercially (Figure 3B). Owing to this high protein contents, it is estimated that 100 g of dried silkworm pupae can provide 75 % of the daily protein requirement of a human body (Singh and Suryanarayana, 2003). Of the total fat content, 66.8 % contains unsaturated fatty acids and the linolenic acid accounts for 25.77 %. The pupa is also a good source of vitamins such as pyridoxal, riboflavin, thiamine, ascorbic acid, folic acid and minerals *viz.*, calcium, iron and phosphorus (Table 2) (Roychoudhury and Joshi, 1995; Singh and Suryanarayana, 2003; Koundinya and Thangavelu, 2005). The carotenoids present in the silkworm pupa contain antioxidants namely, lutein and hexoxanthin (Velayudhan *et al.*, 2008). Several commercial products use pupal protein as raw material for preparing amino acids and flavoured products with high nutritive value (Aruga, 1994). In India, eri pupae are sold in several markets of North-Eastern states (Singh and Suryanarayana, 2003). In Hong-Kong, China, Korea and Japan, the healthy silkworm larvae are sterilized, vacuum dried and sold as commercial food and the cocoon pallade powder is used in soups and sauce preparations (Ramakanth and Raman, 1997). The pupae is used as poultry or fish feed as well (Iyengar, 2002). Feeding of de-oiled pupae to hen improves its egg laying capacity and to carps and fish increases body weight (Aruga, 1994). Fresh water fish fed with silkworm pupae had increased survival and growth rate (Velayudhan *et al.*, 2008).
- d) Pupa oil (Figure 3C), the yellow transparent liquid oil is rich in α -linolenic acid (ALA). Silkworm pupae oil lowers blood sugar, inhibits thrombus and regulates blood fat and liver lipid storage. It is also useful in regulating inflammatory mediators and interleukins to protect liver. An experiment with pupa oil in rats showed that it can reduce serum lipids level and inhibit platelet aggregation (Yang *et al.*, 2002). It is used in cosmetics like hair oil, face powder, creams and body deodorants. The silkworm pupal fat and oil is useful in soap / cosmetology industries as it acts against aging, darkening of body and graying of hair (Velayudhan *et al.*, 2008).
- e) Silkworm excretes profusely during its swift growth and it is reported that approximately 5.7 MT of excreta is generated by rearing silkworm on mulberry leaf harvested from a hectare. Excreta is a tremendous source of very valuable products such as paste chlorophyll, pectin, phytol, carotene, triacontanol *etc.* (Singh and Jayasomu, 2002). Paste chlorophyll is composed mainly of chlorophyll-a and chlorophyll-b in the ratio of 3:1. Apart from paste chlorophyll which is used as raw material in chemical industry, the water soluble sodium copper chlorophyllin is also extracted from excreta. This compound is an antibacterial agent and is also used in manufacturing medicines for hepatitis, acute pancreatitis, chronic nephritis and leukocytopenia. Raghavendra *et al.* (2010) found that partially purified protein from the silkworm excreta with molecular mass of 35 kDa shows considerable hepatoprotection effect in the case of liver damage. In recent years, the possibility has been explored for chlorophyll and chlorophyll derivatives to be used as antimicrobial, antiviral and antitumor agent or as a part of photodynamic therapy. Atansova *et al.* (2013) reported a highly sensitive Near Infrared Spectroscopy technique to determine the chlorophyll and carotenoid in the silkworm excreta. The action of chlorophyllin, a chlorophyll derivative, isolated from silkworm excreta in concentrations of 25-400 $\mu\text{g/ml}$, on

tumor cells was studied by Chiu *et al.* (2003, 2005). They found that chlorophyllin reduced the proliferation of tumor cells by 8.2–95.7 % after 72 hours of incubation of samples on the spectrometer NIRQuest 512.

Pectin is another product which is abundantly available in silkworm excreta (Ichim *et al.*, 2008). It is used as food additive and also used in candy, jelly, jam and in concentrated juice as thickener apart from using in medicines to reduce blood triglyceride and cholesterol.

- f) Carotene, Phytol and triacontanol are the other major products which can be isolated from silkworm excreta (Singh and Jayasomu, 2002). Carotene is the precursor of vitamin A and is used in the manufacturing of medicines used to combat alimentary canal and respiratory tract disorders. Carotene inhibits carcinoma lung and stomach. Phytol is used to synthesize vitamin E and K which are prominent products in the field of medicine. Triacontanol is a product marketed widely as a plant growth promoter which is very effective on rice, wheat, vegetables and lately on mulberry as well.
- g) Production of biomolecules of interest by converting silkworms into bioreactors has great industrial potentials. Foreign genes coding medically important proteins, such as therapeutic proteins, monoclonal antibodies, and vaccines can be inserted into the genome of silkworms with the help of an attenuated recombinant baculovirus or a *piggyBac* transposon-derived vector or both (Tamura *et al.*, 2000; Yamamoto *et al.*, 2004). The transgenic silkworms, thus, produced are capable of synthesising proteins in a large quantity. For instance, transgenic silkworms producing human type III procollagen and feline interferon (Kurihara *et al.*, 2007), and human μ -opioid receptor expressed in the silk glands and fat bodies have already been developed (Tateno *et al.*, 2009). Likewise, human granulocyte-macrophage colony-stimulating factor was successfully expressed in silkworm pupae using *B. mori* nucleopoly-

hedrovirus. The target protein expressed had an apparent molecular mass of 29 kDa and an isoelectric point of 5.1. In another attempt, Kurihara *et al.* (2007) constructed a fibroin H-chain expression system to produce Feline interferon (FeIFN) recombinant proteins in the cocoon of transgenic silkworms using *piggyBac* transposon-derived vector. The transgenic silkworm produced the normal sized cocoons containing each FeIFN/H-chain fusion protein. These studies clearly indicate that silkworm can be used as a convenient and low-cost bioreactor for the production of heterologous proteins (Chen *et al.*, 2006). The expression levels of recombinant proteins in silkworm are generally higher than those in cultured cells (Kato *et al.*, 2010). It has also been shown that using transgenic technique, it is possible to combine the exceptionally high strength of spider dragline silk proteins with the lustrous *Bombyx* silk (Xia *et al.*, 2010). Teule *et al.* (2012) developed a transgenic silkworm using *piggyBac* vectors to produce chimeric silk containing both silkworm/spider silk proteins integrated in a stable manner. This composite silk fiber was as tough as native dragline spider silk fiber.

CONCLUSION

Silk industry till now thrived well in India due to the availability of labour at affordable rates. However, the changed scenario in the economic sector coupled with demographic changes necessitated adoption of labour saving measures and introduction of value adding steps to sustain a healthy growth rate in the sericulture sector. One has to look at silkworm as a wholesome product provider. Merely depending on silk fibers neglecting other products and by-products may cost dearly in future. Similarly, restricting the usage of silk to textile sector alone may fetch less remuneration to the farmers, thereby putting sericulture in the peril of losing to other crops in competition. Thus, for better sustainability, it is necessary to expand the usage of silk into other high value adding areas. Utilizing silk as biomaterials in biomedical fields is one such highly promising and potential area which should be embraced

with both hands. From the above discussions, it is quite obvious that although silkworms give an array of products with varied use to human beings, appropriate strategies are to be adopted to make use of them not only to meet the basic needs of mankind such as clothing, food, health care and employment but also to make sericulture as a versatile and vibrant industry.

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VALEUR AJOUTEE POUR L'ELEVAGE DU VER A SOIE EN EXPLORANT DES VOIES HORS DES SENTIERS BATTUS

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RESUME

La sériciculture, comme toutes les agro-industries, fait face à des défis importants comme l'augmentation du coût de la main-d'œuvre, la conversion des terres pour des raisons industrielles, les changements démographiques, le manque de main-d'œuvre qualifiée et la volatilité des marchés mondiaux. Afin de soutenir la croissance de toute industrie, il faut maintenir la rentabilité à un niveau sain. Ceci est également applicable à la sériciculture. Aussi, est-il non seulement nécessaire d'explorer des moyens et des voies pour réduire le coût de production mais aussi d'ajouter de la valeur par la diversification des produits et des sous-produits. En dehors des produits habituels, il existe des boulevards pour utiliser les sous-produits de la sériciculture qui, s'ils sont empruntés judicieusement et prudemment peuvent aider à accroître la valeur des produits de la sériciculture pour maintenir un niveau sain de profits. Grâce aux récents progrès des sciences biomédicales, la soie a été de plus en plus utilisée comme biomatériau pour fabriquer des implants, des éponges, des films, des gels, des nano-particules et des nano-fibres. Ces produits à haute valeur peuvent entraîner des bénéfices supplémentaires pour l'industrie séricicole. Cette revue tente de mettre en lumière ces progrès dans l'usage de la soie et des autres produits tirés des vers à soie avec pour objectif de changer la perception sur les retours économiques des vers à soie, ceci afin que la sériciculture soit plus attractive et durable.

Mots-clés: Biomatériau, fibroïne, sérécine, soie, ver à soie.



EFFICIENCY OF ENRICHMENT OF FERTILITY AND REGULATION OF SOIL ACIDITY ON GROWTH AND YIELD OF MULBERRY

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ABSTRACT

Rwanda, located in Central Africa between 1°04' and 2°51' latitude south and between 28°45' and 31°15' longitude east, enjoys a tropical temperate climate due to its high altitude (900 ~ 4507 m ASL). It receives an annual rainfall of around 700-1000 mm / year. Almost all soils of Rwanda are reported to be acidic (pH 4.8-5.8), which negatively affects soil fertility and results in 50 % reduction in productivity of all basic grains and root crops. Of late, development of sericulture as a new branch of agriculture has started receiving great attention in Rwanda, as the state has big hopes to increase its export potential through it. Since mulberry plantation being the major economic component in sericulture, the quality of soil indirectly has a profound influence on silk production. Soils with the slightest tinge of acidity (pH 6.8) are ideal for good growth of mulberry plants. Both the lateritic and sandy types of soil observed in Rwanda are characterized by low concentration of K, Mg and other basic vital elements, low water holding capacity and low pH. Hence, administration of suitable soil reclamation measures is an essential step towards raising superior quality mulberry leaf. Usually dolomite limestone or wood ashes are recommended for regulation of soil acidity. Chemical analysis of mulberry wood ash (MWA) has shown that the composition of basic elements, necessary for a plant, except for Ca, Mg and Zn surpass that in lime since the young branches are rich in macro and micro elements. Average calcium carbonate equivalent (CCE) in mulberry wood ash is 43.0 %. Use of mulberry wood ash as fertilizer in combination with other mineral and organic fertilizers improves the soil fertility, regulates acidity and enriches chemical components of soil, incidentally decreasing the incidence of diseases in a mulberry plantation and ultimately improving productivity and quality of leaves.

Key words: Leaf yield, mulberry, mulberry wood ash, soil chemical composition, soil fertility, soil pH.

INTRODUCTION

Soil quality is defined as its ability to perform a specific function within a managed or natural ecosystem that is essential to people and environment. It is known, that almost one fourth of soils on the planet is in a condition of degradation, the main reason of which is soil acidification. Soil acidification is a natural process, which usually takes hundreds, or even thousands of years to occur; however, atmospheric precipitates and incorrect management of the earth resources by humans accelerate this process enormously. For example, Rwanda, located in Central Africa between 1°04' and

2°51' latitude south and between 28°45' and 31°15' longitude east is characterised with almost all stretches of its soils being acidic (pH 4.8-5.8). This condition adversely affects the productivity of all basic grains and root crops.

Recently, sericulture has been progressing as a new branch under the ambit of Agriculture in Rwanda. As in the case of any plantation, the quality of soil of mulberry field has a profound influence not only on the leaf yield, but also on its quality, that ultimately affects growth of silkworms and thereby quantity and quality of cocoons produced. The soil of mulberry

plantation must be capable of maintaining the mulberry plants for prolonged maximum productivity of quality leaves. Though mulberry is tolerant to a wide range of soil conditions, it grows well on highly fertile loamy soil. In general, the soil recommended for mulberry should be deep, well-drained, and clayey loam to loam in texture, friable, porous, and fertile with good water retention capacity. Soils with pH around 6.8 are free from injurious salts and ideal for good growth of mulberry plants. Saline and alkaline soils and also highly acidic soils should be avoided and if not possible, should be suitably reclaimed. The present condition of soils in Rwanda makes it a requirement that suitable reclamation practices are undertaken so that mulberry can be safely grown. The aim of this work is to study the growth efficiency of vegetative organs of mulberry in response to various reclamation measures and fertilizer application under field experimental conditions.

MATERIALS AND METHODS

Study area and treatments

The experimental site is located in the mulberry plantation of Rubona Research Station, in mid-altitude agro-ecological zone in Southern Rwanda, 125 km from Kigali, the capital city. It's a hilly and mountainous station 1700 m above the sea level. It has a subequatorial climate with annual average temperatures around 18-21 °C and rainfall between 1300 -1800 mm/year.

There are basically two types of soil in this region: lateritic and sandy soil characterized with low concentrations of K, Mg and other basic vital elements required for plants, low water holding capacity and low pH.

The experiment was carried out on six rafts in three replications with 4-year-old Diamond (H) mulberry variety under an area of 1200 m². The fertilizer treatments imposed were organic manure (30,000 kg/ha), mulberry wood ash (1125 kg/ha), lime (750 kg/ha) and N₃₀P₂₄K₁₈ (90 kg/ha) in different combinations. Control plantation did not receive any supplementary treatment.

Soil analysis

For interpretation of physiological state of trees in the experiment, the chemical composition of the growth substrate was analyzed in the sample plots after application of different combinations of fertilizers to soil. Soil samples were collected with a steel bore cylinder from depths of 30 cm, taking into account that approximately 80 % of feeder roots of mulberry trees are located in the layer of 10-40 cm depth (Homidy, 2012). Soil sampling was carried out once in every 12 weeks from each 10 m length of plots in four replications per treatment from May 2011 till June 2012. The soils were sieved through a 5 mm screen to remove root fragments and coarse gravel. The nutrient status of the soil upper horizon (30 cm) was determined in the Laboratory of Soil Chemistry of the Uzbek Agriculture Research Institute by adopting standard methods of soil analysis. Concentrations of P and K were determined by the Egner-Riehm double lactate method and that of Ca by the Egner-Riehm-Domingo ammonium acetate-lactate method (ISO/11260. 1995). Total N was determined by the Kjeldahl method (ISO/11261. 1995); Cu, B, Mn, Zn, and Fe were measured using a Shimadzu atomic absorption/flame emission spectrometer (AA-670), and the pH of the soil was measured as the potential acidity in H₂O (1:1 soil: water ratio) (ISO/10390. 1994).

Chemical analysis of mulberry wood ash (MWA)

The characteristics and chemical composition of wood ash may vary depending on the type of raw bio material, incineration technique, additives, and storage conditions (Kofman, 1987). For our experiments, mulberry branches were used as raw material for producing wood ash. Dry wood ash was collected and mixed carefully to get homogeneous material. The same ash was used in all variants and replications. The chemical composition of wood ash was analyzed in the Laboratory of Biochemistry and Artificial Feed of the Uzbek Sericulture Research Institute.

Chemical analysis of mulberry leaves

Plant samples were taken treatmentwise, 4 times during the vegetation as follows:

30.06.2010 (rainy season, after bottom pruning of mulberry plantation), 31.10.2010 (dry season, after top pruning of mulberry plantation), 31.01.2011 (rainy and dry season, after second top pruning of mulberry plantation) and 30.04.2011 (rainy season, after second bottom pruning of mulberry plantation).

Leaves were cut into small pieces and oven-dried at 70 °C for 24 h to stop metabolic activity (Wilde *et al.*, 1979; Landis, 1985). 1.2 g of dried leaves were ground and chemically analyzed in the Laboratory of Biochemistry and Artificial Feed of the Uzbek Sericulture Research Institute. The quantities of mineral elements viz., N, P, K, Ca, and Mg were determined. Concentrations of metallic elements were determined using an atom-adsorption analyzer AAA-1N (Karl Zeiss, Jena). For measuring N, the method of Kjeldahl was used, and P was extracted with vanadium molybdate yellow complex.

Statistical analysis

The statistical processing of the experimental data from each independent experiment with four replicates was done according to standard methods, using Microsoft® Excel 2003 program of Microsoft® Windows'2003. Average values were used for graphic presentation of results, with the significance of differences ($P < 0.05$) calculated by the *t* test (Dospehov, 1985).

RESULTS AND DISCUSSION

Analysis of soil

Various macro and micro elements are necessary for normal growth and development of plants. As known, there are around 20 of such elements, without which plants cannot complete a cycle of development and which cannot be replaced by others. As productivity of a field depends on fertility of soil and more on effective use of fertilizers, it is of great importance to define the type of soil in the field. Availability of nutrients in soil strongly depends on pH. Because of low or too high level of pH, nutrients in soil can be inaccessible for plants. The preliminary analysis of fertility of soil in the mulberry field has shown a low status of all basic vital nutrient elements and no conformity with the optimum level for mulberry (Figure 1).

It was noticed that the amount of nutrient elements in soil (except Ca, Mg and Cu) rose a little during the rainy season, in parallel with the decreased soil acidity. Such change directly depends on intensity of rains and moisture content in the soil. Moisture held in the soil promotes vital processes such as build up of microorganisms and in turn accelerates the splitting process of the biological material. Moreover, heavy rains incidentally lead to soil erosion which decreases acidity of soil.

Restoration of the fertility of soil

Maintenance of soil's fertility and productivity of crops in Rwanda depends to a great extent on prevention

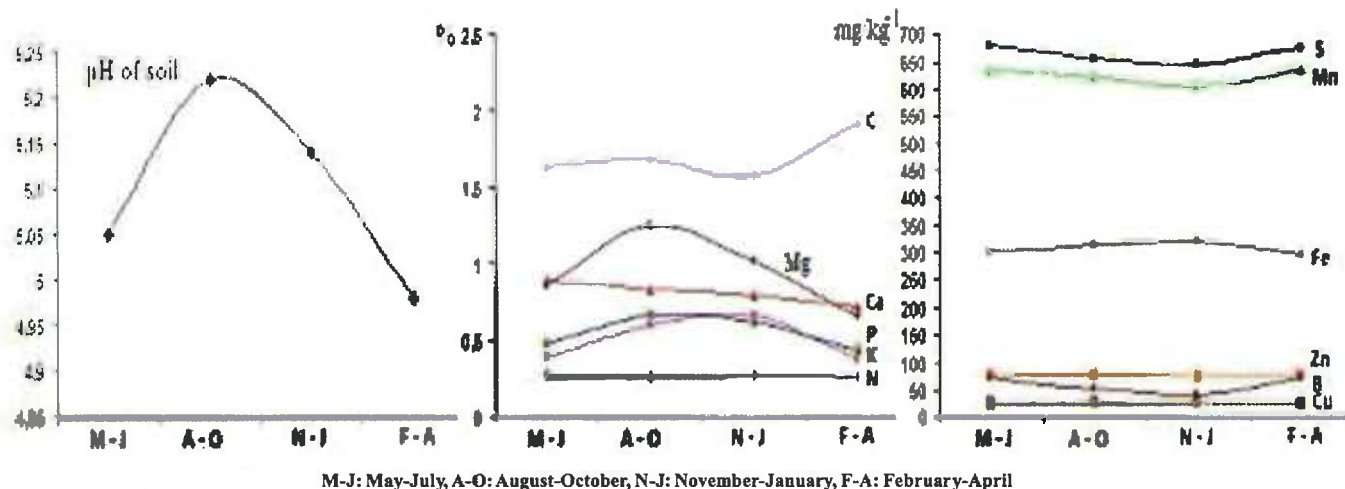


Figure 1: Acidity and chemical composition of soil

of soil erosion and chemicalisation. However, use of fertilizers, being the leading factor in intensification of agriculture, has not reached the required level yet, which can provide steady agriculture crop yield. For restoration of degraded soils' productivity and to receive a minimum yield of crops, *Roose et al.* (1988) recommended massive applications of organic manure (10 t/ha every 2 years), lime (2–4 t/ha every 3 years), and NPK fertilizer (50–100 kg/ha/yr of N, 40–100 kg/ha/yr of P, and 30–200 kg/ha/yr of K). Due to financial constraints, farmers use only one third of the recommended manure and there are practically no actions being taken for correction of soil's acidity. Insufficient doses of fertilizers, unfavourable climatic conditions and unsatisfactory farming standards underline the need to formulate the cheapest and accessible means for promoting restoration of the productivity of degraded soils.

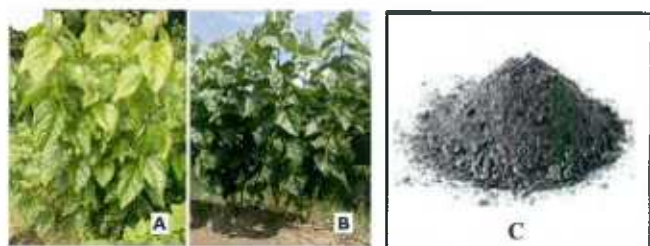


Figure 2 : Mulberry plant grown in (A) acidic and (B) fertile soil; (C) mulberry wood ash

Mulberry plantation is a source of biomass production too. It is possible to receive more than 20 tons of firewood and more than 40 tons of mulberry leaves from one hectare annually, which creates great potential for production of organic fertilizer. The left over mulberry from silkworm rearing are being collected and converted into compost, which is then used as organic fertilizer. It is known that application of organic fertilizers leads to soil oxidation. Typically, deviations in the acidity of soil from slightly acidic or neutral status leads to an imbalance of nutrients available to plants and oppression of beneficial soil microorganisms. Majority of cultivated plants, including mulberry, and useful soil microorganisms grow well at low soil acidity (pH 6.5-7.0) It is recommended to add dolomite limestone into soil for neutralization of its hyper acidity. Practice shows, that application of limestone is very expensive for farmers (around 8000 USD/ha every 3 years). Besides that, the

mineral limestone does not contain enough necessary vital elements for a plant. In this context, the wood ash which has been widely used as a fertilizer since ancient times, especially in those countries, where wood was in abundance, deserves a mention as an alternative to limestone. In our case, after each silkworm rearing, mulberry trees are being pruned, through which enough timber is being collected for production of wood ash (Figure 2).

Chemical analysis of mulberry wood ash

Before using mulberry wood ash as a fertilizer, chemical analysis of samples from different parts of the mulberry tree have been carried out and compared to limestone (Table 1)

As can be seen from the table, quantities of basic necessary elements for a plant, except for Ca, Mg, Na and Zn, in mulberry wood ash surpass those in lime. Chemical analysis of different tissues of mulberry showed the highest content of micro and macro elements in its young organs: shoots and leaves, which corresponds to the earlier literature (*Hakkila, 1989; Wong et al., 2004; Werkelin et al., 2005*). This is primarily due to the ongoing process of metabolism in growing organs of the plant. The increased content of elements in the leaf can be explained by the necessity for the photosynthesis process and accumulation of nutrients. Average calcium carbonate equivalent (CCE) in mulberry wood ash is 43.0 %. As the particle size of mulberry wood ash is much smaller, its acidity reducing effect considerably surpasses that of limestone. Mulberry wood ash contains a few elements that may pose environmental problems. But the heavy metal concentrations are typically low and not in a highly extractable or available form. Hence, taking into account the rich content of macro and micro elements in mulberry wood ash, it can be used as an effective valuable complex fertilizer for the restoration and maintenance of soil fertility.

