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Characterization of Bud Break Process in Mulberry (*Morus alba* L.)

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ABSTRACT

Mulberry leaves are the sole feed for the monophagous silkworm, *Bombyx mori.* Bud break and regrowth are crucial for the timely supply of foliage for sericulture. The regulatory mechanism governing mulberry bud break is poorly understood which is crucial for manipulating the bud break process in a tailored manner. In the present study, an attempt has been made to study the anatomical, biochemical and molecular basis of mulberry bud break. For studying the bud break process, bud growth was classified into four different stages (stage 0 to stage 3) based on morphological features. Scanning electron microscopy (SEM) analysis of mulberry bud anatomy indicated mixed bud features with both vegetative and reproductive primordia in a single bud. Further, hormone profiling using LC-MS revealed that dormant bud (stage 0) had significantly higher ABA content (71.58 ng g^{-1} fresh weight) and its content decreased when bud break was initiated. The transition to active growth was marked with a high GA3 content at stage 2 (9.12 ng g^{-1} fresh weight). Auxin and cytokinin contents also increased during bud break with the highest content in stage 2. Cell size in the buds significantly increased from stage 2 (242.8 μm^2), and the highest cell size was at stage 3 (277.9 μm^2), which coincided with the higher levels of auxin and cytokinin. Expression of a key dormancy regulator gene, *Short Vegetative Phase* (SVP) reduced significantly at stage 2. Our study indicated that ABA is crucial for dormancy induction and maintenance while GA3 triggers bud break and the relative ratios of ABA and GA play a major role in the mulberry bud dormancy cycle.

Keywords : Anatomy, Bud break, Hormones, Mulberry

MULBERRY (*Morus alba* L.) is a perennial tree that is commercially exploited for its leaves to feed monophagous silkworm, *Bombyx mori* in the sericulture industry (Gerasopoulos and Stavroulakis, 1997). In India, mulberry silk accounted for 70.72 per cent (23,860 MT) of the total silk produced in 2020-21 (Anonymous, 2021). The sustainability of the sericulture industry depends on the timely availability of mulberry foliage. Mulberry, for commercial silk production, is maintained as bush by recurrent pruning, which ensures continuous availability of leaves at convenient manageable height (Yamashita 1986 and Suzuki & Kohno, 1987). The emergence of new foliage and growth depends on the bud dormancy break in the main stem. Rapid and uniform bud break is essential for the timely supply of leaves for silkworm rearing. Understanding the mechanism underlying bud break is the key to effective manipulation of the trait associated with foliage production (Dhanyalakshmi and Nataraja, 2018). In mulberry, bud-break can be considered an economically important process, but its molecular mechanisms are poorly understood.

In tree crops, bud dormancy is a survival strategy and the dormancy protects them from harsh environmental conditions. Dormancy is characterized by resting cell cycle, and reduction in respiratory and metabolic activities (Arora *et al.,* 2003). Examination of bud anatomy is crucial to study and comprehend the bud break process and branching pattern. Trees have been shown to exhibit different types of bud features such as only vegetative primordia or floral primordia and mixed buds (both vegetative and floral) (Costes *et al.,* 2014) and each type of bud has different regulatory mechanisms. The bud break or regrowth of bud is regulated by complex interaction of environmental cues and endogenous factors (Dhanyalakshmi *et al.,* 2020 and Azeez *et al.,* 2021). One of the key regulators of bud break is endogenous signals, mainly hormones. Hormones act in isolation and also interact with each

other to activate or inhibit bud break (Singh *et al.,* 2018). The complex hormonal interaction regulates bud break through the regulation of key genes, which acts downstream in the hormonal signal transductions. However, the role of hormones in regulating bud break in trees is not fully elucidated and there is a large gap in the information available (Azeez *et al.,* 2021).