Use of mulberry wood ash for regulating acidity and enriching fertility of soil

Wood ash has long been recognized as a valuable substance. Many centuries ago, ancient Roman scientists and scholars documented the value of returning ash to

Table 1: Chemical composition of the mulberry wood ash and lime

Element	Variant				Lime
	Mulberry wood ash				
	Stems	Branches	Leaves	Average	
Concentration (%)					
Nitrogen	0.13	0.20	0.29	0.21	0.01
Potassium	0.17	0.26	1.21	0.55	0.13
Sodium	0.042	0.029	0.015	0.03	0.07
Calcium	17.32	13.21	11.43	13.98	31.1
Phosphorus	0.089	0.24	0.712	0.35	0.06
Magnesium	0.047	0.073	1.57	0.56	5.1
Carbon	1.02	1.67	2.08	1.59	-
Concentration (mg/kg)					
Iron	40.2	150.2	302.3	164.40	0.29
Sulfur	200.0	355.1	780.9	445.33	-
Manganese	120.0	559.2	789.5	489.57	0.05
Zinc	47.0	56.5	75.5	59.67	113
Copper	20.02	23.09	27.66	23.59	6.87
Aluminum	60.68	40.10	20.3	40.36	0.29
Boron	25.00	27.00	31.50	27.83	-
Pb (Heavy metal)	12.05	8.01	6.21	8.75	-
Other chemical properties					
CaCO ₃ Equivalent (%)	47.00	43.00	39.00	43.00	100
pH	10.80	10.40	9.80	9.80~10.80	9.9
Total solids (%)	78.00	75.00	66.00	73.00	100

the land. In the 18th century, the benefits of ash-derived potash, or potassium carbonate, became widely recognized. There was a time when trees were felled in North America, burned and the ash was exported to Great Britain as "potash fever" hit. In 1790, the newly-independent United States of America's first patented process was a method for making fertilizer from wood ash (U.S. patent number 1: "An improved method of making pot and pearl ash"). USA, Finland, Sweden and Denmark were the pioneers to undertake research on the composition and use of wood ash. In the USA, wood ash is derived from paper industry waste and power generation of which, 90 per cent share of many states goes to landfill. However, in the north-east states, only 15 per cent is land filled, as the remaining 80 per cent is land applied and 5 per cent co-composted with sewage sludge. This practice has reduced the costs of disposal for the producing companies by up to 66 per cent in Maine and New Hampshire (Greene, 1988; Campbell, 1990; Vance, 1996).

In Finland, wood ash has been used as a soil ameliorant for second-rotation conifer stands on drained peats, on sites monitored since 1935 (Hakkila, 1989; Korpilahti *et al.*, 1998). Research on the restoration of cut-over peat using ash as an ameliorant to change pH and restore biodiversity is underway (Näsi *et al.*, 2005). In Sweden, ash is already produced in large quantities from energy generation and studies on recycling ash to forest sites on peats and podzols were undertaken in the 1970s itself (Högbom and Nohrstedt, 2001). Recently, efforts to restore acidified soils in the south of the country has explored the use of both wood ash and lime (Lundström *et al.*, 2003).

In Denmark, ash is produced from community energy projects using mixed organic fuels such as straw, woodchip, green waste and tree thinning. This has resulted in a mixed quality ash of variable chemical content with some high levels of heavy metals and dioxins. As a consequence, 2500 tons of ash per year is disposed to landfill (Serup, 1999; Moller and Ingerslev, 2001).

In Rwanda, mulberry wood ash is used as a fertilizer for the first time. Chemical analysis of soil and determination of its acidity were carried out (Figure 3) every three months (seasonally) and the effectiveness of using mulberry wood ash, was compared with that of organic and chemical fertilizers.

The diagram shows that the use of lime and MWA in conjunction with other fertilizers has led to changes in acidity of the soil and its chemical composition. Adding only organic manure (OM) and NPK could not bring about a significant change. The acidity of the soil after adding lime and MWA linearly decreased and remained static during the year. MWA was proved the most effective component because within a short time after its introduction, the pH of the soil rose from 4.8 to 6.52 (slightly acidic), which appears to be a function of its structure and particle size, enabling its rapid interaction with the soil. It is known that the presence of nutrients in the soil and their dissolution directly depends on the pH level. Both the low or too high pH level are unfavourable to plant growth since the nutrients in the soil remain unavailable to plants. The content of the nutritious elements in soil depends on quality, quantity and combination of applied fertilizers. For example, the quantity of N, P and K in the soil sharply increased as a function of application of mineral fertilizer $N_{30}P_{24}K_{18}$ whereas, application of OM, Lime and especially MWA not only increased the quantity of N, P and K, but also Ca, C, Mg, Fe, Mn and Zn. It has to be taken care that lime or MWA should be applied one month before application of mineral and organic fertilizers.

The soil nutrient elements show a specific response with regard to season and type of fertilizer applied (Figure 3). Some elements viz., K, Mg, Fe, Mn and C achieve their highest activity 6 months (August – October: a dry season) after fertilizing while the others such as P, Ca and Zn are the most active in the 9th month after fertilization (November – January: rainy season) and S and B, on the contrary, are highly concentrated in May-June and February-April, the mild season.

Impact of regulation of acidity (pH) and fertility of soil on mulberry leaf yield

According to modern concepts, fertility refers to the ability of the soil to meet the needs of plants in terms of elements of nutrition, water and, ensuring their root systems of sufficient air, heat, and physical and chemical environment that is conducive to the normal growth and development. There are variety of other factors such as climate, plants, time, activity of the farmer and others, which are also playing a great role (Balloni and Favalli, 1987; Phelan *et al.*, 1995). The main means of regulation of nutrients reserve in soil, in particular forms, accessible to plants, consist of regulation of its acidity and addition of necessary organic and mineral fertilizers (Tisdall and Oades, 1982; Korpilhati *et al.*, 1998) which creates normal conditions for the life of *Azotobacter* and other organisms that assimilate nitrogen from the atmosphere (Balloni and Favalli, 1987; Ledgard and Steele, 1992; Dewes and Hunsche, 1998; Haynes, 1999; Doran and Zeiss, 2000). It makes no sense to saturate the soil with fertilizer and microelements, if the pH of the soil is not at the optimum. This is also verified from our studies (Figure 3).

At soil pH 5.2 (control), growth and development of mulberry was belated, leaves were subjected to various diseases, which led to an overall decrease in quantity (2.5 kg/tree) and quality of fodder for silkworms (Figure 4). Soil fertilization with OM and $N_{30}P_{24}K_{18}$ fertilizer has led to a slight decrease in pH and a slight increase in the incidence of diseases with a marginal increase in leaf yield (Wong *et al.*, 2004). Organic and mineral fertilizer (especially NPK) lead to insignificant oxidation of soil. The role of MWA in enhancing the fertility of soil, has already been explained that its application gradually decreases the acidity of soil, and in a period of half an year, reaches up to pH 6.5 and remains stable at this level for about 1.5 years. Hence, application of mulberry wood ash in comparison with lime, is the most comprehensible and cheap means for regulation of acidity and fertility of soil, which essentially reduces incidence of diseases (leaf spot and chlorosis) in a mulberry plantation, thus leading to 3.5 times improvement in yield and quality of leaves.

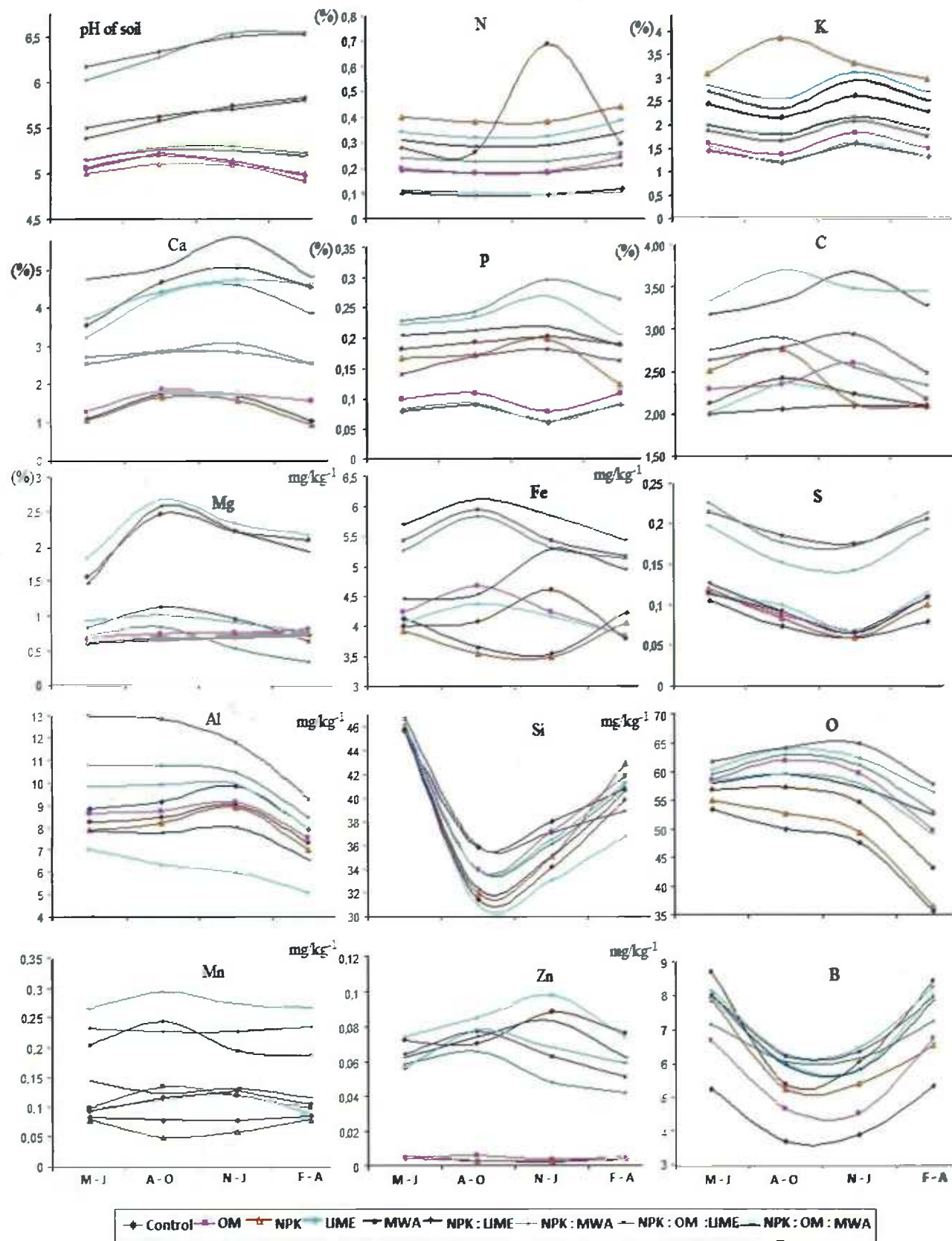


Figure 3: Change in acidity level and contents of chemical elements in soil after treatment with different fertilizers

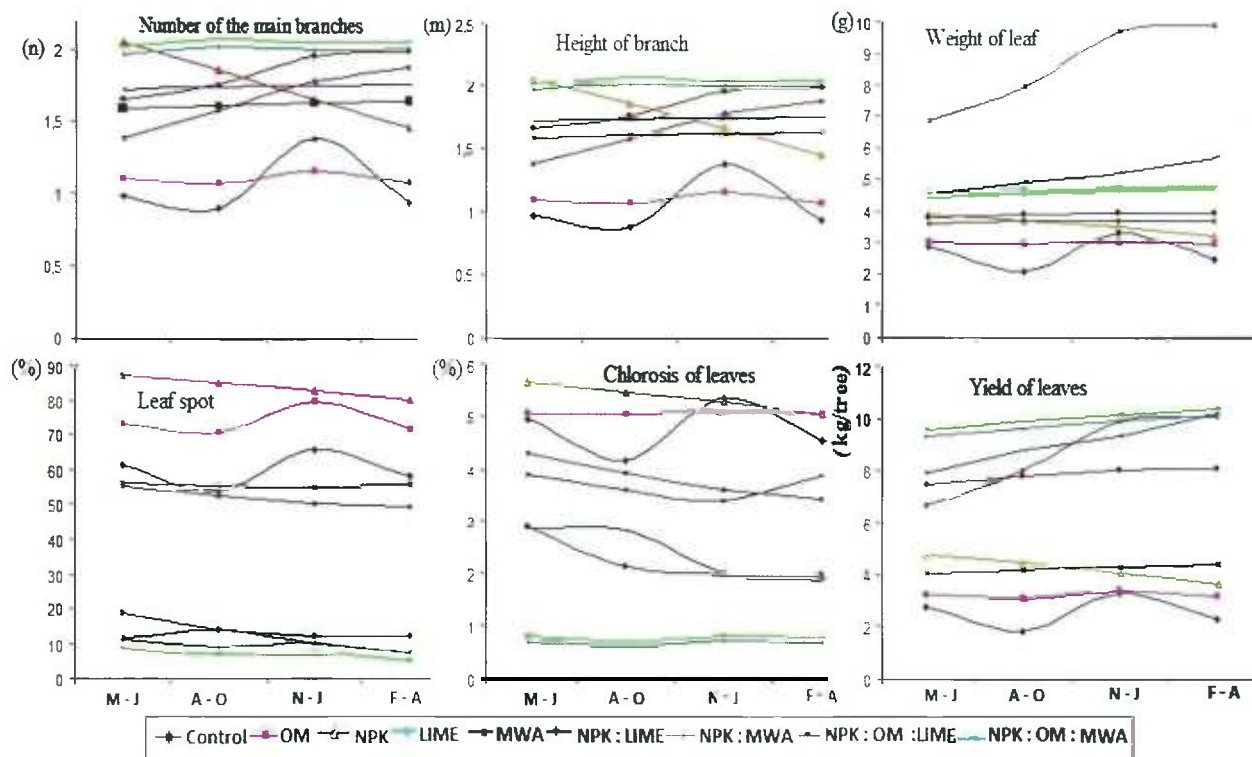


Figure 4: Effect of change of soil acidity (pH) after treatment with different fertilizers on economic parameters and yield of mulberry

CONCLUSION

Problems related to soil's acidity, the means of regulation and its fertility enrichment are central in agriculture. Various methods are being used for these purposes, among which is recommended the application of mulberry wood ash. Mulberry wood ash is richer than lime in terms of all necessary vital elements for plants, and is the cheapest and accessible alternative. It is noteworthy that mulberry wood ash is also alkaline and can cause crop damage if misused. It is imperative that the land owners follow the prescribed application rates and use common sense approaches to prevent decrease of yield and also ensure to avoid environmental contamination.

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EFFICACITE DE L'AUGMENTATION DE LA FERTILITE ET DE LA REGULATION DE L'ACIDITE DU SOL SUR LA CROISSANCE ET LE RENDEMENT DU MURIER

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RESUME

Le Rwanda, situé en Afrique Centrale entre 1°04' et 2°51' de latitude sud et entre 28°45' et 31°15' de longitude est, bénéficie d'un climat tropical tempéré en raison de l'altitude élevée (900 – 4507 m). La pluviométrie annuelle est de 700-1000 mm. Presque tous les sols du Rwanda sont acides (pH 4.8-5.8) ce qui affecte la fertilité du sol et entraîne une réduction de 50 % de la productivité de toutes les récoltes de graines de base et de racines. Le développement de la sériciculture comme nouvelle branche de l'agriculture a démarré sous une grande attention au Rwanda, l'état y mettant de grands espoirs pour accroître son potentiel d'exportation. Puisque la plantation de mûriers est le composant économique majeur de la sériciculture, la qualité du sol a une profonde influence indirecte sur la production de soie. Les sols présentant une faible acidité (pH 6.8) sont idéaux pour une bonne croissance du mûrier. Les sols de type latéritique et sableux observés au Rwanda sont caractérisés par de faibles concentrations en K, Mg et autres éléments vitaux de base, une faible capacité de rétention de l'eau et un pH bas. Aussi, la mise en œuvre de mesures adéquates de mise en valeur du sol est une étape essentielle pour obtenir de la feuille de mûrier de qualité supérieure. Habituellement, la chaux dolomitique et les cendres de bois sont recommandées pour réguler l'acidité du sol. L'analyse chimique des cendres de bois de mûrier montre que la composition en éléments de base nécessaires pour une plante, sauf pour le Ca, le Mg et le Zn est plus élevée que dans la chaux puisque les jeunes branches sont riches en macro et micro-éléments. L'équivalent moyen en carbonate de calcium (CCE) dans les cendres de bois de mûrier est de 43.0 %. L'utilisation de cendres de bois de mûrier comme engrais en combinaison avec d'autres éléments minéraux et organiques améliore la fertilité du sol, régule l'acidité et enrichit le sol en composants chimiques, décroît incidemment l'ampleur des maladies dans une plantation de mûriers et finalement améliore la productivité et la qualité des feuilles.

Mots-clés: Rendement en feuilles, cendres de bois de mûrier, composition chimique du sol, fertilité du sol, pH du sol.



Research Paper

OXIDATIVE STRESS INDICES IN THE LARVA AND PUPA OF WILD TASAR SILKMOTH, *ANTHRAEA PAPHIA* LINN.

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ABSTRACT

The level of oxidative stress in the haemolymph and fat body tissue was studied in the V instar larva and diapausing pupa of tasar silkmoth, *Antheraea paphia* Linn. The concentration of protein, ascorbic acid, reduced glutathione, endogenous level of lipid peroxidation (LPX) and hydrogen peroxide content were analysed. Increase in the level of reduced glutathione towards pupal stage is the indication of maintenance of cellular antioxidant status. Also reduction in the level of LPX in the fat body tissue showed a protective mechanism to limit the tissue oxidation. Decrease in the level of H₂O₂ rendered oxidation of tissue lipids. The results suggest that pupal period challenges more oxidative stress in comparison to the V instar larva of Indian wild tasar silkmoth, *A. paphia*.

Key words: *Antheraea paphia*, antioxidants, larva, oxidative stress, pupa.

INTRODUCTION

Environmental stresses diminish *in vivo* antioxidant status and cause oxidative stress in living organism (Klasing, 1998; Sahin *et al.*, 2001). Oxidative stress is the result of an imbalance between pro-oxidant species and the levels of the defences resulting from the generation of reactive oxygen species (ROS) (Santoro and Thiele, 1997). Reactive oxygen species is related to ageing and life span (Orr and Sohal, 1994) and plays a significant role in the innate immune response of insects (Hao *et al.*, 2003). All aerobic organisms are endowed with a mutually supportive team of defense mechanisms against ROS or the pro-oxidant forces, commonly called as the antioxidant defences. Insects like other animals possess a suite of antioxidant systems for the removal of damaging ROS (Felton, 1995). It has been reported that proteins are vulnerable to oxidative damage (Dean, 1991). Ascorbic acid is redox catalyst which can reduce, and neutralize ROS such as hydrogen peroxide (Padayatty *et al.*, 2003). Glutathione (GSH) is reported as one of the most important cellular antioxidants (Meister and Anderson, 1983). Oxidative stress in the

cells occurs as a result of increased exposure to oxidants or from decreased protection against oxidants, or even from both the events occurring simultaneously (Cadenas, 1989). Acute or chronic oxidative stress may result in uncontrolled lipid peroxidation (LPX) and protein oxidation (Levine *et al.*, 1981), which leads to cell death and impairs cell function. Hydrogen peroxide (H₂O₂) is a common reactive oxygen species which causes oxidative damage in tissues. In phytophagous insects, antioxidant defence mechanisms are of paramount importance because they are exposed to ROS promotive environments as well as allelochemicals present in the host plants (Sahoo *et al.*, 2008).

Tasar silkmoth, *Antheraea paphia* Linn. is exclusively wild and multiplies in nature, on Sal (*Shorea robusta*) plant, as it is the primary food plant of the species. It is the silkworm which produces the highest amount of silk in a cocoon (Dash and Nayak, 1991). In the natural environment, the larvae as well as the diapausing pupae get exposed to adverse physicochemical, environmental or pathological agents

that play an important oxidative regulatory mechanism (Patra *et al.*, 2011b). As the haemolymph (HL) is the chief circulating fluid in the haemocoel and fat body (FB) is the storage tissue of insects they may have some roles in fighting oxidative stress. It has been reported that the haemolymph bathes all tissues and organs in the insect body and transports nutrients, hormones and metabolic wastes (Gilbert and Chino, 1974). It serves as a transient mobile biochemical repository for protein, amino acids, carbohydrates and lipids (David and Ananthakrishnan, 2006). Of all constituents, the haemolymph proteins assume significance as they represent the products of gene expression. Their levels show generic-specific, tissue specific and stage specific variations during insect metamorphosis (Hou *et al.*, 2007, 2010, Bakkappa and Subramanya, 2010). Hence, in this attempt, the antioxidants and oxidative stress indices of haemolymph and fat body tissues of V instar larva and diapausing pupa of wild tasar silkworm, *A. paphia* were studied. As fat bodies are the only reserve source of energy in the insect body for its utilization during diapausing period, its relationship with haemolymph with regard to antioxidant level (increase or decrease) and oxidative stress indices in both the tissues were studied. Further, as the diapause is a non-feeding stage, whatever antioxidants present in V instar larva (last feeding stage before pupation) will be utilized during spinning and to fight adverse conditions throughout the pupal period. Hence, the estimation of antioxidants in the V instar larva has a relevance regarding its utilization at the time of spinning and the left over amount to fight the oxidative stress faced during the pupal diapause. Therefore, these parameters were studied in the early pupal period (described below) in comparison with the antioxidant contents in the V instar larva.

MATERIALS AND METHODS

Animal: The live larvae on the 4th day (mid larval period) of the V instar and day 15 diapausing (early diapause period) pupae of Indian wild tasar silkworm, *A. paphia* were collected from the host plant, Sal (*Shorea robusta*) trees of peripheral zone of Similipal Biosphere Reserve, Odisha, India. The insects grow in nature during July to October where the total V instar larval period is

about one week and pupal diapausing period is usually eight months.

Tissue preparation: Haemolymph was collected from larvae and pupae, in prechilled eppendorf tubes coated with 3 % phenylthiourea to inhibit denaturing or blackening of the tissue (Mishra *et al.*, 2009) and centrifuged at 7,000 x g for 10 minutes at 4 °C to settle the haemocytes. Larvae and pupae were dissected to collect the fat body. After washing in ice-cold physiological saline (0.67 % NaCl), the tissues were retained in ice separately. The tissues were weighed in monopan digital balance and homogenized (10 %) in 50 mM phosphate buffer (pH 7.4) with 1mM EDTA using hand homogenizer under ice (Sahoo *et al.*, 2008). Homogenates were centrifuged at 10,000 x g for 20 minutes at 4 °C. The supernatant was collected for further chemical analyses.

Biochemical estimation: The amount of proteins was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Ascorbic acid (ASA) content was measured according to the method of Jagota and Dani (1982). The reduced glutathione (GSH) content in the tissue samples was determined (Ellman, 1959). The sample after centrifugation was used for the estimation of lipid peroxidation (LPX) by monitoring the formation of malondialdehyde (MDA) by the method of Ohkawa *et al.* (1979). The amount of MDA formed was calculated from the extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Wills, 1969). The hydrogen peroxide (H_2O_2) content in the supernatant of post mitochondrial fraction was determined spectrophotometrically (Pick and Keisari, 1981) using horseradish peroxidases (HRP) and phenol red.

Statistics: To know the difference between means of two independent samples, Fisher t-test and for dependent samples, Paired t-test were employed (Chainy *et al.*, 2008).

RESULTS AND DISCUSSION

As stated earlier, HL is the circulatory fluid of insects which contains all the essential biochemical components supplied to all the tissues whereas, the FB carry reserve food material in the insect for its utilization

during diapause. Therefore, to know the increase or decrease in antioxidant contents in one tissue in relation with the other tissue, antioxidants contents of HL and FB tissues were studied. It was observed that the concentration of the protein, ascorbic acid, GSH, LPX (MDA content) and hydrogen peroxide were not uniform in HL and FB tissues.

Statistical analyses (Fisher t-test) revealed that the protein content of FB was significantly higher ($P < 0.001$) than that of the HL in both V instar larva and pupa (Figure 1). The reverse trend was observed in the case

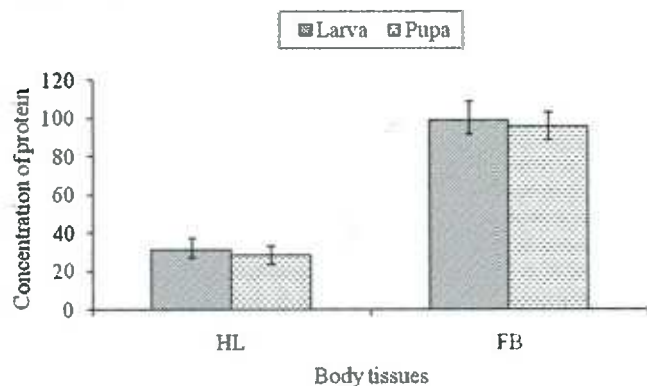


Figure 1: Concentration of protein in haemolymph (mg/ml) and fat body (mg/g) tissues of V instar larva and diapausing pupa of wild tasar silkworm, *A. paphia*. Data are mean $\pm$ SD ($n = 7$ each).

of ASA content where HL tissue was found to have significantly higher amounts ($P < 0.001$) than that of the FB tissue in the V larval instar and pupa (Figure 2). The concentration of reduced glutathione (GSH) was found to be more in FB compared to HL of V larval instar as well as diapausing pupa. However, this was found to be

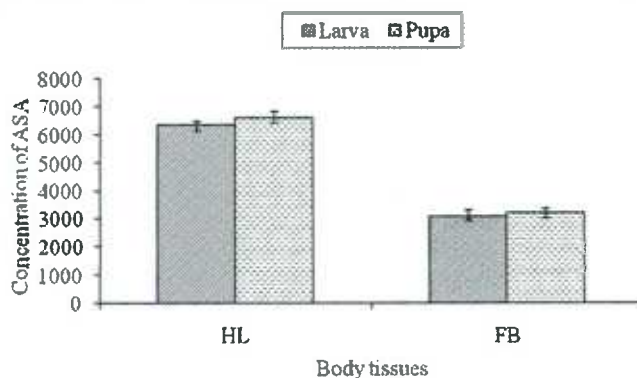


Figure 2: Concentration of ASA (ascorbic acid) ($\mu\text{mol/mg}$ protein) in haemolymph and fat body tissues of V instar larva and diapausing pupa of wild tasar silkworm, *A. paphia*. Data are mean $\pm$ SD ($n = 7$ each).

statistically insignificant (Figure 3). The level of LPX (MDA content formed) in the FB was significantly higher

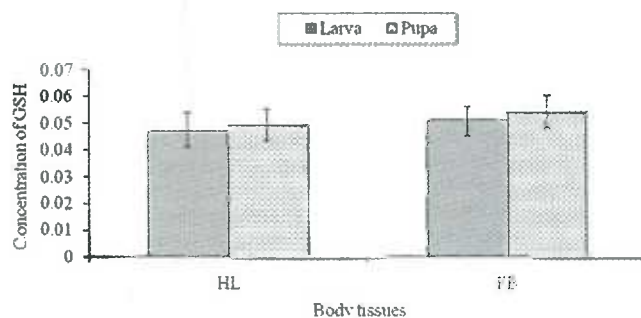


Figure 3: Concentration of reduced glutathione (GSH) ($\mu\text{mol/mg}$ protein) in haemolymph and fat body tissues of V instar larva and diapausing pupa of wild tasar silkworm, *A. paphia*. Data are mean $\pm$ SD ($n = 7$ each).

($P < 0.001$) than that of the HL tissue in both V larval stage and pupa (Figure 4). The H_2O_2 content in the V larval instar was significantly higher ($P < 0.01$) in FB

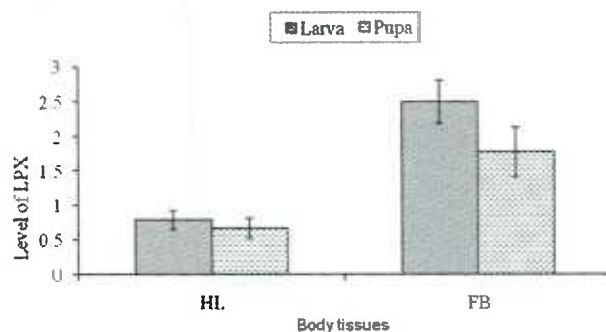


Figure 4: Endogenous LPX level (nmol MDA/mg protein) in haemolymph and fat body tissues of V instar larva and diapausing pupa of wild tasar silkworm, *A. paphia*. Data are mean $\pm$ SD ($n = 7$ each).

than that of the HL (Figure 5). Similar pattern was also observed in the case of pupa where the FB tissue showed

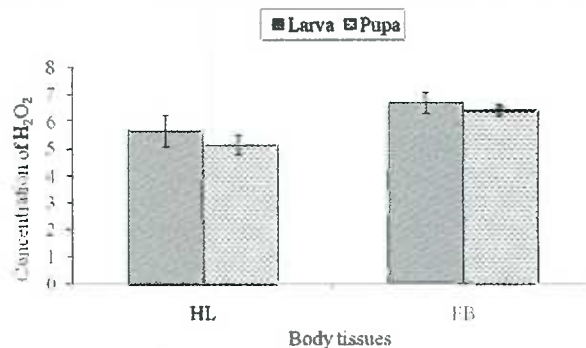


Figure 5: Endogenous H_2O_2 content (nmol/mg protein) in haemolymph and fat body tissues of V instar larva and diapausing pupa of wild tasar silkworm, *A. paphia*. Data are mean $\pm$ SD ($n = 7$ each).

higher ($P < 0.001$) level of H_2O_2 compared to HL tissue (Figure 5).

High protein content in FB indicates that the animal stores its antioxidant components in the respective tissue to maintain its antioxidant defence system. Summers and Felton (1994) observed that ascorbic acid is an effective antioxidant in protecting *Helicoverpa zea* tissues against phenolics prooxidants. Therefore, higher ASA content observed in the HL than FB might be an adaptive antioxidant response against elevated oxidative assault. Jovanovic-Galovic *et al.* (2004) also observed higher ASA content in the pupal tissues of European corn borer in their mid-diapause stage. The reduced glutathione and ASA are important for their protective role against ROS and form a powerful redox couple to neutralize variety of free radicals in biological system (Summers and Felton, 1994). Compensatory function of several other small molecular weight hydrophilic antioxidants such as uric acid, trehalose, polyol *etc.*, may be a reason for low amounts of GSH in the haemolymph. Summers and Felton (1994) also observed that trehalose, the primary carbohydrate in insect haemolymph, scavenges $\cdot OH$ with significantly greater efficiency than glucose and other compounds. The higher concentration of GSH reported in FB tissue corroborates with the findings of Meister and Anderson (1983) that this tissue maintains the cellular antioxidants like GSH. The high level of LPX (MDA) content in FB indicates that this tissue challenged higher rate of tissue oxidation than HL. The high level of H_2O_2 in FB indicates that this tissue faced more oxidative stress in larval as well as pupal stages.

The V instar larval period is the most important and crucial for this insect as it undergoes a significant metamorphosis wherein the larva starts spinning and enters into a distinctive phase of diapause, called pupa. Subsequently, the pupa is transformed to a winged adult moth by the next course of metamorphosis. Moth is the adult form of *A. paphia* which starts the next generation. It implies that the V larval instar and diapausing pupal stages are the crucial period in the life of this insect (Patra *et al.*, 2011a, b).

To compare the antioxidant contents between same tissues of larva and pupa, Paired t-test was employed. It was observed that the concentration of HL protein of

larva was significantly higher ($P < 0.01$) compared to that of pupa (Figure 1). The same trend was also found in the FB protein content where the larva showed significantly greater value ($P < 0.001$) than that of the pupa (Figure 1). The concentration of ascorbic acid in HL of pupa was significantly higher ($P < 0.01$) than that of the larva (Figure 2). Same trend was also found in the FB where the concentration of ASA was higher ($P < 0.02$) in pupa than that of the larval group (Figure 2). In the pupal stage, the GSH content of HL was found to be higher than that of the larva though not significant (Figure 3). On the other hand, in the case of FB, the GSH content was significantly greater ($P < 0.001$) in pupa compared to that of larva (Figure 3). The level of LPX (MDA content formed) in the HL and FB tissues of larva was found to be higher than that of the pupal stage which was statistically insignificant (Figure 4). The H_2O_2 content in both the tissues of larva was significantly higher ($P < 0.001$) than that of the pupa (Figure 5).

Dean (1991) had opined that decrease in protein content of tissues in different pathological conditions is due to oxidative damage. The decrease in protein content towards pupal stage is an indication of oxidative damage in the HL and FB as reported by Dean (1991). The increase in the redox catalyst ascorbic acid in the pupal tissues indicates its role in neutralizing ROS, like hydrogen peroxide in its body. The increase in concentration of GSH content in both the tissues towards the pupal stage indicates the activity of cellular antioxidant to protect the cell from oxidative damage as reported by Meister and Anderson (1983). Reduction of the LPX level indicates a protective mechanism to limit the tissue oxidation during metamorphosis of the post-diapausing pupa into winged adult. The higher level of LPX in V instar larva can be linked to increase in O_2 consumption as reported by Rath *et al.* (2005) for *A. mylitta*. A comparison of levels of LPX in both V instar larva and pupa indicates that larval tissue was challenged with relatively higher level of oxidative threat than that of the pupal tissue. The decrease in level of H_2O_2 towards the pupal stage might be indicative that it has rendered oxidation of tissue lipids, which are more susceptible to ROS-mediated oxidative damages compared to proteins and DNA as reported by Halliwell and Gutteridge (1999). A substantial decrease in the level of H_2O_2 as

observed in the present study supports the above hypothesis.

From the present findings it is concluded that the oxidative stress was comparatively high concomitant with better antioxidant protection in the fat bodies of V instar larva and diapausing pupa of Indian wild tasar silkworm, *A. paphia*. It was further observed that the pupal stage experienced more oxidative assault with simultaneous induction of non-enzymatic antioxidants (GSH and ASA) probably as an adaptive cellular response due to higher rate of oxygen consumption.

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INDEXES DE STRESS OXYDATIF CHEZ LA LARVE ET LA CHRYSALIDE DU VER A SOIE TASAR SAUVAGE *ANTHERAEA PAPHIA* LINN.

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RESUME

Le niveau de stress oxydatif dans l'hémolymph et le corps adipeux a été étudié chez des larves du 5^{ème} âge et des chrysalides diapausantes du ver à soie tasar, *Antheraea paphia* Linn. La concentration en protéines, en acide ascorbique, en glutathion réduit, le niveau de peroxydation des lipides (LPX) et le contenu en peroxyde d'hydrogène ont été étudiés. L'augmentation du niveau de glutathion réduit en avançant vers le stade de pupaison est le signe du maintien du statut antioxydant cellulaire. La réduction du niveau de LPX dans le corps adipeux montre l'existence d'un mécanisme de protection pour limiter l'oxydation du tissu. La baisse du niveau de H₂O₂ permet l'oxydation des lipides tissulaires. Les résultats suggèrent que la période pupale est marquée par plus de stress oxydatif que le 5^{ème} âge larvaire chez le ver à soie tasar sauvage indien, *A. paphia*.

Mots-clés: *Antheraea paphia*, antioxydants, larves, pupes, stress oxydatif.



Research Paper

EFFECTS OF ETHANOLIC PLANT EXTRACTS ON BmNPV INOCULATED SILKWORM, *BOMBYX MORI* IN RELATION WITH MIDGUT ANTIOXIDANT ENZYMES, ACID PHOSPHATASE AND NON-SPECIFIC ESTERASE

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ABSTRACT

Plants containing secondary metabolites such as alkaloids, flavonoids and triterpenoids are normally used against infection caused by virus in animals. A few selected ethanolic plant extracts containing alkaloids (*Aegle marmelos*, *Mormordica charantia* and *Argemone mexicana*), triterpenoids (*Curcuma longa*, *Syzygium cumini*, *Mormordica charantia* and *Euphorbia geniculata*) and flavonoids (*Syzygium cumini*) were tested in the present investigation against Nuclear Polyhedrosis Virus (BmNPV) infection of *Bombyx mori*. LC50 dose of BmNPV (1×10^4 polyhedral inclusion bodies) resulted in 51.66 % mortality in inoculated larvae. But the treatment of ethanolic plant extracts containing antiviral secondary metabolites reduced the mortality in BmNPV inoculated larvae (LC50 dose) up to 8 ~ 21 %. BmNPV infection caused increased oxidative stress in midgut tissue resulting in decreased superoxide dismutase (SOD) activity and decreased catalase (CAT), ascorbate peroxidase (APOx), acid phosphatase (ACP) and nonspecific esterase (NSE) activity in larvae on the sixth day of infection. Treatment of ethanolic plant extracts to BmNPV inoculated larvae at LC50 dose augments the activity of enzymes SOD, APOx, ACP and NSE and decrease CAT activity on sixth day of treatment. Results of the present study confirm antiviral activity of the ethanolic plant extracts against grasserie causing BmNPV of *Bombyx mori*.

Key words: Ascorbate peroxidase, BmNPV, catalase, mortality, plant extracts, superoxide dismutase.

INTRODUCTION

Due to centuries of domesticated life, the silkworm *Bombyx mori* has limited natural resistance and shows neither morphological nor behavioural adaptations to escape from the attack of their natural enemies i.e., disease causing pathogens, parasites and pests. *Bombyx mori* has long been reared as a beneficial insect in sericulture industry and as an experimental model in laboratory. In tropical countries like India, grasserie disease is a major problem which is caused by *B. mori* nuclear polyhedrosis virus (BmNPV) (Khurad *et al.*, 2004). The nuclear polyhedrosis prevails throughout the year especially in summer and rainy seasons (Dandin *et al.*, 2000). Ingestion of food contaminated with viral polyhedra is the mode of infection of BmNPV. Infection

occurs in all larval instars more commonly in fourth and fifth instars causing 20-50 % cocoon crop loss in India (Vidya, 1960; Chitra *et al.*, 1975; Samson *et al.*, 1990; Shivprakasam and Rabindra, 1995; Nataraju *et al.*, 1998).

BmNPV is a baculovirus which affect midgut epithelial cells, tracheal system, haemolymph cells and fat body; the nuclei of silk gland epithelial and tunica intima cells are also invaded by this virus (Khurad *et al.*, 2004). Virions combine with midgut epithelial cells and enter nuclei to start the first cycle of viral production and replication. These processes cause many biochemical changes in larvae which are in turn defended through specific metabolic activities (Etebari *et al.*, 2007). In most of the viral diseases, the pathogens

are extruded by infected silkworms along with gut juice and faecal matter. It can also enter into the rearing environment through breakage of fragile integument. Besides, vertical transmission BmNPV *i.e.*, transovarian and transovum transmission is reported by Khurad *et al.* (2004).

Natural plant products are being used by many researchers to control various diseases of silkworm (Ravi Kumar *et al.*, 2009; Manimegalai *et al.*, 2010). Some plants are having the antimicrobial activity due to the presence of secondary metabolites *viz.*, phenols, tannins, flavonoids, alkaloids, quinons, lectins, glucosides, terpenoids and essential oils which are useful for curing many diseases caused by pathogens including viruses (Cowan, 1999). *Eucalyptus globules* is reported to contain tannins having antibacterial and antiviral effects. *Lantana lawsonia* possess lawsone, a kind of quinone which is effective against Gram-positive bacteria (Suresh *et al.*, 1997). *Curcuma longa* has a terpenoid, curcumin which can act against bacteria and protozoans (Apisariyankul *et al.*, 1995). *Aegle marmelos* is characterized to have marmelide which is the most effective virucidal agent (Badam *et al.*, 2002) and antifungal essential oils (Rana *et al.*, 1997). Seeds and leaves of *Syzygium cumini* contain triterpenoids such as oleanolic acid, eugenin, ellagic acid, betulinic acid and friedelin (Bhatia and Bajaj, 1975; Sah and Verma, 2011) which are reported to be antibacterial (Ahmad and Beg, 2001). *Argemone mexicana* seeds possess alkaloids (Venkata *et al.*, 2010) with antibacterial properties (Kempuraj and Bhat, 2010). In addition, there are several references on terpenes or terpenoids containing plant products which are active against viruses (Fugioaka and Kashiwada, 1994; Hasegawa *et al.*, 1994; Pengsuparp *et al.*, 1994; Sun *et al.*, 1994; Xu *et al.*, 1996).

It is well known that the main forces of cell lysis under intestinal pathogenesis of vertebrates are lipid peroxidation (LPO) and generation of ROS (Mehta *et al.*, 1998; Pavlick *et al.*, 2002). ROS include oxygen ions, free radicals and peroxides both inorganic and organic. These molecules are highly reactive and may generate as natural byproduct of the normal metabolism of oxygen. Besides the harmful effects, they play an

important role in cell signaling and induction of host defense genes (Dalton *et al.*, 1999; Kamata and Hirata, 1999). However, the production of reactive oxygen species (ROS) is increased when the organism is subjected to irradiation, chemicals or infection (Knapowski *et al.*, 2002). Under environmental stress *e.g.*, bacterial infection, ROS levels may increase dramatically, resulting in significant damage to cell structures. This process is known as oxidative stress (Rahman and Macnee, 2000; Wang *et al.*, 2001). Effect of bacterial infection on antioxidant activity and LPO in the midgut of *Galleria mellonella* larvae has been reported by Dubovskiy *et al.* (2008). Oxidative stress during the viral pathogenesis of insect cell lines has been described previously (Wang *et al.*, 2001). Overproduction of ROS damages cellular lipids, nucleic acids, proteins and leads to LPO, genome instability or gene mutation; protein carbonyl formation and enzymatic inactivity resulting in degenerative processes leading to aging (Martin *et al.*, 1996; Berlett and Standtman 1997; Finkel and Halbrook, 2000). The antioxidant enzymes in insects which defend ROS produced in cellular reactions include SOD, CAT, glutathione peroxidase (GPx), glutathione S-transferases (GSTs) and APOx (Ahmad, 1995). SOD, CAT, and GPx form a defensive complex against endogenously produced ROS. SOD catalyzes the dismutation of superoxide radicals to H_2O_2 and molecular oxygen, and appears to be the main response to prooxidant effects of dietary allelochemicals exposure. H_2O_2 is subsequently scavenged by CAT, resulting in the production of water and molecular oxygen (Ahmad and Pardini, 1990). APOx also scavenges H_2O_2 but only at low concentrations which are not normally scavenged by CAT. GPx also reduce H_2O_2 and hydroperoxides, thereby scavenging oxidative radicals in tissues and cell membrane. Because GPx is found only at very low levels in insects, they appear to rely instead on elevated activities of CAT and glutathione S-transferase with peroxidase-like (GSTpx) activity (Krishnan and Kodrik, 2006).

It has been reported that after viral infection, there is increase in lysosomal enzymes in cytoplasm of infected cells. It can readily be understood that excessive release of such hydrolytic enzymes into the cytoplasm could be damaging to cells.

The NSE is a heterogenous and ubiquitous group of cellular carboxyl esterases (Ansley *et al.*, 1971). In insects, there is evidence that the titre of juvenile hormone is regulated by esterases (Kort and Granger, 1981). It is also involved in digestive processes (Kapin and Ahmad, 1980; Jones and Brancoft, 1986), reproduction (Richmond *et al.*, 1980; Mane *et al.*, 1983) and insecticide resistance (Devonshire *et al.*, 1986, Fournier *et al.*, 1993). There is no information available about the behaviour of these enzymes during viral infection in insects. Therefore, the present investigation was undertaken to ascertain the efficacy of a few of ethanolic plant extracts against BmNPV infection by analyzing the effects on antioxidant enzymes such as SOD, CAT and APOx. The oxidative damage was measured by way of estimating LPO. In a similar line, the status of ACP and NSE in response to ethanolic plant extracts was also studied.

MATERIALS AND METHODS

- 1. Plant material:** The selected plant materials such as rhizome of *Curcuma longa*, fruits of *Aegle marmelos*, leaves of *Mormordica charantia* and *Euphorbia geniculata* and seeds of *Argemone mexicana* and *Syzygium cumini* were collected from their natural habitat and shade dried.
- 2. Preparation of ethanolic extracts:** The shade dried plant materials were powdered and kept in ethanol for extraction for 72 hours. The extract obtained was kept at 10 °C for further use.
- 3. Rearing of silkworm:** The silkworm larvae of Pure Mysore (PM) race were reared according to the standard method (Krishnaswami *et al.*, 1979).
- 4. Isolation and purification of PIBs:** The NPV infected silkworm larvae collected from natural rearing were crushed in distilled water and centrifuged at 10,000 g for 20 min. until clear layer of PIBs was obtained. These isolated PIBs were stored in refrigerator until its use.
- 5. Determination of LC50 value:** The isolated PIBs were serially diluted and each concentration was given to 50 larvae to determine the LC50.
- 6. Inoculation of BmNPV and plant extract treatment to silkworm larvae:** The larvae were divided into 8 groups including control with 30 larvae each. On the first day of fifth instar, the larvae were starved for 6 hours. 100 µl of LC50 concentration (1×10^4 PIBs) was spread on one square inch mulberry leaf piece and fed to each larva in compartments. After six hours, the larvae were fed with ethanolic plant extract (100 µl of 10 mg/ml solution) coated mulberry leaves. The plant extract was fed for three days at the same time in the morning. The experiment was repeated thrice.
- 7. Preparation of sample:** On the sixth day of fifth instar, the larvae were dissected and midgut tissue homogenates were prepared in 0.8 % saline and centrifuged. The supernatant was used for estimation of LPO, antioxidant enzymes, acid phosphatase and non-specific esterase activity.
- 8. Estimation of LPO:** LPO was estimated using TCA-TBA-HCl reagent. The reaction mixture contained 1 ml homogenate and 2 ml of TCA-TBA-HCl reagent. The tubes were kept in boiling water bath for 10 min., cooled and centrifuged. Absorbance was measured at 532 nm on spectrophotometer. Amount of malondialdehyde (MDA) was calculated using extinction coefficient, 1.56×10^5 M MDA/cm². Amount of MDA was expressed in nM of MDA/mg protein.
- 9. Estimation of antioxidant enzymes:** SOD was estimated by Beauchamp and Fridovich method (1971). The reaction mixture contained 3.4 ml 100 mM phosphate buffer, 0.3 ml 10 mM EDTA, 1.2 ml 130 mM methionine, 0.6 ml 750 µM nitroblue tetrazolium, 0.1 ml sample and 0.4 ml 60 µM riboflavin. Riboflavin was added last and the tubes were kept in front of 22W fluorescent tube of 45 cm length for 20 min in dark room. One tube without sample and not exposed to light served as blank and one tube without sample exposed to fluorescent light served as control. 50 % inhibition of NBT reduction was considered 1 unit of SOD. The activity was expressed in units of SOD/mg protein/h. CAT activity was estimated by titanium

sulfate method as described by Fugimoto (1965) with some modifications. The reaction mixture contained 0.9 ml substrate buffer [50 mM phosphate buffer, (pH 7.0) containing 0.15 mg $\text{H}_2\text{O}_2/\text{ml}$]. 0.1 ml of sample was added to this and after 1 min, the reaction was hampered by adding 4 ml titanium sulfate reagent (1 % titanium sulfate in 2.5 N H_2SO_4), the yellow colour formed was measured at 410 nm on spectrophotometer. The control tube contained 0.9 ml substrate buffer, 0.1 ml distilled water and titanium sulfate reagent. The blank tube contained 1 ml of 50 mM phosphate buffer (pH = 7.0) and 4 ml of titanium sulfate reagent. The difference between control and sample OD was used to calculate the amount of consumed H_2O_2 and activity of CAT was expressed in mg H_2O_2 consumed/mg protein/min. APOx activity (Nakano and Asada, 1981) was measured in samples using reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid and 10 mM H_2O_2 . The hydrogen peroxide-dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm ($\epsilon = 2.8/\text{mM}/\text{cm}$) and the enzyme activity was expressed in mM of ascorbate oxidized/mg protein/min.

10. **In-gel assay of antioxidant enzymes:** Native gel electrophoresis was done using 10 % separating gel and 4 % stacking gel. Equal amount of sample was loaded in wells. After sufficient running, gel was removed, washed with distilled water and kept in 1 mg/ml NBT solution for 30 min. Then it was incubated in fluorescent light 100 mM phosphate buffer, (pH 7.8) containing 60 μM riboflavin and 130 mM methionine, under fluorescent light till the colourless bands of Mn SOD and Cu/Zn SOD appeared.