The release of bud dormancy in perennial plants has been shown to bear a resemblance to flowering in *Arabidopsis thaliana* (Beauvieux *et al.,* 2018). In tree species, genes encoding MADS-box transcription factors are reported to regulate the dormancy cycle and these genes are called Dormancy-Associated MADS-box (DAM) and these genes are phylogenetically related to Arabidopsis floral regulator genes, Short Vegetative Phase (SVP) and AGAMOUS-like 24 (Jimenez *et al*., 2010). Among these, Short Vegetative Phase - like (SVL) is the key negative regulator of bud break and SVP-like (SVL) has shown to regulate the process through acting on hormonal regulation cascade (Busov, 2019). SVL has been shown to inhibit bud break in hybrid aspen by activation of transcription of abscisic acid (ABA)-related genes such as 9-cis-epoxy carotenoid dioxygenase (NCED3) and ABA receptor (Singh *et al.,* 2018). SVL was also reported to act upon gibberellic acid (GA) signaling by repressing one of the GA biosynthetic genes GA 20-oxidases (GA20ox) in hybrid aspen (Singh *et al.,* 2018). The regulatory genes govern the biochemical and physiological process by regulating several types of downstream genes and associated pathways. The specific biochemical pathways bring about anatomical / developmental changes, which help in the transition from dormant to the active growth of buds. Examination of anatomical changes during bud break could aid in assessing the sequential developmental processes in commercially exploited crops like mulberry. From this context in this study, an attempt was made to monitor the anatomical and biochemical changes in the mulberry bud during bud break.

Material and Methods

Plant Material

Mulberry plants (*Morus alba* var. Dudia White) were grown in 25 kg pots filled with Soil : Sand : FYM at the ratio of 2:1:1 and recommended dose of fertilizers were applied to induce normal growth. One-year old well-established and healthy plants which were regularly pruned to maintain as the mulberry bush were used in the present study.

Classification of Mulberry Bud Break Stages and Scanning Electron Microscopy (SEM) Analysis

Mulberry bud break stages were classified into four stages based on the morphological markers with minor modifications from Sanchez Salcedo *et al.* (2018). For SEM analysis, the buds were harvested, cleaned by washing twice with deionized water. The buds were then fixed in four per cent glutaral dehyde for four hours and post-fixed with 0.1 per cent osmium-tetroxide for two hours (Dwiranti *et al.* 2019). The fixed samples were dehydrated using gradient ethanol wash (60, 70, 80, 90 and 100 per cent each for 10 minutes). The respective buds were longitudinally and cross-sectioned with a sharp sterile blade. The sections of the bud samples were coated with gold-palladium ions using the sputtering device (Quorum - SC7620) for 75 seconds at 12.5 mA discharge current under 8×10^{-2} mbar vacuum pressure. The processed samples were then visualized using SEM (Zeiss Evo, Scanning Electron Microscope, Germany) at 10 to 15 kV. The cell size and other anatomical features were observed and recorded under standardized conditions. Cellular area in respective bud stages was analyzed using ImageJ software (Schneider *et al.,* 2012).

Phytohormone Profiling from Mulberry Bud Stages

Hormone profiling was performed on a LC-MS/MS equipped with an APCI-ESI for ionization (WATERS Acquity UPLC H-Class, Coupled with Acquity-TQD MS / MS). The procedure for extraction and quantification of multiple hormones was followed as described by Pan *et al.* (2010). Briefly, about

The Mysore Journal of Agricultural Sciences

0.5g of samples were homogenized in five ml of 1-propanol-H_2O-concentrated HCl (2:1:0.002, v/v/v) and extracted at 4 C for 12 h. About 10 ml of dichloromethane was added to homogenate and was shaken for 30-minutes and was centrifuged. The bottom layer was transferred to a conical flask containing sodium sulphate and was evaporated using a flash evaporator. Before injecting into LC-MS/MS for further analysis, the dried samples were dissolved in 500 µl methanol-0.05 per cent formic acid (1:1, v/v) and filtered using a nylon filter paper and injected into LC-MS for further analysis. Data acquisition and analysis were done with AB SCIEX Analyst 1.6 software (Foster, CA, USA). The phytohormone content was expressed on a fresh weight (fw) basis.

Analysis of Expression of Short Vegetative Phase (SVP) Gene

Expression of one of the key regulators of bud break in different tree crops *Short Vegetative Phase* (SVP, NCBI GeneID: 1491814060) was studied in different stages of buds to understand its regulatory role.