Electrophoresis for CAT in-gel assay was done by the method of Woodbury *et al.* (1971) as described by Achary *et al.* (2008). In-gel assay was performed using 8 % separation and 4 % stacking gels. The gel was then incubated in 0.025 % H_2O_2 for 5 min, washed in distilled water and stained in a solution containing 1 % (w/v) potassium

ferricyanide and 1 % (w/v) ferric chloride (equal volumes of 2 % solution of each component added sequentially) for 5 min at room temperature. Colourless bands of CAT enzymes appeared on the blue background of the gel. APOx in-gel assay was done according to the method of Mittler and Zilinskas (1993) as described by Achary *et al.* (2008). In-gel assay was run using a 10 % separation and 4 % stacking gel. 2 mM ascorbic acid was added to the electrode buffer and the gel was pre-run for 30 min before the samples were loaded. After electrophoresis, the gel was immersed in a solution of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbic acid for 30 min, changing the solution three times in every 10 min. The gel was then soaked in 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbic acid and 20 mM H_2O_2 for an additional 20 min before washing in 50 mM sodium phosphate buffer of pH 7.0. Finally, the gel was incubated in a solution of 50 mM sodium phosphate buffer, pH 7.8, 28 mM TEMED and 2.45 mM NBT until the gel turned uniformly blue except at positions exhibiting APOx activity. Gel was rinsed when maximum contrast was achieved to stop reaction.

11. **Estimation of ACP activity:** The ACP activity was estimated using the Linhardt and Walter method (1963). The assay mixture contained 0.2 ml of enzyme source and 0.8 ml of citrate buffer (pH 4.0) containing 5.5×10^{-3} M p-nitrophenyl phosphate as substrate. The assay tubes were incubated for 30 min. at 40 °C. The reaction was stopped by adding 4 ml of 0.1 N NaOH. The absorbance was measured at 405 nm. Zero absorbance was adjusted by control devoid of enzyme source.
12. **Estimation of NSE:** The nonspecific esterase activity was estimated by the method of Bier (1955). Each assay mixture contained three tubes. To each test tube added 5 ml ice cold water, 2 ml 0.66 M phosphate buffer, 1 ml homogenate and 2 ml working substrate solution containing 1.0×10^{-4} M p-nitrophenyl acetate. The tubes were shaken vigorously and centrifuged for 5 min. at 2000 g.

Readings of assayed tubes were taken at zero hour as reference control. The tubes were taken at 400 nm. Both control and incubated tubes were measured against distilled water as blank. The activity was expressed in μg p-nitrophenol/mg protein/h.

13. **Estimation of Protein:** Soluble protein from sample was estimated by Lowry's method (1951).
14. **Statistical analysis:** Student – t test was implemented for statistical analysis.

RESULTS AND DISCUSSION

The ROS are highly reactive hydroxyl radical. While most ROS do not diffuse into more than a few form, the lipid peroxides resulting from the ROS-induced peroxidation of membrane phospholipids, such as malondialdehyde, can transverse the circulation and cell membranes, with resultant dysfunction of vital cellular processes such as membrane transport and mitochondrial respiration (Halliwell, 1987). Thus, ROS are often viewed as etiologic in producing cellular injury. The LPO is caused by the oxidation of lipids by highly reactive ROS. In the present investigation, levels of LPO estimated in midgut tissue (Figure 1.) was found to be decreased in BmNPV inoculated group. Similar results are reported in *Galleria mellonella* larvae two days post infection by *Bacillus thuriangiensis*. *M. charentia* treated larval group also showed decrease in LPO with low significance as compared with control group (Figure 1).

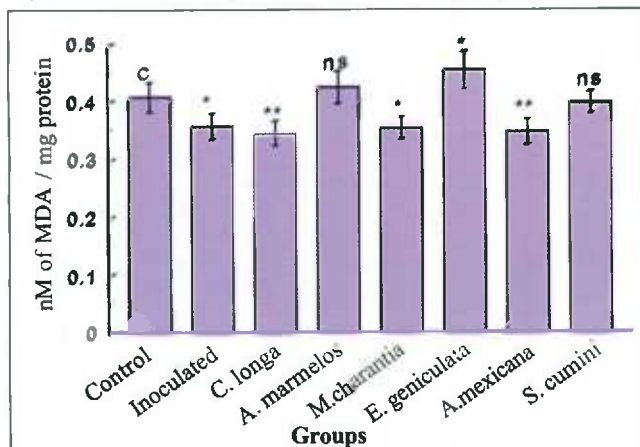


Figure 1: LPO in midgut tissue of silkworm

Data are mean $\pm$ SD (n = 3). ***, ** and * indicate high ($p < 0.001$), low ($p < 0.01$) and very low ($p < 0.05$) significance respectively. ns indicates non-significant change ($p > 0.05$) over the control group.

But there was no significant change observed in larvae treated with *A. marmelos* and *S. cumini*, whereas more significant decrease in LPO were observed in groups treated with *C. longa* and *A. mexicana* as compared with control group.

Generation of $\text{O}_2^{\bullet-}$ in haemolymph plasma of lepidopteran insect *Pseudeletia separata* (Arakava, 1994) and high SOD activity in the larval haemocytes of cabbage looper *Trichoplusia ni* (Ahmad *et al.*, 1991) have been reported which leads to prediction that insects produce a deleterious flux of superoxide in the haemolymph prior to phagocytosis and encapsulation similar to that of mammals (Krishnan *et al.*, 2002). Increase in oxidative stress has been reported as a result of viral infection to cell lines of insects, *Spodoptera frugiperda* Sf-9 (Sf-9) and *Trichoplusia ni*, BTI-Tn-5B1-4 (Tn-5B1-4) (Wang *et al.*, 2001). In insects, there are two isozymes of SOD i.e., mitochondrial Mn-SOD and cytoplasmic Cu/Zn SOD. In the present work, there was a highly significant ($p < 0.001$) decrease in SOD activity observed in BmNPV inoculated group as compared with control (Figure 2). Increase in SOD activity was observed only in group treated with *S. cumini*. There was a non significant change ($p > 0.05$) in groups treated with *A. marmelos* and *M. charentia*. Highly significant decrease in activity of SOD was also evident in groups treated with *E. geniculata* and *A. mexicana*. *C. longa* treated group showed decrease in activity with low significance ($p < 0.01$) as compared with control group of larvae. In - gel activity assay of SOD

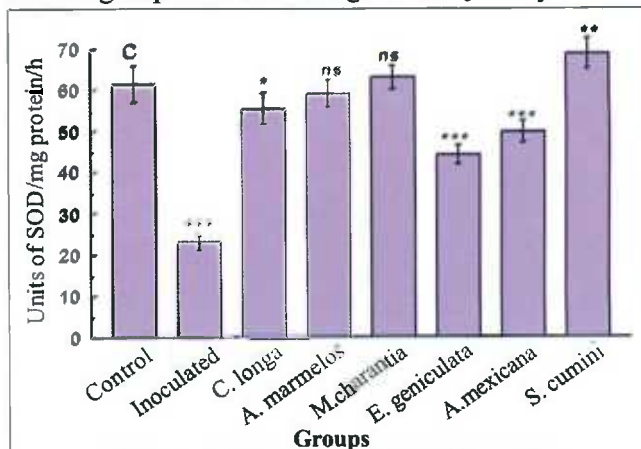
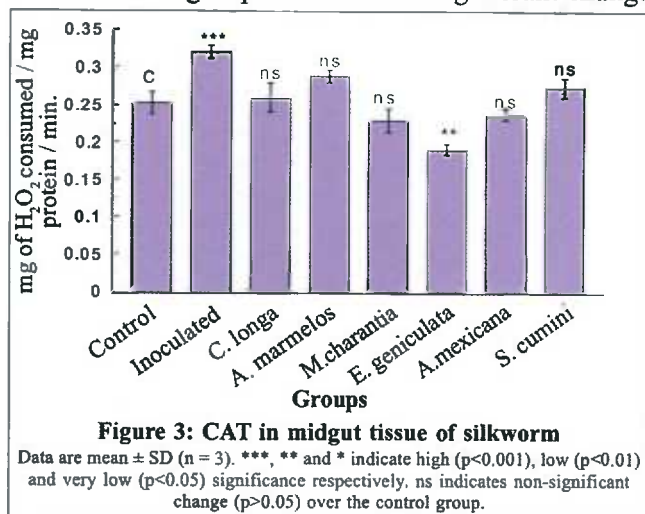


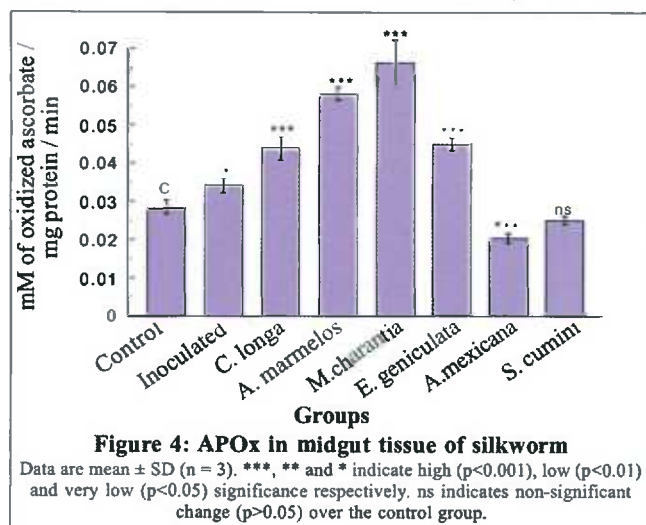
Figure 2: SOD in midgut tissue of silkworm

Data are mean $\pm$ SD (n = 3). ***, ** and * indicate high ($p < 0.001$), low ($p < 0.01$) and very low ($p < 0.05$) significance respectively. ns indicates non-significant change ($p > 0.05$) over the control group.

showed that the Mn SOD was active only in control group. In other groups, it showed weak contrast staining. In all groups, the Cu/Zn SOD activity bands were moderately unstained (Figure 8A). Significant increase in CAT activity was observed only in inoculated group whereas other groups exhibited no significant change



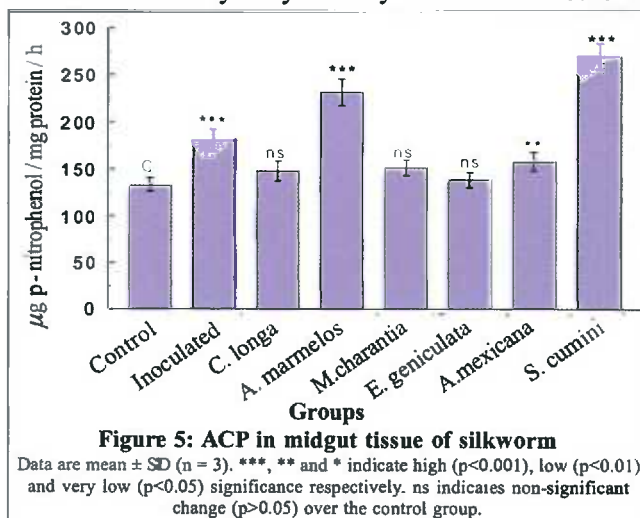
except *E. geniculata* treatment which showed significant decrease in CAT activity (Figure 3). The same results were evident from in-gel assay of CAT (Figure 8B). APOx is a novel antioxidant enzyme which catalyses the reduction of H₂O₂ to water by using ascorbic acid as specific electron donor. As a result of viral infection, there was very low APOx activity as compared with control group. APOx activity was higher in groups treated with *C. longa*, *A. marmelos*, *M. charantia* and *E. geniculata* (Figure 4, Figure 8C). In group treated with *A. mexicana*, there was significant decrease and non significant change in *S. cumini* treated group. It has



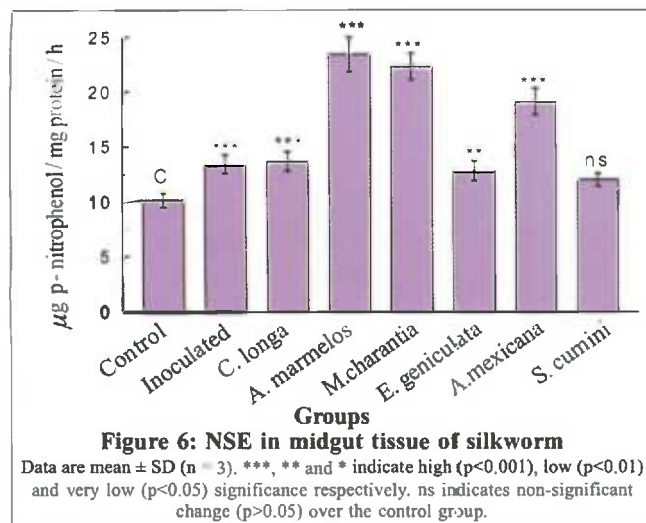
been reported that viral infection to insect cell lines viz., *Spodoptera frugiperda* Sf-9 (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5B1-4) showed gradual increase in activity of APOx post infection (Wang *et al.*, 2001). Previous studies on *Fenneropenaeus indicus* showed similar results after infection with white spot syndrome virus (WSSV). The significant decrease in the antioxidant enzyme activities indicates an increased level of oxidative stress due to the viral infection and an imbalance between pro-oxidants and antioxidants (Mohankumar and Ramasamy, 2006).

Acid phosphatase is a lysosomal hydrolytic enzyme. The activity of ACP was found to be significantly increased in inoculated group as a result of BmNPV infection (Figure 5). The lysosomal enzymes are found to be increased in cytosol of virus infected liver cells of mice (Allison and Brustone, 1963). There are evidences that animal viruses are taken into cell by pinocytosis and disintegrated in pinocytotic vacuoles. There is activation of lysosomes around pinocytotic vacuoles. The lysosomal enzymes are also involved in breakdown of host cell polynucleotide which results in a markedly increased pool of acid-soluble nucleotides in infected cells (Newton *et al.*, 1962). Activity of phosphatase, and another lysosomal enzyme, phosphoprotein phosphatase (Paigen and Griffiths, 1959) might contribute to the increased intracellular pool of phosphate and phosphate acceptor, and concomitant enhancement of glycolysis, which is a common feature of virus-infected cells (Cohen, 1959).

Esterases are a group of highly polymorphic and multifunctional hydrolytic enzymes. Four classes of

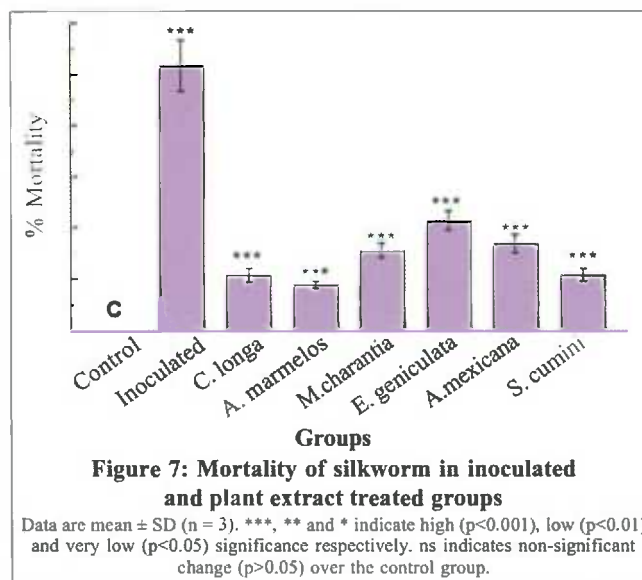


esterases are now recognized: arylesterases, acylesterases, carboxylesterases and cholinesterases. Each class has been defined by its substrate specificity, sensitivity to different types of inhibitors, and active site of amino acid residues. The NSE activity was increased significantly in all groups except group treated with *S. cumini* (Figure 6). The increase was highly significant

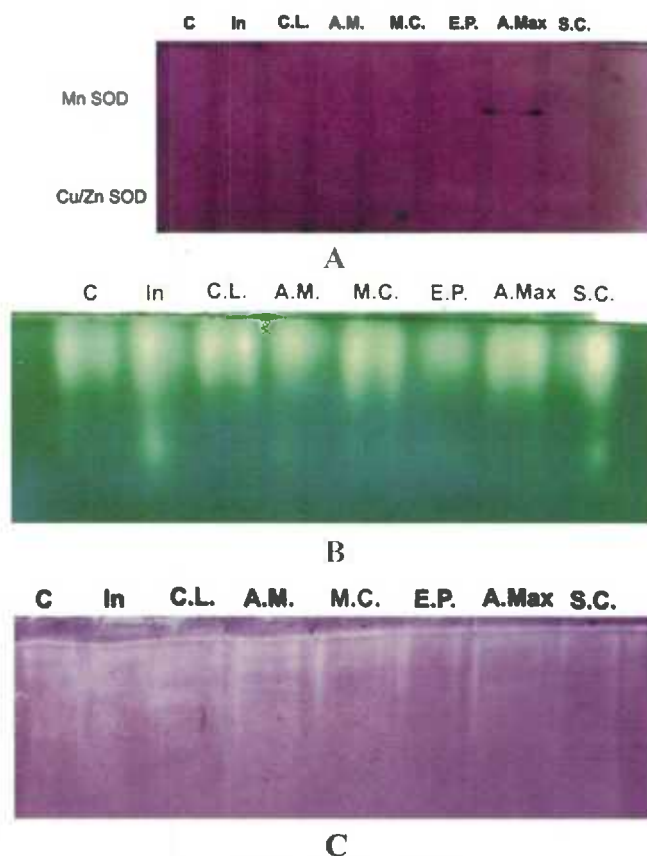


in BmNPV inoculated, *C. longa*, *A. marmelos*, *M. charantia* and *A. mexicana* treated groups. However, increase in activity was less significant in *E. geniculata* treatment.

Plants secondary chemicals can alter susceptibility of insect herbivores to naturally encountered pathogens (Cory and Hoover, 2006). Caterpillar mortality, for example, can differ by as much as 50-fold depending on the species of host plant upon which baculoviruses or *Bacillus thuringiensis* subsp. *kurstaki* (BtK) are consumed (Keating *et al.*, 1988; Duffey *et al.*, 1995; Hoover *et al.*, 1998; Farrar and Ridgeway, 2000; Kouassi *et al.*, 2001; Ali *et al.*, 2004). Phytochemicals can bind to or deactivate virus occlusion bodies in the larval midgut (Felton and Duffey, 1990), or reduce cell permissiveness to infection (Foster *et al.*, 1992; Ali *et al.*, 1998; Cory and Hoover, 2006). In the present experiment, ethanolic plant extract treated larval groups showed decreased mortality rate as compared with inoculated group (Figure 7). *A. marmelos* and *S. cumini* showed the lowest mortality as compared with other plants used. Fruit of *A. marmelos* contains marmelide which is very effective against viruses and is found to influence on the early stages of replicative cycle such



as adsorption, penetration *etc.* (Badam *et al.*, 2002) while seeds of *S. cumini* contains pentacyclic triterpenoid - friedelin, tannins and essential oil (terpenes, limonene and dipentene) (Bhatia and Bajaj, 1972; Jagetia *et al.*, 2002; Kokate *et al.*, 2002). *E. geniculata* treatment



recorded higher mortality than other plant extracts. From the present investigation, it is clear that different plant extracts have variable effects on BmNPV infections. The exact role of these extracts in BmNPV infection can be visualized only after isolation of the active compound.

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EFFETS D'EXTRAITS ETHANOL DE PLANTES SUR LE VER A SOIE *BOMBYX MORI* INOCULE AVEC BMNPV EN RELATION AVEC DES ENZYMES ANTIOXYDANTES, LA PHOSPHATASE ACIDE ET UNE ESTERASE NON SPECIFIQUE DE L'INTESTIN MOYEN

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RESUME

Des plantes contenant des métabolites secondaires tels que des alcaloïdes, des flavonoïdes et des triterpénoïdes sont habituellement utilisées contre les infections virales chez des animaux. Un petit nombre d'extraits éthanol de plantes contenant des alcaloïdes (*Aegle marmelos*, *Mormordica charantia* et *Argemone mexicana*), des triterpénoïdes (*Curcuma longa*, *Syzygium cumini*, *Mormordica charantia* et *Euphorbia geniculata*) et des flavonoïdes (*Syzygium cumini*) ont été testés ici contre l'infection de *Bombyx mori* par le virus de la polyèdrose nucléaire (BmNPV). La LC₅₀ de BmNPV (1x10⁴ corps d'inclusion polyédriques) entraîne une mortalité de 51.66 % chez les larves inoculées. Le traitement par des extraits éthanol de plantes contenant des métabolites secondaires antiviraux réduit la mortalité jusqu'à 8 à 21 % chez les larves inoculées avec BmNPV (dose LC₅₀). L'infection par le BmNPV cause la formation d'espèces réactives à l'oxygène (ROS) dans les tissus intestinaux qui entraînent la baisse de l'activité superoxyde dismutase (SOD) et augmentent l'activité catalase (CAT), ascorbate peroxydase (APOX), phosphatase acide (ACP) et esterase non spécifique (NSE) chez les larves au sixième jour d'infection. Le traitement des larves inoculées par le BmNPV à la dose de la LC₅₀ par des extraits éthanol de plantes augmente l'activité des enzymes SOD, APOX, ACP et NSE et baisse l'activité CAT au sixième jour de traitement. Nos résultats confirment l'activité antivirale des extraits éthanol de plantes contre la grasserie causée par le BmNPV de *Bombyx mori*.

Mots-clés: Ascorbate peroxydase, BmNPV, catalase, mortalité, extraits de plantes, superoxyde dismutase.



Research Paper

ZONATING LEAF SPOT CAUSED BY *GONATOPHRAGMIUM* SP. : A NEWLY REPORTED FOLIAR DISEASE OF MULBERRY IN THE PHILIPPINES

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ABSTRACT

This is the first report of a fungus under the genus *Gonatophragmium*, causing zonating leaf spot (ZLS) of mulberry in the Philippines. Disease symptoms consist of localized irregular, zonate, pale brown, large spots on the lamina of infected leaves. Fungal identification was based largely on Ellis' (1976) Dematiaceous Hyphomycetes keys and confirmed by the Department of Plant Pathology, University of the Philippines, Los Baños (DPP-UPLB). Pathogenicity test could develop typical symptoms of zonating leafspot disease when inoculated with a spray of newly prepared spore suspension consisting of conidia or ascospores on the lower leaf surface. Incubation period was about 17-19 days at 24-29 °C under greenhouse (open) condition. Field surveillance revealed susceptibility of 13 mulberry varieties to ZLS caused by *Gonatophragmium* sp. S54 and Batac were severely infected. On the otherhand, ZLS infection on CRR and K2 was moderately severe, while Papua, King and S36 revealed slightly severe condition. Interestingly, J-unlobed, C6, S61, Malba, Hakikkalu and S3 were occasionally infected with the disease whereas, Alfonso, Kosen, Mlocal, MR2, S13 and SRDC2 remained resistant and free from infection.

Key words: *Gonatophragmium* sp., mulberry, zonating leaf spot.

INTRODUCTION

Mulberry production is an integral part of silk industry. Since mulberry (*Morus* spp.) foliage is the only food of silk-producing insect (*Bombyx mori*), its quality is an important factor determining the grade of silk cocoons. But like any other high valued crop, mulberry is also prone to several diseases caused by fungi, bacteria, viruses and nematodes. Some of the serious foliar diseases are leaf rust, leaf spot, powdery mildew, twig blight and bacterial blight (Govindaiah and Gunasekhar, 1992).

Information generated on mulberry diseases are quite limited and the area still remain unexplored in Philippines. In the late nineties, Telan and Gonzales (1998) detected a disease with almost similar symptoms

as reported in this study. They described it as "*Culvularia* leaf spot of mulberry". The present study is a re-examination of this which served as a gateway to identify the causal organism of zonating leaf spot in mulberry observed in the Philippines.