RNA isolation and qPCR : Bud break was induced as described by Dhanyalakshmi *et al.* (2020). Total RNA was isolated from all stages of the buds using Spectrum Plant Total RNA kit (Sigma-Aldrich) as per the manufacture's protocol. RNA samples were treated with DNase (DNase I, Thermo Scientific™) to remove the genomic DNA contamination. About 3 µg of total RNA was used to synthesize the first-strand cDNA in an oligo-dT primed reaction using Molony-Murine Leukaemia Virus Reverse Transcriptase enzyme (MMLV-RT; MBI Fermentas, Hanover, MD, USA). Real-time qPCR was performed with SYBR® Green master mixes (Bio-Rad) using a CFX96 Touch Real-Time PCR Detection System (BIO-RAD, CFX96 TOUCH™). Relative expression levels of SVP were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *Morus alba* Elongation factor 1 was used as the endogenous reference gene for the normalization of the expression levels of the SVP. The sequences of primers used for the study are listed in Table 1. Data were analyzed using XL-STAT software and Duncan's test was used to analyze the data at the significance level of $P<0.05$.

TABLE 1

List of primers used for qRT-PCR experiment (Expression analysis of SVP gene)

Primer	Sequence (5' – 3')
Ma Elongation factor (Forward)	TCTCAAGCGTGGGTTTGTTGCT
Ma Elongation factor (Reverse)	TTGACAGCAATGTGGGAGGTGT
Ma SVP (Forward)	GTGACTGATATTCCAGCAACTAC
Ma SVP (Reverse)	ACAGGCAGTCCTAGATTGTAAAGC

RESULTS AND DISCUSSION

Mulberry Bud Development and Anatomy

Information on crop phenology is essential for the implementation of strategic agronomic practices for manipulating plant behavior. Sánchez-Salcedo *et al.* (2017) classified *Morus* sp. bud development into five stages based on BBCH-scales, which are used to identify the phenological development stages of plants. In the present study, slight modifications in morphology and nomenclature of bud stages have been made for monitoring internal bud anatomical changes associated with molecular and biochemical processes. As shown in Fig. 1, stage-0 depicts the dormant stage of the bud with scales covering the miniature bud. The advancement towards stage-1 is the transition to active growth wherein it shows a small green portion at bud apices. Further, stage-2 is characterized by buds that emerged out of scales and are enlarged, while stage-3 has the small immature leaves that emerged out of the buds (Fig. 1). The SEM imaging of mulberry buds indicated mixed bud features with vegetative and reproductive primordia in a single bud (Fig. 2). The leaflets were positioned in the periphery while the floral primordia were located in the central position of buds. This kind of bud anatomy was also reported in species like apple wherein it determines shoot architecture and branching pattern (Costes *et al.,* 2014). Better understanding

Stage - 0 Stage - 1 Stage - 2 Stage - 3

Fig. 1 : Representative image of different stages of mulberry bud

mulberry bud anatomy can help to schedule the pruning for better vegetative growth.

Hormonal Regulation of Bud Break

Complex hormonal interactions have been shown to influence bud break in different perennial crop systems. These hormones in turn trigger biochemical and anatomical changes leading to dormancy release (Singh *et al.,* 2018). ABA, GA, Cytokinin (6- benzyl aminopurine and zeatin trans-isomer) and Auxin (indole acetic acid and indole butyric acid) differed significantly during bud-break and further development. In this study, the highest ABA content was observed at stage-0 (71.58 ng g^{-1} fw), *i.e.,* the dormant stage (Fig. 3). In many species, bud dormancy is associated with increased levels of ABA (Cooke *et*

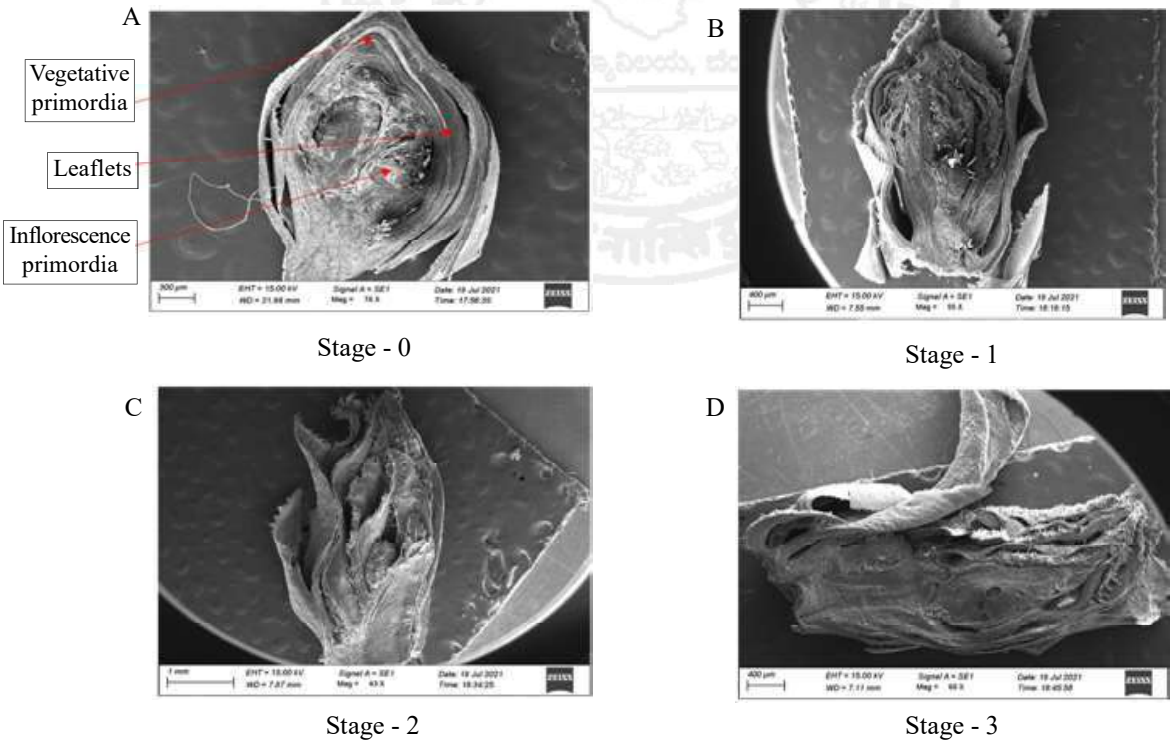

Fig. 2 : Scanning electron microscopy showing the the anatomy of different stages of mulberry buds (A to D) revealing the mixed bud feature (both vegetative and reproductive primordia in a single bud)

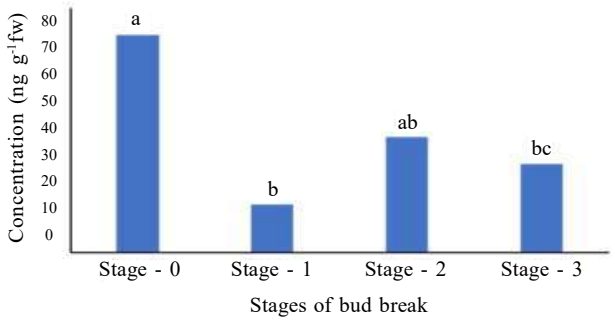

Fig. 3: Abscisic acid content (ng g^{-1}fw) in different stages of mulberry buds (Bar graph with the same letter indicate no significant difference at $p<0.05$ (DMRT)