MATERIALS AND METHODS

Infected leaves were collected from the experimental station of Sericulture Research and Development Institute (SRDI) and brought to the laboratory for examination. The disease symptoms were recorded. The fungal structures from young lesions were picked up carefully with a dissecting needle, transferred into a drop of plain lactophenol on a clean glass slide and covered with a cover slip. The morphology of the fungal structure was examined using a compound

microscope under oil immersion magnification and the measurements made using micrometer.

Conidia or ascospores were extracted from heavily infected mulberry leaves showing symptoms of ZLS and with the aid of an improvised sprayer, the spores were inoculated to the 3-month old mulberry saplings late in the afternoon. The saplings were observed for disease development.

A disease scale was formulated wherein the different grades of disease were assigned with scores to denote the percentage of leaf area infected. The scores represent classes of the different grades of infection so that disease severity could easily be differentiated.

A preliminary assessment of the disease was conducted under natural field condition during the season of peak incidence of ZLS at the SRDI experimental station, DMMMSU, Bacnotan, La Union. All existing mulberry varieties maintained in the germplasm plantation were covered under the assessment.

RESULTS AND DISCUSSION

Symptomatology: Localized, large and irregularly zonating circular brown spots on the surface of medium to mature leaves were commonly observed in the field (Figure 1a). The large spots sometimes coalesce and form shot holes. The margins of zonate spots are distinctly darker with brown shade. The fungal structure of the pathogen forms grayish brown, powdery to velvety patches under the surface of infected leaf tissue (Figure 1b). The same symptoms on plant parts were reported by Takahashi and Teramine (1986).

Pathogenicity test: In the field, disease occurrence and conidial production were recorded during August and noticed as very prevalent again in the months, October until January. Periodical examinations of the fallen leaves were carried out to determine the primary infection source of the fungus. Conidia of the fungus that were still profound under the surface of fallen leaves were used in inoculating healthy leaves of mulberry. Pathogenicity test showed typical symptoms of ZLS disease. This was displayed by the mulberry plants after

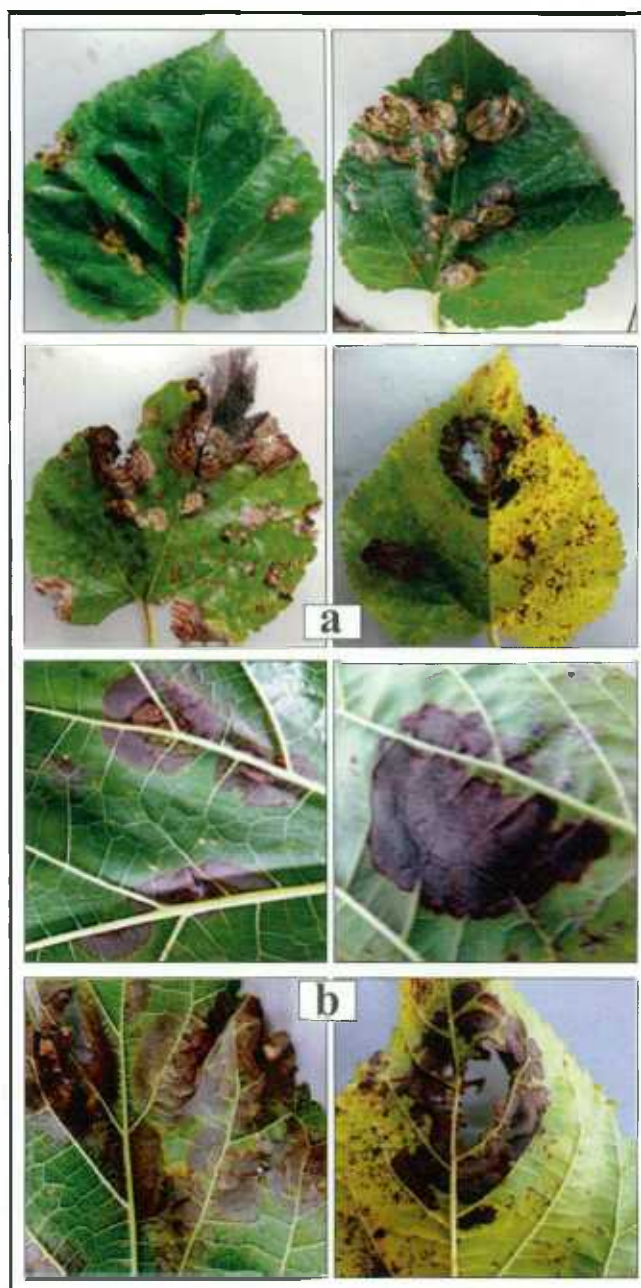


Figure 1: ZLS symptoms on mulberry leaf surface caused by *Gonatophragmium* sp.: (a) symptoms on surface; (b), symptoms and signs under surface

inoculation with the newly prepared spore suspension including conidia or ascospores when sprayed on the lower leaf surface. Incubation period was about 17-19 days at 24-29 °C under greenhouse (open) condition.

Morphology of the fungus: Conidia were borne on denticles which arise from swollen ends of the conidiophores branches (Figure 2 d). They were 0-3 septate, 18.6 µm long and 4.12 µm thick, straight or slightly

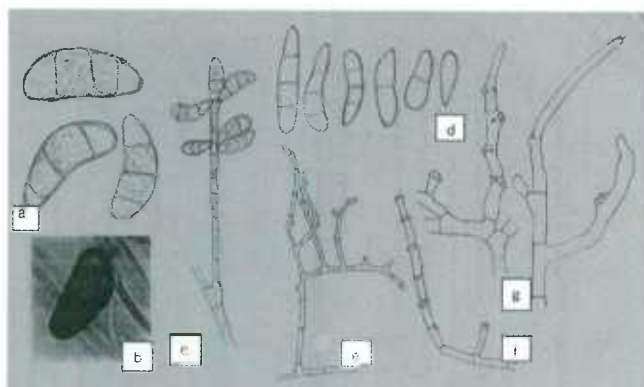


Figure 2: Fungal spp. extracted from infected mulberry leaf: (a) conidia of *Curvularia* spp., (b) conidium in semi-permanent mount (oil immersion magnification), (c) conidiophores with intact conidia of *Curvularia*, (d) conidia of *Gonatophragmium* spp., (e) hyphae, (f) conidiophores, (g) conidiogenous cell

curved, less cylindrical, sometimes clavate, often constricted near the middle, with protuberant hilum, very light olivaceous brown, dry and smooth. Mycelial hyphae were hypophyllous, effuse, brown, velvety and septated (Figure 2e). Conidiophores were macronematous and septated with sympodial type of branching and nodose with terminal and intercalary swellings (Figure 2f, g). Nodose swelling often proliferate as short lateral branches of 48.05 μm length and 4.32 μm thickness with swelling at the ends of the branches. Conidiogenous cells were denticulate, terminal and intercalary, polyblastic, acroauxic, holoblastic and mostly integrated, denticles varied, mostly not in pairs (Figure 2d).

Table 1: Comparative morphology of the two fungal species as studied by Telan (1998) and Gonzales (2003)

Fungal structure	Genus	
	<i>Curvularia</i> sp.	<i>Gonatophragmium</i> sp.
Conidium	Acropleurogenous, strongly curved, clavate, ellipsoidal or obovoid with 3 or more septa, pale to dark brown (Figure 2a, b), often the end cells are paler than the middle cells.	Borne on denticles which arise from swollen ends of the conidiophore branches (Figure 2g), 0-3 septate, 18.6 μm long and 4.12 μm thick, straight or slightly curved, less cylindrical, sometimes clavate, often constricted near the middle, with protuberant hilum, very light olivaceous brown, dry and smooth (Figure 2d).
Mycelial hyphae/ Conidiophore	Macronematous, straight, slightly nodose, brown and septate (Figure 2c)	Macronematous and septated, sympodial type of branching, nodose with terminal and intercalary swellings (Figure 2g), nodose swellings often proliferating as short lateral branches up to 48.05 μm long and 4.32 μm thick (Figure 2f), swelling at the ends of the branches, pale to olivaceous brown, effuse, brown, velvety and septated (Figure 2e).
Conidiogenous cell	Polytretic, sympodial, occasionally swollen, cicatrized (scars) (Figure 2c)	Denticulate, terminal and intercalary, polyblastic, holoblastic and mostly integrated, denticles vary, mostly not in pairs (Figure 2d).

Identification and characterization of fungi requires accurate description of fungal structures and skill in illustrating and describing them appropriately. Knowledge and correct identification of the causal organism is of vital importance to formulate effective management strategies.

Disease scale for assessment

Standardized key for the assessment of disease

severity of ZLS on mulberry caused by *Gonatophragmium* sp. is presented in Table 2.

Disease scale/key has been designed for easy and rapid scoring of zonate leaf spot on the leaves of mulberry in farmers' field, leaf yield loss assessment, resistance studies and other basic studies on chemical efficacy in the future. It is standardized to relate the intensity of disease incidence quantitatively (Figure 3).

Table 2: Field key for the assessment of disease severity of ZLS of mulberry

Grade/Rating	Description (% leaf area affected)
0	No infection
1	<1
2	1-5
3	6-10
4	11-25
5	26-50
6	51-75
7	>75

Varietal reaction to disease

Table 3 shows the varietal reactions of 19 mulberry varieties against ZLS caused by *Gonatophragmium* sp. Among the 19 varieties assessed, S54 and Batac were severely infected. Likewise, ZLS infection on CRR and K2 was moderately severe, while Papua, King and S36 were slightly severe, and J-unlobed, C6, S61, Malba, Hakikkalu and S3 were occasionally infected with the disease. Alfonso, Kosen, Mlocal, MR2, S13 and SRDC2 remained resistant and free from disease/infection.

Table 3: Varietal reaction of mulberry to ZLS

Mulberry variety	Reaction/Incidence
Batac	++++
S54	++++
CRR	+++
K2	+++
Papua	++
King	++
S36	++
J-Unlobed	+
C6	+
S61	+
M alba	+
Hakikkalu	+
S3	+
Kosen	-
M Local	-
Alfonso	-
MR2	-
S13	-
SRDC2	-

++++	Severe	+	Occasional
+++	Moderate	-	Nil
++	Slight		

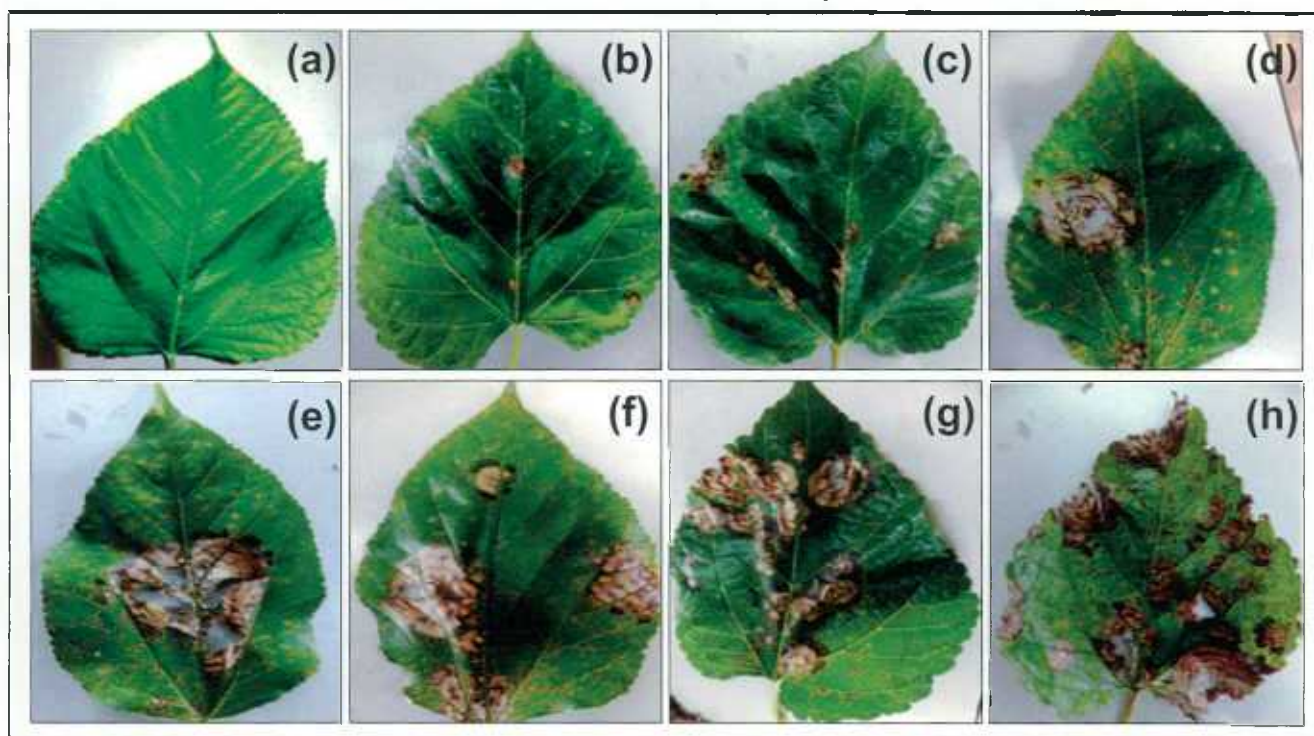


Figure 3. ZLS symptoms caused by *Gonatophragmium* fungus on mulberry leaves with different degrees of disease severity: Grades: (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, (f) 5, (g) 6, (h) 7.

CONCLUSION

Gonatophragmium genus was identified to be the causal organism of zonate brown leaf spot of mulberry. The fungus is a denticulate hyphomycete. Incubation period was about 17-19 days at 24-29 °C under greenhouse (semi-open) condition. Preliminary morphological characterization for fungal identification was based largely on Ellis' (1976) Dematiaceous and Hyphomycetes keys and confirmed by the Department of Plant Pathology, University of the Philippines Los Baños.

The disease assessment key formulated is fundamental to crop loss studies and many types of disease prediction and management systems. Further studies are necessary on epidemiological and plant breeding aspects and assessment of chemical efficacy.

Studies on etiology, characterization of the life cycle of the pathogen and disease management shall also be highly recommended in the context of addressing issues related to quality leaf production.

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LA TACHE ZONALE DE LA FEUILLE CAUSEE PAR GONATOPHRAGMIUM SP. : UNE NOUVELLE MALADIE FOLIAIRE DU MURIER OBSERVEE AUX PHILIPPINES

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RESUME

Ceci est la première observation aux Philippines d'un champignon du genre *Gonatophragmium*, responsable de la tache foliaire zonale (ZLS) chez le murier. Le symptôme de la maladie est la présence de larges taches irrégulièrement réparties, zonées et brun pâle sur la lamina des feuilles infectées. L'identification a été basée sur la flore des Hyphomycètes Dematiaceous de Ellis (1976) et confirmée par le Département de Pathologie des Plantes de l'Université des Philippines à Los Banos (PPD-UPLB). Le test de pathogénicité développe les symptômes de la maladie (ZLS) lorsqu'un spray de suspension fraîchement préparée de conidies ou d'ascospores de la surface foliaire inférieure est inoculé. La période d'incubation est de 17 à 19 jours à une température de 24 à 29°C sous serre (ouverte). La surveillance des plants permet de constater que 13 variétés de mûrier sont sensibles à la ZLS causée par *Gonatophragmium sp.* S54 et Batac sont sévèrement infectées. D'autre part, l'infection de la ZLS sur CRR et K2 est modérément sévère alors que l'infection chez Papua, King et S36 est peu sévère. De manière intéressante, J-unlobed, C6, S61, Malba, Hakikkalu et S3 sont occasionnellement infectées alors que Alfonso, Kosen, M local, MR2, S13 et SRDC2 sont résistantes et restent indemnes d'infection.

Mots-clés: *Gonatophragmium sp.*, mûrier, tache foliaire zonale.



Research Paper

PHYSIOLOGICAL AND BIO-CHEMICAL MARKERS ASSOCIATED WITH ROOT ROT RESISTANCE IN MULBERRY

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ABSTRACT

The sole purpose of mulberry (*Morus* spp.) cultivation is for rearing silkworms (*Bombyx mori* L.) to produce lustrous silk. Of late, due to the introduction of high yielding varieties, mulberry has become vulnerable to many species of phyto-pathogens affecting the quality and quantity of leaves. Until recently, only the foliar diseases such as leaf spot, leaf rust, powdery mildew and leaf blight were considered as serious contributing to the reduction of leaf yield by 10 - 12 % besides deteriorating the nutritional quality. But diseases of the root system pose more serious problems than foliar diseases during mulberry cultivation. Among soil borne diseases, root rot disease has become more alarming because of its epidemic nature and propensity to kill the plant completely. Root rot, which was earlier considered to be a minor disease in India, has become a serious one in recent years particularly in South India. The outbreak of the disease at field level is increasing day by day due to the complex nature of soil borne pathogens and associated edaphic factors. Though short term management technologies have been evolved, a permanent solution to this problem is to evolve resistant mulberry varieties. As an initial step in disease resistance breeding, a study was taken up to identify dependable morpho-physiological and biochemical markers associated with the infection of pathogens to screen the mulberry germplasm genotypes against root rot disease and identify the tolerant and susceptible genotypes for disease resistance breeding. Based on the results, leaf temperature, phenol content and total carbohydrates were identified as physiological and biochemical markers for screening of mulberry genotypes for root rot resistance.

Key words: Biochemical markers, germplasm, *Morus* spp., physiological markers, root rot resistance.

INTRODUCTION

The evolution of high yielding mulberry varieties by different research institutes in India has resulted in maximizing the production of quality mulberry leaves to the tune of 50–60 tonnes/ha/yr. This in turn has increased the cocoon productivity and reduced the leaf-cocoon ratio in southern states of India (Dandin *et al.*, 2003).

After the introduction of high yielding varieties with wide genetic diversity followed by intensive cultivation practices, of late, mulberry has become vulnerable to many species of phyto-pathogens. Mulberry disease is a manifestation of the complex interaction of host,

pathogen and environment and is a major limiting factor for mulberry cultivation (Sastry, 1984). The diseases of mulberry, which are prevalent under the different agro-climatic conditions of South India, drastically affect the quality and quantity of leaves causing severe loss in leaf yield (Philip *et al.*, 1997). Mulberry being a perennial crop, the pathogens seem to perpetuate easily and spread quickly to cover extensive areas (Rangaswami *et al.*, 1976).

Until recently, only the foliar diseases such as leaf spot, leaf rust, powdery mildew and leaf blight were considered as serious contributing to the reduction of

leaf production by 10 - 12 % besides deteriorating the nutritional quality of the leaf (Philip *et al.*, 1997). But diseases of the root system pose more serious problems to mulberry cultivation than foliar diseases. Root knot, root rot and nursery diseases affect the crop to a greater extent. Root knot caused by a nematode *Meloidogyne incognita*, reduces the leaf yield by about 12 % (Govindaiah *et al.*, 1991). It was estimated that about 30 - 35 % loss of cuttings occur due to nursery diseases (Gupta *et al.*, 1997). The management of soil borne diseases in mulberry is more difficult compared to that of foliar diseases because of the complex nature of soil. Among all soilborne diseases, root rot was reported to be causing extensive damage to mulberry plant. This disease has become more alarming because of its epidemic nature and propensity to kill the plant completely. White and Violet root rot diseases caused by *Helicobasidium mompa* and *Rosellinia necatrix*, respectively, created a major problem earlier for mulberry cultivation in Japan, China, Russia and Thailand (Aoki and Matsu, 1959; Kakuliya and Gogeliya, 1970; Aoki, 1971). *Armillaria mellea* causing mulberry root rot is also prevalent in Japan from June to October (Takahashi, 1975). Root rot, which was earlier considered to be a minor disease in India, has become a serious one in recent years particularly in South India. *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina* and *Botryodiplodia theobromae* were reported to cause the root rot disease in mulberry in different parts of India (Philip *et al.*, 1995; Radhakrishnan *et al.*, 1995). Preliminary studies indicated 30 % mortality of plants and about 14 % reduction in leaf yield by wilting, defoliation and drying of plants due to root rot disease in mulberry (Philip *et al.*, 1995). The outbreak of the disease at field level is increasing day by day due to the complex nature of soil borne pathogens and associated edaphic factors (Chowdary and Reddy, 2004). Though short term management technologies have been evolved, a permanent solution to this problem is to evolve root rot resistant varieties.

As an initial step to disease resistance breeding, a study was taken up to screen the germplasm genotypes of mulberry for morpho-physiological and bio-chemical

markers associated with the infection of root rot pathogens for identification of dependable markers and shortlist the tolerant and susceptible genotypes for disease resistance breeding.

MATERIALS AND METHODS

Plant material

Initially, 150 accessions of mulberry germplasm were screened and 40 genotypes consisting of 8 exotic (Xuan-10, Mizusawa, Thai Peach, Thai Beelad, Thai ♂, Philippines, Hungarian and *Morus multicaulis*) and 32 indigenous (Local ♂, K-2, S-30, S-36, S-54, S-13, S-34, MR-2, V-1, RC-1, RC-2, V-4, G-2, G-4, RFS-135, RFS-175, AR-12, Cuckpilla, Ber.S1, BR-2, BR-4, Ber.C763, Ber.C 776, Ber.S-799, S-1635, BC-259, Ber.-20, LF-2, Sujampur-5, Himachal Local, Almora Local and Punjab Local) were shortlisted. The saplings were raised in nursery beds by adopting recommended package of practices.

Root rot pathogens

The purified isolates of *F. solani*, *F. oxysporum*, *M. phaseolina* and *B. theobromae* were mass multiplied on split maize sand medium as recommended by Monga and Raj (1994). The split maize sand medium was prepared and filled up to 3/4th in 1000 ml conical flasks and autoclaved. The isolates were inoculated @ 5 cm² on maize sand meal medium and incubated at 30 ± 1°C for 15 days. Healthy saplings were transplanted to earthen pots after artificially inoculating the soil with root rot pathogens viz., *F. solani*, *F. oxysporum*, *B. theobromae* and *M. phaseolina* at 5 % inoculum level having a spore load of 2.9 × 10⁷/g soil. The soil without inoculum served as control. Five replications were maintained for each treatment.

Disease scoring

The intensity/severity of root rot disease was estimated based on foliar and root infection (%). The foliar/root infection percentage was estimated by using the following formulae.

$$\text{Foliar infection (\%)} = \frac{\text{No. of wilted leaves}}{\text{Total no. of leaves}} \times 100$$

$$\text{Root infection (\%)} = \frac{W1 - W2}{W1} \times 100$$

where, W 1 = weight of whole root mass

W 2 = weight of healthy root mass

Further, based on the foliar/root damage, the mulberry genotypes were categorized as having different levels of disease indices following Gray and Achenbach (1996). This system was adopted with slight modifications (Sharma and Gupta, 2005) by using the following five-point scale and assessing the status of the disease severity.

Disease Index	Foliar infection	Root infection	Severity
1	No wilting	No rotting	Disease free (Healthy)
2	0.1 to 25 % wilting	0.1 to 25 % rotting	Mild
3	25.1 – 50 % wilting	25.1 – 50 % rotting	Moderate
4	50.1 – 75 % wilting	50.1 – 75 % rotting	Severe
5	More than 75.1 % wilting	More than 75.1 % rotting	Very severe

Survival of pathogen in the soil

The pathogen population in the soil was estimated at 15 days interval after artificial inoculation by serial dilution plate technique (Waksman and Fred, 1922) using Potato Dextrose Agar (PDA) medium and expressed as Colony Forming Units (CFU)/g soil. Data at 15 days interval were collected.

Morpho-physiological parameters

Data on morpho-physiological parameters were recorded sixty days after inoculation of the pathogens in both healthy and inoculated plants.

Average plant height (cm) and total number of leaves, leaf yield (g), shoot yield (g) and fresh biomass yield (g) were recorded.

SPAD chlorophyll meter was standardized with an ambient sunlight condition in the field. After standardization of the instrument, chlorophyll content was measured in terms of SPAD units by hand operation.

Index leaves (5th leaf from the tip) were used for measuring the SPAD readings.