al., 2012). ABA has been shown to induce and maintain dormancy in different tree crops (Wang *et al*., 2016; Li *et al*., 2018) and its content is reported to decline during the dormancy release (Zheng *et al*., 2015). ABA levels decreased significantly at stage 1 (15.73 ng g^{-1} fw) marking the release of dormancy. Contrastingly, GA3 content was significantly lower in the dormant bud, while its level increased significantly at dormancy release and transition to active growth, *i.e.,* stage 2 (Fig. 4). The highest level of GA was at stage 2 (9.12 ng g^{-1} fw), while the lowest was in stage 0 and stage 1. As reported earlier, GA activates growth (Eriksson *et al*., 2000) and a dormancy-releasing effect was observed in different crops (Shafer and Monson, 1958; Eagles and Wareing, 1964; Zhuang *et al*., 2013). The data on mulberry indicate that ABA and GA act as negative and positive regulates of bud break, respectively, and probably, relative ratio governs the bud-break process. In poplar, ABA can induce dormancy by blocking the symplastic intercellular communication through plasmodesmata (Tylewicz *et al*., 2018). GAs are predominantly important for the growth renewal process during dormancy release by triggering the energy metabolism pathways and hydrolytic enzymes (Zhuang *et al*., 2015).

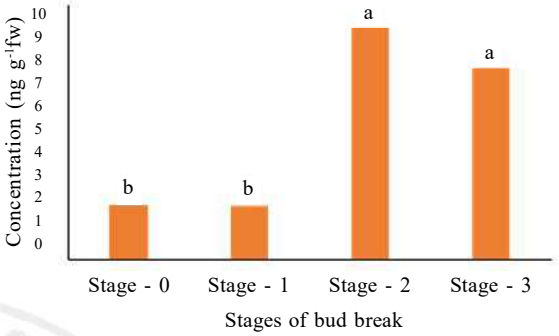

Fig. 4: Gibberellic acid (GA3) (ng g^{-1}fw) content in different stages of mulberry buds (Bar graph with the same letter indicate no significant difference at $p<0.05$ (DMRT)

In this study, the Cytokinin (6- Benzyl Amino purine and zeatin trans-isomer) was significantly more at stage 2 (Fig. 5), while the lowest level was observed in the stage 0 and 1. The 6- Benzyl Aminopurine and zeatin trans-isomer content were 574.0 and 21.36 ng g^{-1} fw, respectively at stage 2 (Fig. 5 a-b). Similarly, indole acetic acid and indole butyric acid was drastically increased at stage 2 (Fig. 6) and the higher concentration of indole acetic acid was observed at stage 3 (58.52 ng g^{-1} fw) (Fig. 6a) while indole butyric acid was significantly more at stage 2 (209.785 ng g^{-1} fw) (Fig. 6b). Auxin and cytokinin have been shown to play role in dormancy release by acting on cell

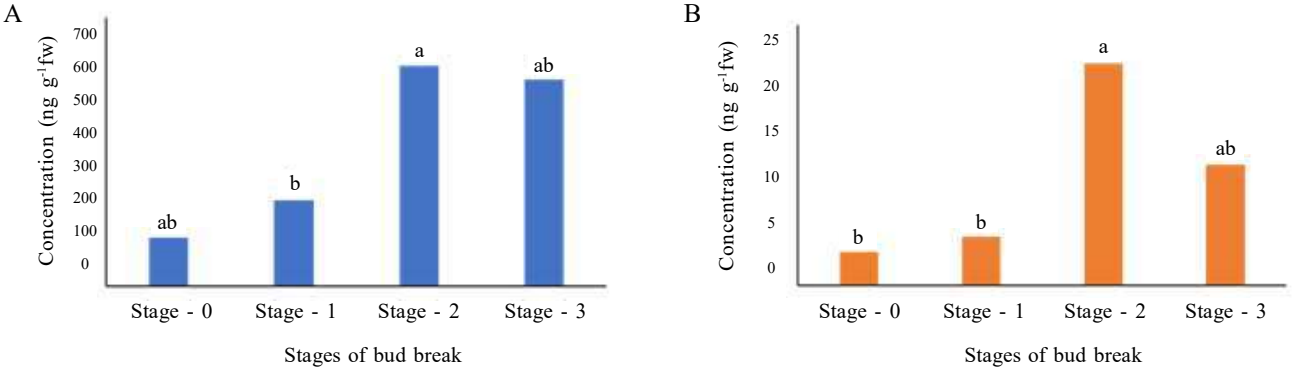

Fig. 5: Cytokinin content (expressed as ng g^{-1}fw) in different stages of mulberry buds. (A) represents 6- Benzyl amino purine content and (B) represents Zeatin trans trans-isomer content (Bar graph with the same letter indicate no significant difference at $p<0.05$ (DMRT)