Data on transpiration rate and leaf temperature were recorded in both artificially inoculated and healthy control plants using a portable steady state porometer (LICOR instruments; Model 1600, USA) as per the recommended procedure (Rajagopal *et al.*, 1990). The gas exchange parameters were recorded between 10 a.m. and 12 noon under natural photoperiodic conditions.

Biochemical parameters

Leaf samples were collected randomly from pot cultures of each genotype after sixty days of pathogen inoculation. Healthy leaf samples were also collected from each genotype. The samples were dried at room temperature for three days. The dried leaf samples were kept in an oven at 65 to 70 °C till constant weight was obtained and powdered using a grinder. The leaf powder thus obtained was used for studying various biochemical parameters.

The standard protocols were adopted for estimating different parameters *viz.*, crude proteins (Lowry *et al.*, 1951), total carbohydrates (Shirlaw, 1967), total phenol (Bary and Thorpe, 1954) and micronutrients (Gupta, 1993).

RESULTS AND DISCUSSION

The gradual increase of pathogen population in soils recorded is presented in Table 1. The gradual increase may be attributed to the time lag required for the acclimatization and establishment of the pathogen population in the soil amongst the competitors due to the

Table 1: Survival of root rot pathogens in soil

Days after inoculation	Population of pathogens (CFU x 10 ⁷ / g soil)*
1	2.9
15	3.1
30	3.1
45	3.2
60	3.2
75	3.2

* Mean of three replications. CD (P ≤ 0.05) - 0.51



Foliar infection



Root infection

Figure 1: Wilting of mulberry sapling due to root rot infection

soil microbiological complexity. On 75th day of inoculation of root rot pathogens (2.9×10^7 /g soil), the above and below ground level symptoms were observed as fully wilted foliage and rotten roots (Figure 1).

Table 2: Response of mulberry genotypes to root rot disease under artificial inoculated conditions

Sl.No.	Variety / Genotype	Sex	Infection (%)	Severity	Disease index
A. Exotic					
1.	Xuan-10	♀	75.25	Very severe	5
2.	Mizusawa	♀	76.84	Very severe	5
3.	Thai Peach	♀	62.15	Severe	4
4.	Thai Beelad	♀	72.35	Severe	4
5.	Thai male	♂	76.13	Very severe	5
6.	Philippines	♂	64.73	Severe	4
7.	Hungarian	♀	12.76	Mild	2
8.	<i>Morus multicaulis</i>	♀	78.23	Very severe	5
B. Indigenous					
1.	Local male	♂	17.25	Mild	2
2.	K-2	♀	76.15	Very severe	5
3.	S-30	♀	87.45	Very severe	5
4.	S-36	♀	54.40	Severe	4
5.	S-54	♀	81.63	Very severe	5
6.	S-13	♂	18.45	Mild	2
7.	S-34	♂	52.64	Severe	4
8.	MR-2	♀	26.25	Moderate	3
9.	V-1	♂	83.52	Very severe	5
10.	RC-1	♀	52.75	Severe	4
11.	RC-2	♀	55.23	Severe	4
12.	V-4	♀	32.65	Moderate	3
13.	G-2	♀	58.43	Severe	4
14.	G-4	♀	61.25	Severe	4

Cont'd

S. No.	Variety / Genotype	Sex	Infection	Severity	Disease index
15.	RFS-135	♂	34.25	Moderate	3
16.	RFS-175	♂	24.95	Mild	2
17.	AR-12	Triploid	45.32	Moderate	3
18.	Cuckpilla	Triploid	20.56	Mild	2
19.	Ber-S1	♂	52.75	Severe	4
20.	BR-2	♀	54.36	Severe	4
21.	BR-4	♀	42.65	Moderate	3
22.	Ber-C763	♂	65.72	Severe	4
23.	Ber-C776	♂	88.65	Very severe	5
24.	Ber-S799	♂	52.65	Severe	4
25.	S-1635	Triploid	45.25	Moderate	3
26.	BC-259	♀	36.69	Moderate	3
27.	Ber-20	♀	16.23	Mild	2
28.	LF-2	♂	24.86	Mild	2
29.	Sujanpur-5	♀	85.75	Very severe	5
30.	Himachal Local	♀	15.42	Mild	2
31.	Almora Local	♂	16.85	Mild	2
32.	Punjab Local	♀	24.15	Mild	2

Among the exotics, the maximum infection percentage was recorded in *M. multicaulis* (78.23 %) and minimum in genotype Hungarian (12.76 %). Among the indigenous accessions, the maximum infection

was recorded in Ber. C 776 (88.65 %) and minimum of 15.42 % in Himachal Local (Table 2).

The variations in morpho - physiological parameters is presented in Table 3. Pooled data of three trials

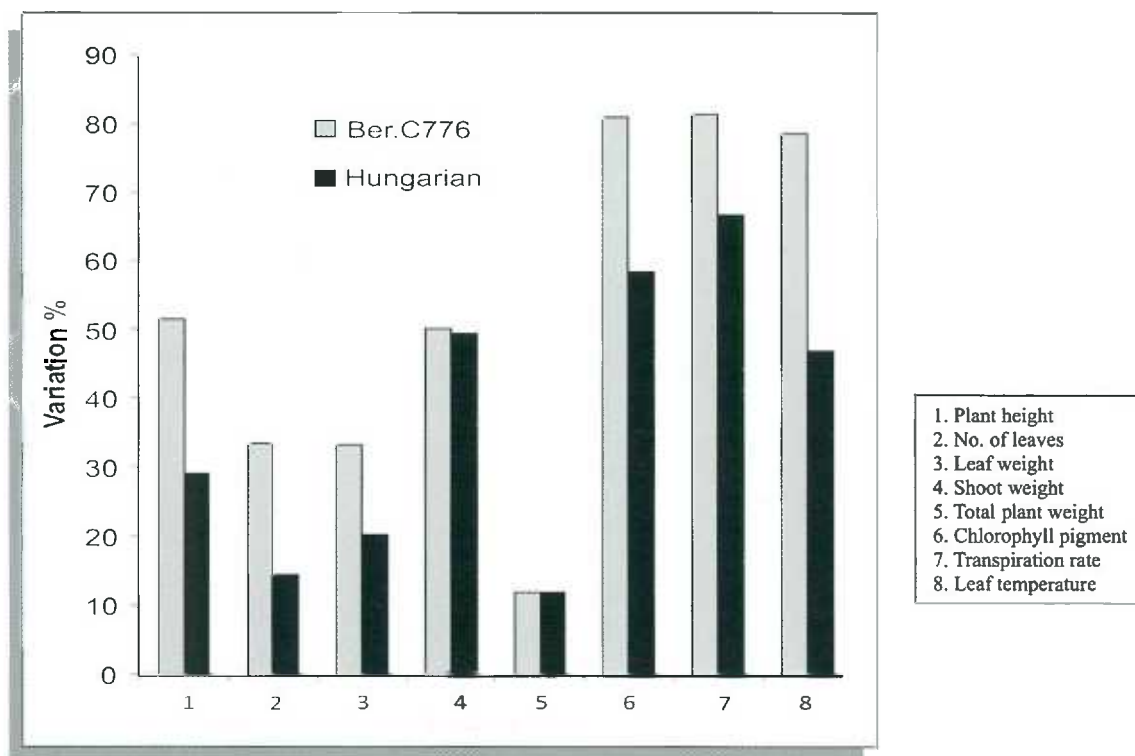


Figure 2 : Root rot induced morpho-physiological variations in susceptible (Ber.C776) and resistant (Hungarian) mulberry varieties

Table 3: Variation in morpho-physiological parameters among mulberry genotypes under healthy and root rot inoculated conditions

Parameter	Control	Inoculated	% decrease / increase over control	't' test
Plant height (cm)	22.0 - 56.0	10.7 - 39.8	28.93 - 51.45	**
Number of leaves	8 - 18	7 - 12	14 - 33	**
Leaf weight (g)	5.0 - 125.0	2.7 - 26.7	46.80 - 78.67	**
Shoot weight (g)	5 - 75	1.7 - 14.0	66.0 - 81.33	**
Total plant weight (g)	20.0 - 200	8.3 - 38.3	58.35 - 80.83	**
Chlorophyll pigment (SPAD values)	27.5 - 37.1	18.4 - 32.0	20.0 - 33.1	**
Transpiration rate (g cm ⁻² S ⁻¹)	93.2 - 109.3	46.6 - 55.4	49.31 - 49.99	**
Leaf temperature(°C)	29.1 - 31.0	32.5 - 34.7	11.68 - 11.84	**

** Significant at 1 %.

indicated that there was a reduction of 28.9 – 51.5 % in plant height, 14 - 33 % in number of leaves, 58.4 - 80.8 % in total plant weight, 66.0 – 81.3 % in shoot weight and 46.8 – 78.7 % in leaf weight. The variation percentage in morpho-physiological parameters between the two most contrasting, susceptible (Ber. C - 776) and resistant (Hungarian) genotypes is presented in Figure 2.

The genotypes studied exhibited a wide variation in different physio-biochemical parameters recorded. There was a reduction in chlorophyll pigment content (20.0 – 33.1 %), transpiration rate (49.3 – 50.0 %) and an increase in leaf temperature (11.7 – 11.8 %). There was a significant reduction in total chlorophyll content of leaves in diseased plants. Variation in chlorophyll content in different varieties of mulberry in response to foliar pathogens has been reported by various workers (Vidyasagar and Kotresha, 2003).

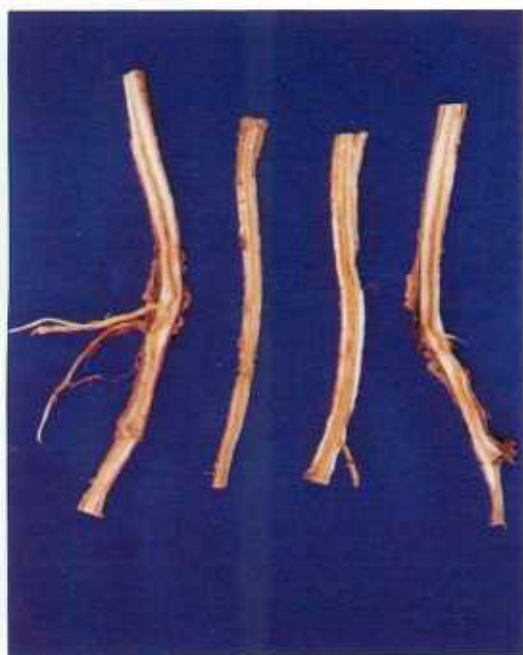
The variations recorded in biochemical parameters is presented in Table 4. In biochemical parameters, there was increase in phenol content (tannins) in inoculated plants (21.1 to 26.7 %) compared to that of healthy plants. There was decrease in the content of other biochemical parameters viz., crude protein (15.9 to 27.4 %), total carbohydrates (28.7 to 54.4 %), zinc (16.2 to 56.1%), iron (23.4 to 52.1 %) and calcium (21.7 to 45.1 %) in inoculated plants compared to that of healthy plants.

Total protein content of leaves of infected plants showed a significant decrease in comparison to that of leaves harvested from healthy plants. Chattopadhyay and Bhattacharjya (1968) reported reduced nitrogen content in *F. solani* and *M. phaseolina* infected shoots and roots of guava plants. In mulberry, several workers have reported the reduction in protein content of leaves due to foliar pathogens (Vidyasagar and Kotresha, 2003).

Table 4: Variation in biochemical parameters among mulberry genotypes under healthy and root rot inoculated conditions

Parameter	Control	Inoculated	% decrease / increase over control	't' test
Crude protein (%)	14.84 - 26.98	12.48 - 19.60	15.90 - 27.35 (21.62)	*
Total carbohydrates (%)	14.14 - 25.94	10.08 - 11.84	28.71 - 54.36 (41.53)	**
Zinc (ppm)	22.60 - 47.80	18.95 - 20.98	16.15 - 56.11 (36.13)	*
Iron (ppm)	220.50 - 519.86	168.95 - 248.88	23.37 - 52.12 (37.74)	*
Calcium (%)	0.92 - 1.44	0.72 - 0.79	21.74 - 45.14 (33.44)	*
Total phenols (mg/g)	3.13 - 5.98	3.79 - 7.58	21.09 - 26.75 (23.92)	*

* Significant at 5 %, ** Significant at 1 %; (Figures in parentheses indicate the average % of decrease / increase.



Longitudinal sections



Transverse sections

Figure 3: Rot infected mulberry roots exhibiting vascular browning

Total carbohydrate content decreased significantly in the leaves of the infected plants indicating the involvement of root rot pathogens in the biochemical pathways of carbohydrate metabolism in mulberry. Invasion of the phloem eventually inhibited the translocation of carbohydrates towards root tip (Ayes, 1986). Plant pathogens interfering with biochemical pathways in plants might be the cause for significant reduction in total carbohydrate content of leaves due to infection.

Vidyasagar and Kotresha (2003) also reported reduction in total carbohydrates in mulberry leaves of various varieties infected with *Phyllactinia corylea*. The total phenol content of the diseased plants showed a significant increase and resulted in vascular discoloration in the infected root tissues (Figure 3). Vidhyasekaran *et al.* (1992) have reported that under severe disease conditions, toxins produced by the pathogen may suppress the phenolic metabolism of plants. Secondary phenolic compounds or phytoalexins are low molecular weight anti microbial compounds that accumulate in plant cells in response to pathogen infection (Kuc, 1995). The accumulation of

phenol is known to be associated with biochemical disease resistance.

There was reduction in zinc (Zn), iron (Fe) and calcium (Ca) contents in the leaves of diseased plants. Shree *et al.* (2005) have reported varietal variation in decrease/ increase of Zn in mulberry leaves due to leaf rust disease and decrease in Ca content in some exotic mulberry varieties and increase in indigenous varieties due to leaf rust disease. The decrease in Zn, Fe and Ca contents in leaves of infected plants may be due to the interference in the biochemical pathways of host due to fungal parasitism resulting in the reduced physiological activity of roots under biotic stress leading to interference in translocation of nutrients to the leaves through xylem.

Based on the results, three parameters *viz.*, leaf temperature, phenol content and total carbohydrates have been identified as physiological and biochemical indicators for screening of mulberry genotypes for root rot resistance. Further, 5 tolerant (Hungarian, Himachal local, Ber. 20, Almora local & Local male) and 5 susceptible (Ber. C 776, S 30, Sujampur 5, V 1 & S 54) mulberry genotypes have been identified, which will be utilized as parents for root rot resistance breeding.

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MARQUEURS PHYSIOLOGIQUES ET BIOCHIMIQUES ASSOCIES A LA RESISTANCE A LA ROUILLE DE LA RACINE CHEZ LE MÛRIER

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RESUME

Le seul objet de la culture du mûrier est l'élevage des vers à soie (*Bombyx mori* L.) pour produire de la soie brillante. En raison de l'introduction de variétés à haut rendement, le mûrier est devenu vulnérable à de nombreuses espèces de phytopathogènes qui affectent la quantité et la qualité des feuilles. Jusqu'à récemment, seules les maladies foliaires telles que la tache de la feuille, la rouille foliaire, le mildiou poudreux et le charbon ont été considérées comme sérieuses entraînant une réduction du rendement de 10 à 12 % tout en détériorant la qualité nutritionnelle des feuilles. Mais les maladies du système racinaire pose des problèmes plus sérieux que les maladies foliaires à la culture du mûrier. Parmi les maladies liées au sol, la rouille de la racine est devenue plus alarmante en raison de sa nature épidémique et sa propension à tuer les plants. La rouille de la racine qui a été considérée comme une maladie mineure en Inde, est devenue une maladie sérieuse dans les années récentes particulièrement en Inde du sud. L'impact de la maladie dans les plantations s'accroît de jours en jours en raison de la nature complexe des pathogènes du sol et des facteurs édaphiques associés. Bien que des technologies de management à court terme aient été élaborées, une solution permanente à ce problème est d'obtenir des variétés de mûrier résistantes. Comme étape initiale à la reproduction de la résistance à la maladie, une étude a été entreprise pour identifier des marqueurs morpho-physiologiques et biochimiques associés à l'infection de pathogènes afin de cribler les génotypes de mûrier contre la maladie de la rouille de la racine et d'identifier les génotypes sensibles et résistants pour la reproduction.

Mots-clés: Marqueurs biochimiques, germoplasmes, *Morus* spp., marqueurs physiologiques, résistance à la rouille de la racine..



Research Paper

VARIATION IN BIOCHEMICAL COMPOSITION OF DIFFERENT HOST PLANTS OF MUGA SILKWORM, *ANTHRAEA ASSAMENSIS* HELFER

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ABSTRACT

Four different host plants of muga silkworm, *Antheraea assamensis*, viz., Som (*Persea bombycina*), Soalu (*Litsea polyantha*), Diglotti (*L. salicifolia*) and Mejankari (*L. citrata*), were evaluated for leaf biochemical constituents during different seasons. For all the seventeen nutrient constituents analysed from the leaves as a whole, Som was superior over other host plants irrespective of season and type of leaves, followed by Soalu. Tender leaves of Som possessed significantly the highest amounts of total mineral, crude protein, reducing sugar, TSS, β -sitosterol, total phenol, ascorbic acid, chlorogenic acid and tannins. Soalu tender leaves were found to contain significantly the highest amounts of moisture, soluble protein and lignin content, whereas, crude fibre, chlorophylls and ADF contents were at par in both Som and Soalu leaves.

Key words: *Antheraea assamensis*, Diglotti, host plants, Mejankari, muga silkworm, Soalu, Som.

INTRODUCTION

Antheraea assamensis Helfer is a polyphagous insect and feeds on a wide range of host plants. While 'Som' (*Persea bombycina*) and 'Soalu' (*Litsea polyantha*) are considered as the primary food plants, there are a number of other food plants such as *Actinodaphnae obovata*, *A. anquistifolia*, *Celastrus monosperma*, *Cinnamomum glaucescens*, *C. glanduliferum*, *Gmelina arborea*, *L. citrata*, *L. salicifolia*, *Magnolia sphenocarpa*, *Michelia champaca*, *Xanthozylum rehsta*, etc. Muga silkworm is multivoltine in nature, i.e., it can be done 5-6 times in a year. Muga silkworms reared during autumn (October – November) and spring seasons (April-May) which are termed as 'Kotia' and 'Jethua' crops, respectively in Assamese, produce cocoons with heavier shell (0.5-0.6 g) compared to silkworms reared during other seasons (0.3-0.4 g). The cocoons produced during these two seasons are used for producing yarn commercially and

hence, known as commercial crops. 'Kotia' commercial crop rearing is preceded by one rearing during June-July which is called 'Aherua' pre-seed crop and the subsequent rearing during August-September is called 'Bhodia' seed crop. Similarly, 'Jethua' commercial crop rearing is preceded by 'Jarua' (December-January) pre-seed crop rearing followed by 'Chatua' seed crop rearing. The pre-seed and seed crops of muga usually fall under adverse seasons of extreme summer and winter and the productivity sometimes slashes down to 10-20 %.

Over the last six decades, production of muga raw silk in the region has been fluctuating (24 -127 MT) with a gradual increasing trend. Muga silk production over the years has been hovering due to certain inherent problems associated with the culture. Non-availability of the required quantity of quality muga silkworm eggs (seeds) during commercial seasons has been identified as a major constraint of the industry.

Rearing of muga silkworms is done outdoor allowing them to feed on leaves of standing host plants, till they mature and then later collected by rearers to form cocoons inside cocooning halls. Thus the survivability of worms as well as harvesting of ripened worms depends on several factors, out of which host plant has a major role. The relative contribution of such factors responsible for a successful crop harvest has been estimated as: host plant: 38.2 %, climate: 37.0 %, rearing technique: 9.3 %, silkworm race: 4.2 %, silkworm egg: 3.1 % and other factors: 8.2 % (Choudhury, 1992).

Growth and development of silkworms and the cocoon crop yield are considerably influenced by the nutritive value of leaf as feed. The importance of good nutrition in mulberry silkworm rearing has been widely recognized (Takeuchi, 1960; Parpiev, 1968; Krishnaswami *et al.*, 1970; Fonseca *et al.*, 1993; Sarkar *et al.*, 1997). Hence, several attempts have been made to assess the nutritional potential of popular mulberry varieties by bioassay (Bongale and Chahuvachari, 1995). Hazarika *et al.* (1995) studied the association of morphological and biochemical characters of Som (*Machilus=Persea bombycina*) with the feeding behaviour of muga silkworm. Chakravorty *et al.* (2004) studied the preferential feeding and moulting behaviour (up to 2nd instar) of muga silkworm on different food plants.

Literature on the biochemical composition of the host plants of muga silkworm, *A. assamensis*, is very meager. In the present investigation, composition of primary as well as secondary metabolites of four host plants *viz.*, Som (*Persea bombycina*), Soalu (*Litsea polyantha*), Diglotti (*L. salicifolia*) and Mejankari (*L. citrata*) was estimated to know the nutritional status of the leaves at different maturity levels.

MATERIALS AND METHODS

1. Host plants of muga silkworm

Four host plants of muga silkworm *viz.*, Som (*P. bombycina*), Soalu (*L. polyantha*), Diglotti (*L. salicifolia*) and Mejankari (*L. citrata*) were chosen for the experiments. Tender (1-2 months old), semi-mature (3-4 months old) and mature (more than 4 months old)

leaves from all the four host plants were harvested during spring and autumn seasons for analysis of the biochemical constituents. Constituents such as moisture, chlorophylls, phenols, ascorbic acid and chlorogenic acid were estimated on fresh weight basis whereas the others were assessed on dry weight basis following standard procedures.

2. Biochemical estimation

2.1. Preparation of leaf samples

Leaf samples of three different types (tender, medium and mature) were collected separately from the host plants, properly cleaned and then dried in hot air oven at a temperature of 80-90 °C for several hours till dried completely. The dried leaves were powdered using an electric mixer grinder. The powdered leaf samples were kept separately in polypropylene containers which were subsequently used for analysis.

2.2. Methods of biochemical estimations

Total nitrogen from the powdered samples was estimated by the method of Vogel (1978) and Crude protein was estimated by multiplying the estimated value of the total nitrogen by 6.25; total phenol was determined in fresh leaves by the method of Malik and Singh (1980). Moisture and crude fibre were estimated following the method of A.O.A.C (1970). Anthrone method (Yem and Willis, 1954) was followed to estimate reducing sugar and total carbohydrate while starch content in the leaf samples was estimated by the method described by Chopra and Konwar (1976). From the fresh leaf samples, Chlorophylls a & b contents were estimated by the colorimetric method described by Arnon (1949) and ascorbic acid, by the method of Sadasivam and Manickam (2005). β -sitosterol content in the leaves of the muga silkworm host plants was estimated following the method of Katayama *et al.* (1974). Phytic acid and chlorogenic acid contents were estimated following the methods of Wheeler and Ferrel (1971) and Michael *et al.* (1978), respectively. The Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL) contents in the leaf samples were estimated by the A.O.A.C. method (1975). The Folin-Denis method described by Schanderl (1970) was adopted to estimate the amount of tannin.

2.3. Statistical analysis

Data recorded during the course of investigation were statistically analyzed using "Analysis of Variance" technique described by Snedechor and Cochran (1967). The significance of differences was calculated by 'F' test.

RESULTS AND DISCUSSION

Biochemical analysis of leaves of different host plants

1. Moisture, total mineral and crude fibre

The moisture, total mineral and crude fibre content of leaves of different host plants of muga silkworm according to type of leaves in two different seasons are presented in Table 1. It is observed that moisture content decreased significantly with the maturity of leaves. Regardless of leaf type, moisture content differed significantly ($P < 0.05$) in respect of host plant and season. Leaves of Soalu possessed the highest amount of moisture content: 77.11 % in tender leaves, 65.97 % in semi-mature leaves and 63.01 % in mature leaves.

The total mineral content varied significantly depending on leaf type, season and host plants. An increasing trend of mineral content was observed with the advancement of leaf age. Som leaves possessed significantly highest mineral content in all types of leaves, being 5.52 % in tender leaves, 11.47 % in semi-mature and 16.10 % in mature leaves. In tender and semi-mature

leaves, significantly lowest amount of mineral content was found in Mejankari, being 3.85 % and 6.40 %, respectively, whereas, in the case of mature leaves, it was recorded in the leaves of Diglotti (8.53 %).