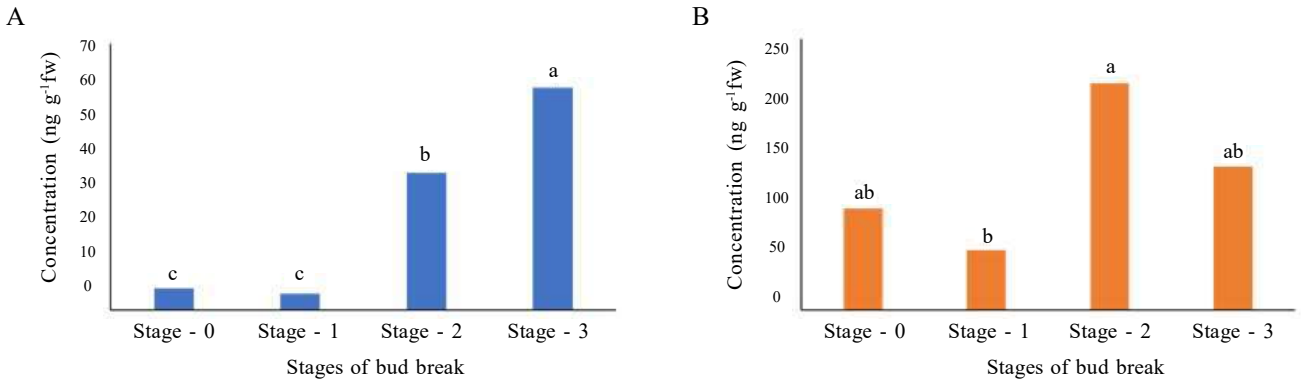

Fig. 6: Auxin content (expressed as ng g^{-1}fw) in different stages of mulberry buds. (A) indicates Indole acetic acid (IAA) content and (B) indicates Indole butyric acid (IBA) content (Bar graph with the same letter indicate no significant difference at $p<0.05$ (DMRT)

cycle regulation (Takatsuka and Umeda, 2014). These hormones are also reported to trigger cell division and cell enlargement (Majda and Robert, 2018). In the present study, the SEM showed increased cell size, which correlated with the changes in the hormone levels at different stages. A steady increase in cell size of buds was noticed from dormancy to active growth transition with the drastic increase in Stage 2 (Fig. 7). The highest cell size was observed in stage 3 (277.9154 μm^2). Dormancy release in tree buds is generally coupled with the restart of the cell cycle and cell division (Azeez *et al.,* 2021). Cell division and cell expansion indicate the regrowth after the dormancy release and are essential for the exertion of leaf whorls out of the buds (Fadon *et al.,* 2018). This typical phenomenon was correlated between the morphology of stage 2 and stage 3 and its corresponding cell size.

SVP Negatively Regulates Bud Break

There are many regulatory genes associated with bud dormancy and bud break, some of them can regulate multiple developmental processes. Amongst many upstream regulators, the (SVP) gene has been shown to be a negative regulator of flowering as well as bud break (Singh *et al.,* 2019 and Busov, 2019). In mulberry, in the present study, the expression of SVP significantly decreased from stage 1 to stage 2. A decrease in fold change of SVP expression at stage 2 proves that it plays a similar negative regulatory role in the bud break process in mulberry. Studies have shown that SVP expression