Data on crude fibre content in the leaves of different host plants showed that it increased significantly with the advancement of leaf age irrespective of host plant and season. Tender and semi-mature leaves of Som and Soalu possessed significantly highest crude fibre content, whereas its content in mature leaves of Som, Soalu and Diglotti were at par, the lowest being in Mejankari leaves (21.12 %).

2. Crude protein and soluble protein

Figure 1 represents the crude protein and soluble protein contents according to leaf type of different host plants in two different seasons. In the case of crude

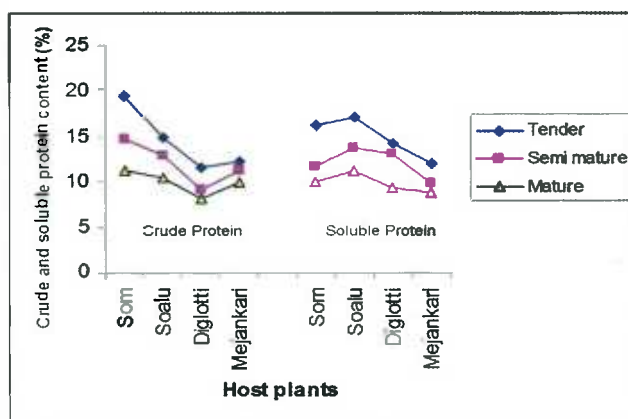


Figure 1: Crude and soluble protein content (%) in the leaves of different host plants at different maturity levels

Table 1: Moisture, total mineral and crude fibre content in the leaves of different host plants at different maturity levels

Host plant	Moisture (%)			Total mineral (%)			Crude fibre (%)		
	Tender	Semi - mature	Mature	Tender	Semi - mature	Mature	Tender	Semi - mature	Mature
Som	74.94b	65.69a	61.87b	5.52a	11.47a	16.10a	6.04a	10.01a	23.42a
Soalu	77.11a	65.97a	63.01a	5.19b	10.13b	13.40b	6.29a	9.36a	23.30a
Diglotti	72.21c	64.97ab	61.24b	4.45c	7.33c	8.53d	5.47b	6.99b	23.45a
Mejankari	63.87d	63.43bc	61.63b	3.85d	6.40d	11.26c	4.93c	6.81b	21.12b
Host plant	S.Ed. (±)	0.69			0.11			0.24	
	CD _{0.05}	1.38			0.23			0.49	
Season	S.Ed. (±)	0.49			0.08			0.17	
	CD _{0.05}	0.98			0.16			0.35	

Figures followed by common alphabets do not differ significantly.

Table 2: Reducing sugar, total soluble sugar and starch content in the leaves of different host plants at different maturity levels

Host plant	Reducing sugar (mg/g)			Total soluble sugar (%)			Starch (%)		
	Tender	Semi - mature	Mature	Tender	Semi - mature	Mature	Tender	Semi - mature	Mature
Som	2.92a	2.56a	2.51a	8.17a	6.72a	5.74a	6.27c	7.02c	8.30d
Soalu	2.50b	2.29b	1.65b	5.27c	4.71c	4.25c	8.55a	9.78a	12.16a
Diglotti	2.14c	1.64c	1.11c	5.17c	4.15d	3.47d	7.69b	9.56a	11.58b
Mejankari	1.94d	1.57c	0.51d	6.58b	5.69b	4.72b	7.56b	7.93b	10.07c
Host plant	S.Ed. ($\pm$)		0.07		0.10			0.11	
	CD _{0.05}		0.13		0.19			0.23	
Season	S.Ed. ($\pm$)		0.05		0.07			0.08	
	CD _{0.05}		0.10		0.14			0.16	

Figures followed by common alphabets do not differ significantly.

protein, Som leaves were superior among all the host plants irrespective of leaf maturity and season. Its content was recorded as 19.46 % in tender, 14.71 % in semi-mature and 11.12 % in mature leaves of Som. Significantly lowest crude protein content was recorded in Diglotti leaves: 11.55 % in tender, 8.97 % in semi-mature and 8.12% in mature leaves.

Unlike crude protein, Soalu leaves were superior in respect of soluble protein among all the host plants irrespective of leaf maturity and season. Its content was recorded as 17.17 mg/g in tender, 13.67 mg/g in semi-mature and 11.19 mg/g in mature leaves of Soalu. Significantly lowest soluble protein content was recorded in Mejankari leaves: 12.13 mg/g in tender, 9.93 mg/g in semi-mature and 8.86 mg/g in mature leaves.

3. Reducing sugar, total soluble sugar and starch

Reducing sugar, total soluble sugar (TSS) and starch content in the tender, semi-mature and mature leaves of different host plants in spring and autumn seasons are presented in Table 2. At all maturity levels, Som leaves possessed significantly highest reducing sugar content, being 2.92 mg/g in tender, 2.56 mg/g in semi-mature and 2.51 mg/g in mature leaves. Significantly lowest reducing sugar content was recorded in Mejankari leaves.

The TSS content varied significantly due to leaf type, season and host plants. A decreasing trend of its content has been observed with the advancement of leaf age. Som leaves possessed significantly highest TSS

content in all types of leaves, being 8.17 % in tender leaves, 6.72 % in semi-mature and 5.74 % in mature leaves. Significantly lowest amount of TSS content was found in Diglotti, 5.17 % in tender, 4.15 % in semi-mature and 3.47 % in mature leaves.

Soalu leaves were superior among all the host plants irrespective of leaf maturity and season in respect of starch content (8.55 % in tender, 9.78 % in semi-mature and 12.16 % in mature leaves). Starch content in semi-mature leaves of Diglotti was at par with Soalu leaves. Significantly lowest starch content was recorded in Som leaves: 6.27 % in tender, 7.02 % in semi-mature and 8.30 % in mature leaves.

4. β -sitosterol, total phenol and ascorbic acid

Data on β -sitosterol, total phenol and ascorbic acid content in the leaves of different host plants are presented in Table 3. β -sitosterol content decreased significantly with the advancement of leaf age irrespective of host plant and season. Tender leaves of Som possessed significantly highest β -sitosterol content (1.06 %); in semi-mature leaves, it was recorded 0.69 % whereas its content in mature leaves of Som and Soalu were at par (0.49 and 0.48 %, respectively). Significantly lowest β -sitosterol content in all types of leaves was recorded in Mejankari (0.49 % in tender, 0.36 % in semi-mature and 0.27 % in mature leaves).

A large variation in the total phenol content in leaves of different host plants according to leaf maturity was

Table 3: β -sitosterol, total phenol and ascorbic acid content in the leaves of different host plants at different maturity levels

Host plant	β -sitosterol (%)			Total phenol (mg/100 g)			Ascorbic acid (%)		
	Tender	Semi - mature	Mature	Tender	Semi - mature	Mature	Tender	Semi - mature	Mature
Som	1.06a	0.69a	0.49a	119.79a	47.26b	53.12b	1.50a	1.56a	0.83d
Soalu	0.82b	0.60b	0.48a	40.41b	101.39a	34.30c	1.38b	1.30b	2.07a
Diglotti	0.69c	0.44c	0.38b	31.10d	47.51b	101.97a	0.58d	1.53a	1.58b
Mejankari	0.49d	0.36c	0.27c	34.51c	15.50c	28.43d	0.90c	0.46c	1.39c
Host plant	S.Ed. ($\pm$)	0.017			1.49			0.05	
	CD _{0.05}	0.034			2.99			0.11	
Season	S.Ed. ($\pm$)	0.012			1.05			0.04	
	CD _{0.05}	0.024			2.12			0.07	

Figures followed by common alphabets do not differ significantly.

observed. Results revealed that, tender leaves of Som contained significantly the highest total phenol content (119.79 mg/100 g) compared to other host plants. Whereas, its content in semi-mature leaves of Soalu was significantly the highest (101.39 mg/100 g), but significantly the highest content of phenol in mature leaves was recorded from Diglotti (101.97 mg/100 g).

Tender and semi-mature leaves of Som were superior among all the host plants irrespective season in respect of ascorbic acid content (1.50 % and 1.56 %, respectively). Its content in mature leaves of Soalu was recorded as 2.07 % which was significantly the highest. Ascorbic acid content in semi-mature leaves of Diglotti was at par with that of Som leaves.

5. Chlorophyll a and b

Data on Chlorophyll a and b content in the leaves of different host plants are presented in Figure 2. Chlorophyll a content increased significantly with the advancement of leaf age irrespective of host plant and season. Tender leaves of Som and Soalu possessed significantly highest Chlorophyll a content (4.17 mg/g and 3.97 mg/g, respectively); in semi-mature and mature leaves, it was recorded significantly the highest in soalu leaves (12.24 mg/g and 21.79 mg/g, respectively). Minimum content of Chlorophyll a in tender leaves was recorded from Diglotti (1.39 mg/g), and in semi-mature and mature leaves, it was recorded minimum from Mejankari leaves (6.45 mg/g and 6.95 mg/g, respectively).

Chlorophyll b content also increased significantly with the advancement of leaf age irrespective of host plant and season. Soalu possessed significantly highest

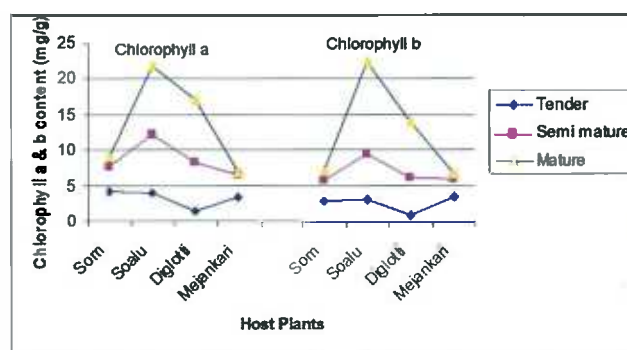


Figure 2: Chlorophyll a & b content in the leaves of different host plants at different maturity levels

Chlorophyll b content (3.10 mg/g in tender; 9.39 mg/g in semi-mature and 22.39 mg/g in mature leaves). Tender leaves of Mejankari recorded the highest Chlorophyll b content (3.48 mg/g) which was at par with that of Soalu. Minimum content of Chlorophyll b in tender leaves was recorded from Diglotti (1.06 mg/g), and in semi-mature and mature leaves, it was recorded minimum from Mejankari leaves (5.90 mg/g and 6.64 mg/g, respectively).

6. Chlorogenic acid and tannin

Table 4 represents the chlorogenic acid and tannin content according to leaf type of different host plants in two different seasons. Results revealed that, tender and semi-mature leaves of Som were superior among all the host plants irrespective season in respect of chlorogenic

Table 4: Chlorogenic acid and tannin content in the leaves of different host plants at different maturity levels

Host plant	Chlorogenic acid (%)			Tannin (%)		
	Tender	Semi-mature	Mature	Tender	Semi-mature	Mature
Som	1.81a	2.06a	1.45b	2.71b	2.88b	1.65b
Soalu	1.01b	0.87c	0.65c	0.90c	1.03c	1.30c
Diglotti	0.34c	0.09d	0.05d	4.75a	5.00a	3.07a
Mejankari	0.18d	1.04b	1.82a	0.20d	0.22d	0.21d
Host plant	S.Ed. ($\pm$)	0.044			0.059	
	CD _{0.05}	0.089			0.119	
Season	S.Ed. ($\pm$)	0.031			0.042	
	CD _{0.05}	0.063			0.084	

Figures followed by common alphabets do not differ significantly.

acid content (1.81 % and 2.06 %, respectively). Its content in mature leaves of Mejankari was recorded as 1.82 % which was significantly the highest. Significantly lowest chlorogenic acid content in tender leaves was obtained from Mejankari (0.18 %) and from semi-mature and mature leaves of Diglotti (0.09 % and 0.05 %, respectively).

In respect of tannin content, significantly highest values were recorded for Diglotti (4.75, 5.00 and 3.07 % in tender, semi-mature and mature leaves, respectively). Mejankari recorded the lowest tannin content in all types of leaves (0.20, 0.22 and 0.21 %, respectively).

7. ADF and lignin

Data on ADF content in the leaves of different host plants are presented in Figure 3. Results showed that ADF content also increased significantly with the advancement of leaf age irrespective of host plant and season. Soalu possessed significantly highest ADF content (17.77 % in tender; 27.87 % in semi-mature and 41.00 % in mature leaves). Tender leaves of Diglotti recorded the highest ADF content (17.86 mg/g) which was at par with that of Soalu. Minimum content of ADF in tender leaves was recorded from Mejankari (14.61 %), and in semi-mature leaves, it was recorded minimum from Diglotti and Mejankari leaves (18.28 % and 18.96 %, respectively), which were at par. Significantly lowest level of ADF content in mature leaves was recorded from Mejankari (21.38 %).

Results revealed that, Soalu leaves were superior among all the host plants irrespective of season in respect of lignin content (10.99 % in tender, 14.70 % in semi-mature and 16.32 %, in mature leaves). Significantly lowest lignin content in tender, semi-mature and mature

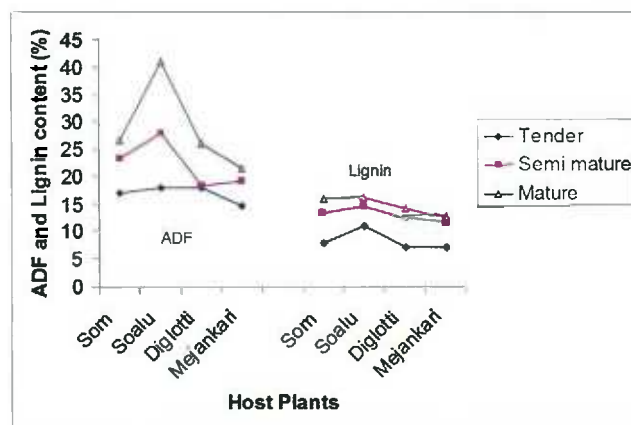


Figure 3: ADF and lignin content in the leaves of different host plants at different maturity levels

leaves was obtained from Mejankari (7.21 %, 11.88 % and 12.66 %, respectively).

Dietary water plays a very important role in silkworm metabolism as it regulates the rate of food ingestion. Moisture acts as an olfactory and gustatory stimulant (Ito, 1963) and its content is known to influence the metabolic activities related to food consumption, digestion and utilization (Reddy, 1981). Waldbauer (1968) and Scriber (1978) also highlighted the importance of moisture content of the feed in the case of other phytophagous insects. Parpiev (1968) reported that, higher moisture content in mulberry leaves favourably

affected not only edibility but also assimilation of nutrients in food and serves as a criterion in estimating the leaf quality. Hazarika *et al.* (1994) found that, higher the moisture content of leaves, higher the blood volume in different instars of muga silkworm body, but lower the total haemocyte count and *vice versa*. Singh *et al.* (2000) reported an average of 64.20 to 73.00 % moisture content in eight different morphotypes of Som. In the present investigation, a declining trend in moisture content was observed from tender to mature leaves and Soalu leaves recorded higher moisture content in all levels of maturity among the host plants. Similar trend of result was obtained earlier for the leaves of mulberry (Sinha *et al.*, 1993, 2003) and Som and Soalu plants (Yadav and Goswami, 1992). Yadav and Goswami (1992) reported moisture content in tender, medium and mature leaves as 74.00, 65.50 and 56.20 %, respectively in the case of Som and 75.40, 64.00 and 62.80 %, respectively in Soalu. Jolly and Dandin (1986) opined that 70 % moisture or more is optimum for silkworm rearing. The tender and semi-mature leaves are succulent, contain higher moisture and as the mandibular structures in the young larvae are underdeveloped, they therefore prefer tender leaves for feeding.

One of the most important components of silkworm feed is the minerals which also influence their growth and survival. Minerals *viz.*, phosphorus and calcium are essential for gaining body weight by the larvae. There are similarities in dose requirements by each element among insect species, and mulberry leaves contain the adequate amount of minerals to maintain good growth of silkworm, *B. mori* (Horie, 1978). Sinha *et al.* (1992, 2003) reported that the contents of total minerals and crude fibre increases with the maturity of leaves in all the primary food plants *viz.*, *Terminalia arjuna*, *T. tomentosa* and *Shorea robusta* of tasar silkworm, *A. mylitta* Drury. In the present study also, similar trend was observed in the case of total mineral content (Table I). It may be due to the translocation of minerals from the soil and accumulation in the comparatively aged leaves for a longer duration (Sinha *et al.*, 2003).

Crude fibre is the ash free material and reduction in total mineral and fibre content had been established

as an advantage for better silkworm crop yield (Vasuki and Basavanna, 1969). Fibre is not grouped under nutrients, but its intake along with diet is essential because of regulatory function and to maintain the normal peristaltic movement of the intestine. Bose *et al.* (1991) reported that succulent mulberry leaves with less fibre and higher mineral contents stimulate the metabolic activities in silkworm resulting in quantitative improvement in cocoon and silk. In the present study, crude fibre content exhibited an increasing trend with maturity of leaves, and higher crude fibre content was recorded in the leaves of Som and Soalu plants.

The role of proteins and amino acids in silkworm nutrition has been emphasized by Fukuda *et al.* (1959) and Takeuchi (1960). In fact, they appear to be involved practically in the structural units and functions of cells (Mallette *et al.*, 1960). Nitrogen as protein and non-protein nitrogenous matter present in the food plant leaves are responsible for healthy growth of silkworm as silk substances consists of protein. Yadav and Goswami (1992) reported 16.188 % crude protein content in the tender leaves of Som and 15.540 % in medium and mature leaves; 20.720 % in the tender, 18.170 % in medium and 15.540 % in mature leaves of Soalu. Singh *et al.* (2000) reported 9.65 to 11.88 mg/100 g protein content in eight different morphotypes of Som. In the present study, crude protein content was maximum in tender and minimum in mature leaves; and Som was superior over other host plants in this respect.

Carbohydrates, particularly reducing sugars are very important for growth and development of silkworms. Some sugars possess a gustatory stimulation effect on larval feeding (Ito, 1960). The carbohydrates are generally the most effective in increasing fat body glycogen. The rate of increase of fat body glycogen and haemolymph trehalose is also dependent on the content of carbohydrate in diet (Horie, 1978). Yadav and Goswami (1992) reported an average of 4.85 % and 4.71 % total sugar content in the leaves of Som and Soalu, respectively, being higher in medium leaves. In the present investigation, a decreasing trend of total soluble sugar content was observed for all the host plants. This may be due to variation in different factors such as

season, variety of host plant used, place *etc.* Similar trend was observed in the case of starch content of the leaves.

Sterol compound, β -sitosterol and β -d-glycoside of β -sitosterol present in mulberry leaf plays the role of a biting factor (Goto *et al.*, 1965; Hamamura *et al.*, 1962). First instar muga silkworms were reported to be more attracted towards tender leaves of *P. bombycina* (Chakravorty *et al.*, 2004) as these contain higher β -sitosterol content. This compound acts as a biting factor for silkworm, *B. mori* too (Hamamura *et al.*, 1962; Hamamura, 2001). Rajanandh and Kavitha (2010) reported that β -sitosterol in *Moringa oleifera* may be responsible for hypolipidemic, and as well as antioxidant properties. In the present study, β -sitosterol content exhibited a decreasing trend from tender to mature leaves of host plants with the maximum recorded in tender leaves of Som.

Ascorbic acid is an essential nutrient for several plant feeding insects for their normal growth, development and fertility (Vanderzant *et al.*, 1962; Chippendale and Beck, 1964), including *B. mori* (Ito and Arai, 1963). El-Karaksy and Idriss (2009) studied the effect of ascorbic acid as a food additive on mulberry silkworm, *B. mori* and reported that ascorbic acid at different concentrations (0.25, 0.5, 1 and 2 %) increased significantly the weights of both larvae and pupae. In addition, ascorbic acid proved to exhibit a significant effect on increasing the fecundity of emerged females, CA volume and the juvenile hormone level. Ascorbic acid content of the four host plants of muga silkworm exhibited significant variation. Its content was found higher in tender and semi-mature leaves of Som and semi-mature leaves of Diglotti and in mature leaves of Soalu (Table 3).

Secondary metabolites are organic compounds that often play an important role in plant defense against herbivores and other interspecies defenses. They contribute towards the adaptation of plants to the changing environment and in overcoming stress constraints. Importance and biological relevance of secondary metabolites on the cocoon production of mulberry silkworm, *B. mori* is well documented. Terpene compounds, terpinyl acetate, linalyl acetate, linolool and

citral act as attractants for *B. mori* (Hamamura, 2001). Chlorogenic acid, a phenolic acid, is reported to have strong growth promoting action and a role in moulting of *B. mori* larvae.

Phenols comprise the largest group of plant secondary metabolites present in both edible and non-edible plants. Hazarika *et al.* (1995) reported that good quality plants (on the basis of rearing performance) have higher total phenol contents, which range from 1.98 % in the least preferred to 6.26 % in the most preferred genotypes of *P. bombycina*. Apart from acting as biting factors, morin and chlorogenic acid enhance the rate of development, especially in the early stages (Kato, 1978). In the present investigation, chlorogenic acid content in the leaves of Som was more in medium leaves (2.06 %) compared to tender or mature leaves.

Lignins are phenolic polymers present in the cell walls of plants which are responsible together with cellulose, for the stiffness and rigidity of plant stems. It acts as a physical barrier against invading pathogens. Tannins are secondary metabolites of plants, non-nitrogenous, phenolic in nature and are present in all plant materials. It gives immunity to seed attack by birds and diseases; they on the other hand display impaired nutritional quality, lower digestibility and reduction of food consumption. In the present study, leaves of Diglotti at all growth stages possessed significantly higher tannin content. Soalu leaves of all maturity levels contained significantly higher lignin content.

In the present investigation, it has been revealed that, for all the nutrient constituents as a whole, Som (*P. bombycina*) exhibited superiority over other host plants irrespective of season and type of leaves, followed by Soalu (*L. polyantha*). Tender leaves of Som possess significantly the highest amount of total mineral, crude protein, reducing sugar, TSS, β -sitosterol, total phenol, ascorbic acid, chlorogenic acid and tannins content. Soalu tender leaves have been found to contain significantly the highest levels of moisture, soluble protein and lignin content; whereas, crude fibre, chlorophylls and ADF contents were at par in the case of both Som and Soalu leaves.

Table 5: Leaves of host plants at different maturity levels containing significantly higher level of chemical constituents

Sl. No.	Chemical constituent	Maturity level		
		Tender	Semi-mature	Mature
1.	Moisture	Soalu	Som & Soalu	Soalu
2.	Total mineral	Som	Som	Som
3.	Crude fibre	Som & Soalu	Som & Soalu	Som & Soalu
4.	Crude protein	Som	Som	Som
5.	Soluble protein	Soalu	Soalu	Soalu
6.	Reducing sugar	Som	Som	Som
7.	Total soluble sugar	Som	Som	Som
8.	Starch	Soalu	Soalu	Soalu
9.	β - Sitosterol	Som	Som	Som & Soalu
10.	Total Phenol	Som	Soalu	Diglotti
11.	Ascorbic acid	Som	Som & Diglotti	Soalu
12.	Chlorophyll a	Som & Soalu	Soalu	Soalu
13.	Chlorophyll b	Som, Soalu, Mejankari	Soalu	Soalu
14.	Chlorogenic acid	Som	Som	Mejankari
15.	Tannins	Diglotti	Diglotti	Diglotti
16.	Acid Detergent Fibre	Som & Soalu	Soalu	Soalu
17.	Lignin	Soalu	Soalu	Soalu

Semi mature leaves of Som were found to be superior in respect of total mineral, crude protein, reducing sugar, TSS, β -sitosterol and chlorogenic acid content, whereas, Soalu leaves were superior in respect of soluble protein, total phenol, chlorophyll a, ADF and lignin content. Som and Soalu semi-mature leaves were at par with respect to the contents of moisture, crude fibre and chlorophyll b, while that of Diglotti were significantly higher in respect of tannin content. Starch content of Soalu and Diglotti and ascorbic acid content of Som and Diglotti semi-mature leaves were at par.