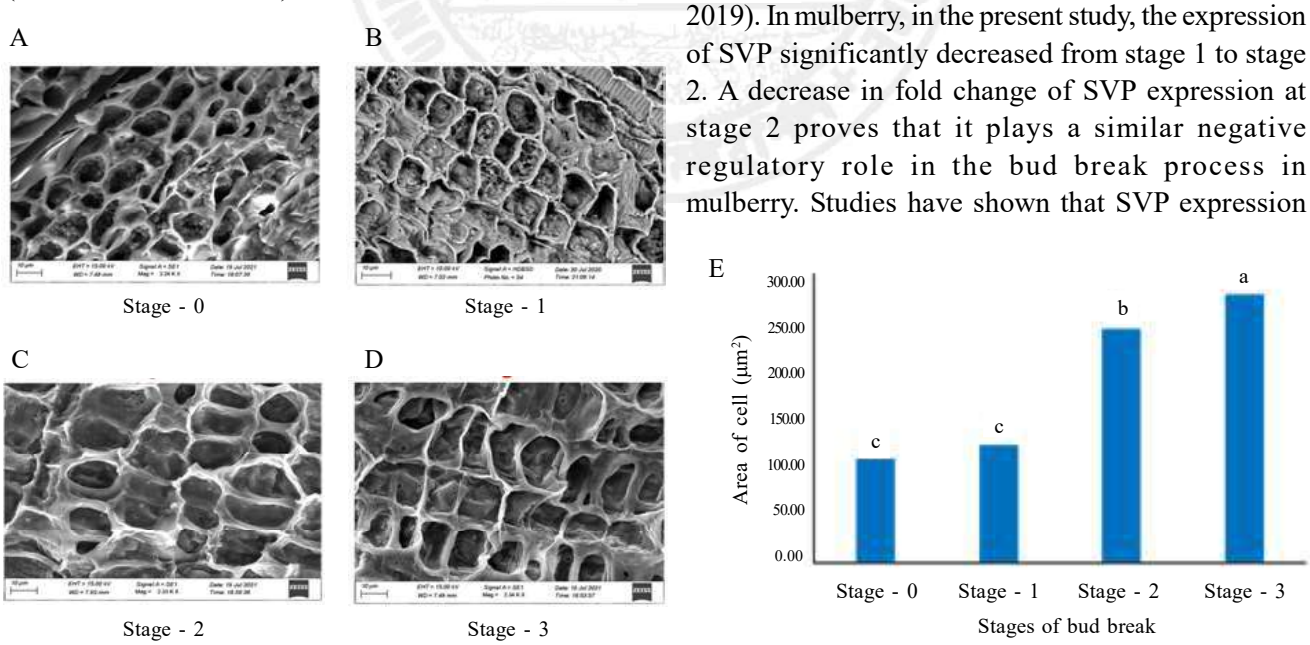

Fig. 7: Scanning electron microscopy (SEM) analysis of internal cellular anatomy corresponding to different bud break stages (A to D). (E) denotes cell size of different stages of buds represented as represented ass area (μm^2) (Bar graph with the same letter indicate no significant difference at $p<0.05$ (DMRT)

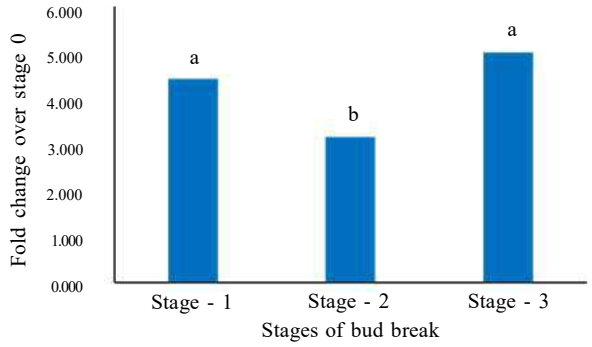

Fig. 8: Relative expression of *Short Vegetative Phase (SVP)* gene at different stages of bud break (Bar graph with the same letter indicate no significant difference at $p<0.05$ (DMRT)

and GA content acts antagonistically (Singh *et al.,* 2018). Our data is also in line with the earlier reports wherein Stage 2 has the highest GA3 content with the lowest SVP gene expression levels.

Mulberry bud break is an important trait for the timely production of foliage. The bud characteristically contains both vegetative and reproductive primordia within a single bud. The transition of bud from dormancy to active growth is mainly associated with levels of GA and ABA with corresponding anatomical changes. ABA and GA act antagonistically in induction and release of bud dormancy, respectively. Altering the levels of these hormones through agronomic practice or genetic manipulation can help in tailoring bud break in mulberry.

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The Mysore Journal of Agricultural Sciences

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