In respect of mature leaves, Soalu was superior over others containing significantly the highest values of moisture, soluble protein, starch, ascorbic acid, chlorophylls, ADF and lignin content. This was followed by Som with significantly the highest level of total mineral, crude protein, reducing sugar and TSS content. Som and Soalu were at par in respect of crude fibre and β -sitosterol content. Total phenol and tannin content were significantly the highest in the mature leaves of Diglotti, whereas chlorogenic acid content of Mejankari mature

leaves was significantly the highest over all the other host plants (Table 5).

This study gives an insight into the quality parameters of the leaves of different host plants in terms of the important nutrient constituents. Considering the assessment parameters as a whole, Som was ascertained superior to other host plants irrespective of season and type of leaves, followed by Soalu. This underlines the increased preference shown by muga silkworms and the resultant higher survival rate on Som. Visual field observation of the feeding behaviour of the worms also supports the observation.

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VARIATIONS DANS LA COMPOSITION BIOCHIMIQUE DE DIFFERENTES PLANTES HÔTES DU VER A SOIE MUGA, *ANTHRAEA* *ASSAMENSIS* HELFER

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RESUME

Nous avons évalué les constituants biochimiques foliaires pendant quatre saisons de quatre plantes hôtes différentes du ver à soie muga, *Antheraea assamensis*, à savoir Som (*Persea bombycina*), Soalu (*Litsea polyantha*), Diglotti (*L. salicifolia*) et Mejankari (*L. citrata*). Pour les dix sept constituants des feuilles analysés, au total Som est supérieure aux autres plantes hôtes quels que soient la saison et le type de feuilles, suivie par Soalu. Les feuilles tendres de Som contiennent les plus grandes quantités de minéraux, de protéines, de sucres réduits, de TSS, de B-sitostérol, de phénols, d'acide ascorbique, d'acide chlorogénique et de tannins. Les feuilles tendres de Soalu contiennent les plus hauts niveaux d'humidité, de protéines solubles et de lignine alors que les fibres brutes, la chlorophylle et les ADF sont à un niveau à peu près égal à la fois chez les feuilles de Som et celles de Soalu.

Mots-clés: *Antheraea assamensis*, Diglotti, plantes hôtes, Mejankari, ver à soie muga, Soalu, Som.



Research Paper

ENERGY EFFICIENT SILK REELING PROCESS USING SOLAR WATER HEATING SYSTEM AND USHMA SHOSHAK UNIT IN MULTIEND SILK REELING UNIT

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ABSTRACT

A new system for energy efficient management of silk reeling process has been formulated with solar flat plate collector water heating system and Ushma Shoshak unit as additional components for multiend silk reeling unit. The energy consumption for cooking, reeling and re-reeling operations was derived and compared with that of conventional process. On an average, 570, 1000, 100 and 375 l of water is consumed for cocoon cooking, reeling, re-reeling and boiler operations, respectively for production of 10 kg multivoltine raw silk (20/22 denier) in 10 basin multiend silk reeling unit, leading to considerable amount of firewood consumption to produce heat energy for cocoon processing. The new, energy efficient management process with the combined use of solar water heating, Ushma shoshak unit and boiler yields 46 % saving in firewood consumption compared to conventional process with 2.26 years payback period on investment.

Key words: Cocoon cooking, firewood, heat recovery, insulated hot water tank, silk reeling, solar water heating system, Ushma Shoshak Unit.

INTRODUCTION

India has achieved the unique distinction of being a producer of all the five commercially traded varieties of natural silk namely, mulberry, tropical tasar, oak tasar, eri and muga. The bulk of the commercial silk produced in the world is mulberry silk of *Bombyx mori* L. with India having its share of 20,410 MT in the year 2010-11. Around 35,000 kcal of heat energy is generated by burning 22-25 kg firewood to convert 6-8 kg of green mulberry cocoon into 1 kg of reeled raw silk (Nagaraj *et al.*, 2008). It is estimated that 145,000 MT fuel wood and 170,000 MT of other biomass are consumed per year by the silk reeling industry in India for cocoon reeling due to the higher price and non-availability of commercial fuels such as coal, CNG and LPG (Dhingra, 1998). Silk industry consumes high energy, but with efficiency as low as ~10 % (Kumar and Kumar, 2005).

The dried timber or firewood holds 1,200 kg CO₂/MT and this CO₂ gets released into the atmosphere

when it is burnt (rapid oxidation) or allowed to rot on the forest floor (slower oxidation). On the contrary, when a tree grows, the equal amount of CO₂ is removed from the atmosphere by photosynthesis which gets held into it until further oxidized. Thus the effect of carbon footprint on atmosphere is maintained neutral naturally. But the human induced emission over the last two centuries has added to carbon footprints mainly by fossil fuel burning and deforestation. To minimize the detrimental effect on environment, the burning of firewood has to be brought to an acceptable level so that the CO₂ removed is held in the form of forestation.

Solar energy is the vital form of renewable energy that can provide practical solutions mitigating many of the energy related risks to our economy and is a silver lining that helps to reduce the greenhouse effect thereby helping to restore balance in our ecosystem (Brown, 1988). India has an abundant solar resource with more than 300 days of sunshine every year, which is equal to

over 5000 trillion kWh/year (Subramanya, 2008). The application of solar energy in energy intensive silk industry has huge potential (Kumar, 1997). Reeling being a cottage industry located mostly in villages and towns, depends mainly on firewood and other forms of agriculture residues for energy needs (Somashekar and Kawakami, 2002), causing serious ecological implications and depriving the soil of valuable nutrients and organic conditioning material (Kumar and Kumar, 2005).

In order to contribute towards prevention of deforestation, this study has attempted to develop a new, energy efficient management process by tapping solar energy to heat water and increase energy efficiency by Ushma Shoshak unit to reduce consumption of firewood. This yields ecological and economical benefits in multiend silk reeling process.

MATERIALS AND METHODS

Conventional system of energy management in silk reeling process

The conventional system in multiend silk reeling unit consists of a 100 kg capacity boiler for 10 basins multiend silk reeling unit (Sonwalkar, 1991). Firewood is used in the boiler to generate steam for all the activities of reeling process such as to heat water in reeling basins, cook cocoon in boiling water and heat air in re-reeling machine. The boiler needs 220-250 kg firewood to generate steam for production of 10 kg raw silk (20/22 denier) per day (8 h) in 10 basins unit. Since tamarind wood contains higher calorific value of more than 4,000 kcal/kg, silk reeling clusters in south India prefer it for silk reeling.

Cooking: Steam was used to bring the water temperature of open pan cooking vessels to 80-85 °C. Four vessels containing 9 l of water each were used to cook the cocoon for production of 10 kg raw silk in a day. The cooking water of vessels was replaced 3-4 times a day with fresh water to avoid the effect of turbidity on cooking performance and raw silk quality. The energy consumption by 4 cocoon cooking vessels in conventional process was ascertained by allowing boiling water at 90°C to cool down naturally. The

temperature of cooling water was recorded after every 2 min by digital thermometer.

Reeling: Ten basins each of 40 l capacity, requires 400 l hot water at 40-45 °C during the entire reeling process. Steam energy was required to raise temperature of entire water of reeling basins to that of reeling status and maintain it during the reeling process. The natural cooling of hot water takes place in all the basins and steam was used to augment the effect of cooling. By continuous reeling of cocoon, the concentration of dissolved sericin becomes more in basin water, affecting the colour of the raw silk produced. To avoid this effect of turbidity, fresh water was filled in basins twice a day.

Re-Reeling: Re-reeling of the silk was done in a hot compartment that ensured drying of the moist raw silk so as to transfer it on to standard reel to make skeins for easy transportation. In high-end machines like multiend reeling package, the re-reeling compartments are heated by conduction and convection through 5 rows of steam pipe surfaces running in the compartments. To maintain quality of re-reeled silk, it is suggested to maintain the temperature and humidity at 33 °C and 60 % inside the re-reeling compartments and at 27 °C and 45 % for room ambience, respectively. Water was used to maintain dampness of the silk on reel for easy re-reeling process.

Boiler: Two shell boiler of 100 kg capacity was used in a 10 basin multiend silk reeling unit for supply of steam energy to reeling activities such as heating of water in reeling basin, cocoon cooking and maintenance of re-reeling temperature.

New system of energy efficient management in silk reeling process

A schematic line diagram of the new energy efficient management set up for silk reeling process is shown in Figure 1. The components of the system comprise of 100 kg firewood boiler along with 1000 LPD solar water heating system (SWHS) having 10 flat plate solar collectors of 2 m² each, Ushma Shoshak unit and an additional 600 l insulated hot water tank. The renewable solar energy and conventional energy (wood fuel) are efficiently utilized through the Ushma Shoshak

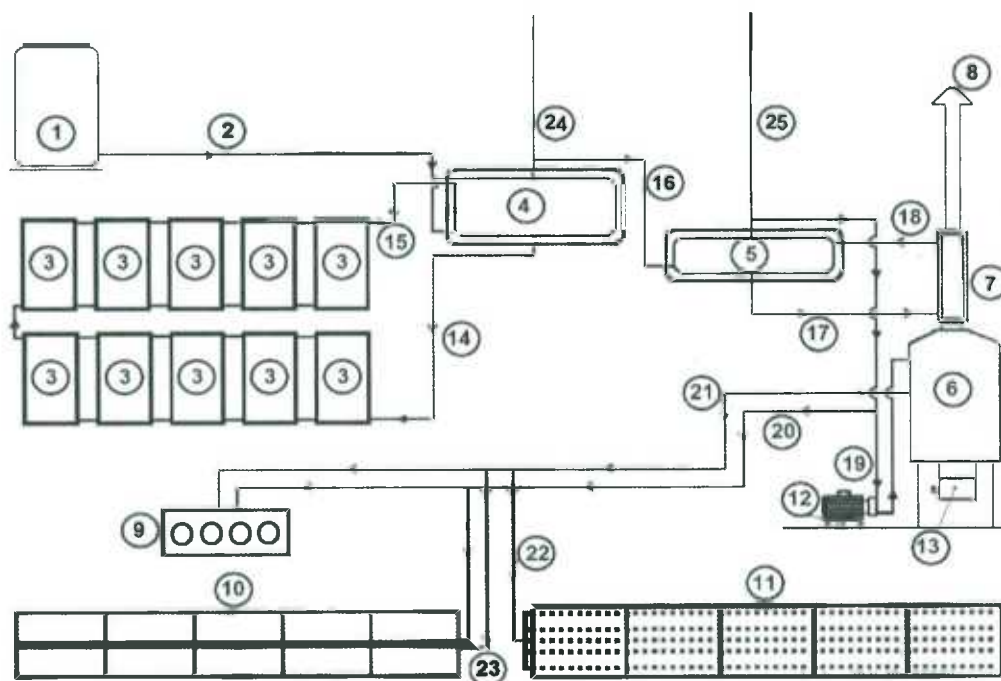


Figure 1: Line diagram of new, energy efficient management system in 10 basin multiend silk reeling unit

1-Overhead water tank, 2-Cold water supply 3-Flat plate solar collectors (each 2 m²), 4-Insulated solar water tank (1000 LPD), 5 - Additional insulated hot water tank (600 l), 6-100 kg steam boiler, 7-Ushma Shoshak Unit (USU), 8-Boiler chimney, 9-Cocoon cooking bench, 10-Ten basin multiend reeling machine, 11- Re-reeling machine, 12-Water pump, 13- Boiler firebox, 14-Water supply to solar collectors, 15-Solar hot water, 16-Solar hot water supply, 17-Solar hot water supply to USU, 18-USU hot water to 1000 l tank, 19-Hot water supply to boiler, 20-Hot water supply to cooking and reeling, 21-Boiler steam supply, 22-Steam supply to re-reeling machine, 23-Steam supply to reeling machine, 24 & 25-air vent

unit fabricated and fitted on to the boiler (Figures 2, 3 and 4).

As shown in Figure 1, the overhead tank supplies cold water to 1000 LPD insulated solar tank and by gravitational circulation through 10 solar collectors, water gets heated up to 65-85 °C by its passive thermodynamic property, in one full day sunshine, whereas, 10 basin multiend reeling unit consumes around 2000 l of water for production of 10 kg raw silk. The incidence of solar radiation on each solar panel is 4-6 kW/m²/day. More number of solar panels may also be added for better performance of the solar water heating system.

The hot water in 1000 LPD solar tank was drawn into 600 l additional insulated tank when silk reeling process starts. The inlet and outlet points of the additional insulated 600 l tank was connected to Ushma Shoshak unit in a manner that additional heating of water takes place by way of its passive thermodynamic properties. Ushma Shoshak unit was mounted on the boiler chimney

so that boiler flue gas should pass through it. The firewood burning process transforms around 55 % of heat energy into steam energy in the boiler and the rest 45 % gets lost into the atmosphere in the form of carbon dioxide, carbon monoxide *etc.* Ushma Shoshak Unit was designed to allow circulation of water into its jacket



Figure 2: 600 l additional insulated tank and Ushma Shoshak unit fitted to the boiler chimney passage



Figure 3: 600 l additional insulated tank with 1000 LPD solar water heating system installed on the roof of multiend silk reeling unit

through inlet and outlet valves, whereas, its inner surface facilitates to recover the heat from the flue gas emitted into the boiler chimney while burning firewood. The recovered heat energy gets transferred on to the water in the jacket of Ushma Shoshak Unit, the hot water then rises up and enters into the 600 l additional insulated tank. At the same time, equal amount of cold water from additional insulated 600 l tank enters into Ushma Shoshak Unit. This continuous water circulation between Ushma



Figure 4: Ushma Shoshak unit fitted on the 100 kg capacity boiler

Shoshak Unit and 600 l additional tank facilitates further heating of water. Another outlet of the 600 l additional tank supplies hot water for different activities of silk reeling (Figure 1).

RESULTS AND DISCUSSION

The studies on fuel consumption in conventional system of energy management and new, energy efficient silk reeling processes were conducted in a commercial reeling unit under the prevailing natural condition (Tables 1 and 2).

Table1: Energy consumption in conventional energy management system

Silk reeling activity	Energy consumption (kcal)	Firewood consumption (kg)
Cocoon cooking	123,840	89.35
Heating water for reeling basin	33,440	24.13
Re-reeling	119,920	86.52
Invisible loss	27,720	20.00
Total	304,920	220.00

Table 2: Energy and firewood consumption in new, energy efficient management system with solar water heating and Ushma Shoshak unit

Silk reeling activity	Energy consumption (kcal)	Firewood consumption (kg)
1000 LPD Solar water heating system-10 flat plate solar collectors of 2 m ² at 80 % efficiency of 5KW/m ² /day	80,000	-
Heat recovery by Ushma Shoshak unit at 38.76 % efficiency	58,600	-
100 kg Boiler	166,320	120
Total	304,920	120

In the natural condition, there are variable factors which influence the quantity of fuel wood consumed for raw silk production. It is very difficult to establish standard or ideal condition to conduct studies on fuel consumption in the commercial silk reeling process. The natural variable factors those influence fuel consumption are, ambient temperature (higher the temperature, lower the fuel consumption), humidity (lesser moisture *i.e.*, RH %

Table 3: Economical benefits of new, energy efficient management process and rate of return on investment

Sl. No.	Particulars	Year				
		1 st	2 nd	3 rd	4 th	5 th
1	Price of the fuel per kg (₹)	3.80	4.00	4.20	4.30	4.40
2	Initial Capital Investment (₹)	281,000				
3	Savings from fuel (₹)	114,000	120,000	126,000	129,000	132,000
4	Rate of Return on initial capital investment (%)	44%	46%	48%	49%	51%
5	Average savings from fuel (₹)	124,200				
6	Payback period (years)	2.26				

in the atmosphere results in lesser fuel consumption), quality of cocoons (inferior cocoons need more time for cooking and reeling), fineness of raw silk (finer denier raw silk production needs higher energy), moisture content in the fuel (fuel wood retains up to 50 % of moisture of its own weight; higher moisture generates more smoke leading to energy loss), improper insulation of steam transmission pipes which leads to higher invisible energy loss, interruption in electricity supply, idle machine, skill of the worker, under-utilization of unit capacity, hot water wastage, boiler maintenance, water quality and work culture.

Under the above prevailing natural conditions, firewood consumption for conventional energy management system was ascertained as 22-25 kg for 10 basin multiend reeling unit for production of 1 kg multivoltine raw silk (20/22 denier). Similarly, the study on innovative usage of Ushma Shoshak unit and Solar water heating system was conducted for firewood consumption under the same prevailing natural conditions. This new, energy efficient management process yielded a reduction of 12-14 kg in fuel wood consumption per kg multivoltine raw silk (20/22 denier) production.

In the conventional energy management process, 220 kg of firewood was burnt for cooking, reeling and re-reeling activities for the production of 10 kg (20/22 denier) multivoltine raw silk in 10 basin multiend silk reeling unit and for similar production under new, energy efficient management system, it is

reduced to 120 kg, resulting in a saving of 46 % in firewood consumption. Thus, the adoption of energy efficient management process in silk reeling in turn prolongs deforestation and thereby bring down firewood burning to an acceptable level. This paves the way for extended holding of CO₂ in the form of forestation.

The outcome of promoting the new, energy efficient management process was studied and the economical benefit worked out for the reeler is presented in Table 3. It was assumed that 10 basin multiend reeling unit runs in single shift for 300 days in a year, raw silk production is 10 kg per day; fuel (firewood) consumption in conventional energy management process is 22 kg per kg raw silk (20/22 denier) production and 12 kg in new, energy efficient management process. It is evident from the data that the total expenditure incurred in the new, energy efficient management process is ₹ 281,000/-, which includes installation of flat plate solar collectors water heating system (₹ 150,000), Ushma Shoshak unit (₹ 41,000), additional 600 l insulated water tank (₹ 60,000) and plumbing items and labour charges (₹ 30,000). The average savings from firewood consumption in five years due to use of new energy efficient management process will be ₹ 124,200/-, which is 45.5 % of that required for conventional energy management system. Thus, the recovery period on investment works out to be 2.26 years by considering the total investment in the new energy efficient management process with respect to the amount saved in the consumption of firewood.

CONCLUSION

India produces 20,000 MT of raw silk per annum by using conventional energy sources in the form of an estimated 145,000 MT fuel wood/year and 170,000 MT of other biomass/year. The installation of solar water heating system and Ushma shoshak unit in multiend silk reeling sector increases the efficiency of energy management, helping to bring down firewood burning to an acceptable level and prolong holding CO₂ in the form of forestation thus contributing to ecological well being and economical benefit of the silk producer.

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PROCEDE DE DEVIDAGE DE LA SOIE ENERGETIQUEMENT EFFICACE UTILISANT UN SYSTEME SOLAIRE DE CHAUFFAGE DE L'EAU ET UNE UNITE USHMA SHOSHAK D'UNE FILATURE MULTIBOUTS

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RESUME

Un procédé nouveau, énergétiquement efficace a été développé avec un système de chauffage de l'eau par panneau solaire et une unite Ushma Shoshak comme composants ajoutés à une unité de dévidage multibouts. La consommation d'énergie pour la cuisson, le dévidage et le reflottage a été mesurée et comparée avec celle du procédé conventionnel. En moyenne, 570, 1000, 100, et 375 l d'eau sont consommés par la cuisson des cocons, le dévidage, le reflottage et le chauffage respectivement, et ceci pour la production de 10 kg de soie grège polyvoltine (20/22 deniers) dans une unité de dévidage de 10 bassines multibouts, ce qui entraine une consommation importante de bois de chauffe pour produire l'énergie nécessaire au traitement des cocons. Le nouveau procédé énergétiquement efficace avec l'utilisation combinée du chauffage solaire de l'eau et d'une unité Ushma Shoshak avec chaudière conduit à une réduction de 46 % de la consommation de bois comparée au système conventionnel et le retour sur investissement est de 2.26 années.

Mots-clés: Cuisson des cocons, bois de chauffe, tuyau de cheminée, récupération de la chaleur, réservoir d'eau chaude calorifugé, dévidage de la soie, système solaire de chauffage de l'eau, unite Ushma Shoshak

THE EC AND XXIII CONFERENCE OF ISC



The Executive Committee meeting and XXIII Conference of International Sericultural Commission were held at Hotel Leela Palace, Bangalore, India on 5th & 6th December, 2013. Delegates from Japan, Egypt, France, Thailand, Madagascar, Romania and India had actively participated in this Governing Council meeting of ISC. The major agenda items for discussions were:

- Report of the Secretary General's activities for the period from 1st January to 30th November, 2013
- Progress on organizing the next ISC Congress-2014 in Bangalore, India
- Budget for the year 2014
- Discussion on the amendment of ISC Statutes

Bangalore, India
5th & 6th, December, 2013





In the afternoon of 2nd day of the Conference, the delegates were taken to the newly established ISC Secretariat and also to the sericulture and silk facilities of Central Silk Board, Ministry of Textiles, Government of India situated in Bangalore. The 3rd and 4th day were devoted for post-conference tour to Hassan which is about 150 km from Bangalore. The delegates were taken to the magnificent temple of Bahubali situated in Vindhyagiri hill top of Shravanabelagola, the Channakesavaraya temples in

Mosale, the submerged church in Hemavathi Dam, the magnificent temples of Hosysala kingdom in Beluru and Halebidu.

XXIII Congress of ISC

The XXIII Congress of the International Sericultural Commission is scheduled to be held between 24th and 27th November 2014 at Hotel Leela Palace, Bangalore, India.

Announcement

The International Sericultural Commission (ISC), Bangalore, India, an inter-Governmental Organization engaged in the development of sericulture and silk industry, has been conferring the prestigious Louis Pasteur Award on persons who have made outstanding contributions to the development of silk industry in their respective countries. The award is given away once in three years for three persons selected from across the globe. The Louis Pasteur Award consists of a Citation and a Medal, which will be presented at the time of ISC Congresses held triennially.

International Sericultural Commission hereby invites nominations for the Louis Pasteur Award - 2014 from the ISC Member Countries (Brazil, Egypt, France, Greece, India, Indonesia, Iran, Japan, Madagascar, Romania, Syria, Thailand and Tunisia). The nominated candidates should have involved with the development of sericulture industry in their country or across the world. The nominations of each candidate should be prepared on plain paper and contain the following details:

- Bio-data, incorporating Name, sex, present position held, present address, date of birth, qualifications, experience in the field of sericulture, list of relevant publications, if any (grouped into primary authorship and co-authorship), etc.
- Brief self appraisal, not exceeding 5 pages, chronologically listing their contributions to the development of sericulture industry.
- One page write-up indicating why they consider themselves fit for consideration to the award.
- A self certified declaration that the information given is true and correct.

The nominations along with all relevant details (also in soft copy) from the Member Countries should reach ISC office not later than 30th June 2014, to the Secretary General, International Sericultural Commission, CSB Complex, B.T.M. Layout, Madiwala, Bangalore - 560 068, India; E-mail: iscbangalore@gmail.com

Nominations received after the last date will not be considered.



INTERNATIONAL SERICULTURAL COMMISSION

An intergovernmental organization instituted in 1960 and registered with United Nations; Reg. No. 10418

APPLICATION FOR ASSOCIATE MEMBERSHIP

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Submission of a paper to SERICOLOGIA implies that it has not previously been published, that it is not under consideration for publication elsewhere and that, if accepted in SERICOLOGIA, it will not be published elsewhere in the same form without the written consent of the Chief Editor.

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The typescript should contain the following features:

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- **Authors** full name, address, mobile/fixed line numbers, and email/alternate email address should be in 10 point font and centered underneath the title. The first address should be of the centre at which the study was conducted. In the case of multiple ownership, the authors may indicate the correspondence address.
- **Abstract** must be the gist of the paper which should explain the background, aims, methods, results & conclusion in a single paragraph not exceeding 200 words.
- **Key words** should follow the abstract, subject to a maximum of five, arranged in alphabetic order.
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Ceci S. J., Williams W. M. and Barnett S. M. (2009) Women's underrepresentation in science: Sociocultural and biological considerations. *Psychol. Bull.*, 135: 218-261.

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