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Optimizing Cultivation Technique of *Silybum marianum* (L.) Gaertn. under Tropical Climate

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ABSTRACT

Background: A high prevalence of global liver diseases compels the availability of affordable drugs with fewer side effects. *Silybum marianum* (L.) Gaertn. is being used as a traditional medicine for centuries to treat a range of liver and gallbladder disorders. In tropical regions especially in Indonesia, *S. marianum* is scarcely cultivated. Therefore, cultivation on a large scale to initiate the independency of providing domestic medicinal raw materials demand is needed. This research was conducted to find out a suitable method in producing silymarin from *S. marianum* by optimizing cultivation technique by using foliar fertilizer (FF) applied to different planting periods and various spacing protocols.

Methods: The experiment of nine treatment packages consisted of a combination plant spacing (S) and foliar fertilizer/FF dosage (D) factors, also applied time of planting (P), were examined to obtain the highest silymarin content. This field study was designed using a randomized complete block design (RCBD).

Result: The results showed that a combination of the plant spacing of 140 × 140 cm, FF of 2 mg per plant and planting period in May, obtained the best results in the silymarin content of 1.52±0.07%. It also gave the highest estimated silymarin production per unit area that was 13.79±1.42 kg.ha^{-1}.

Key words: Foliar fertilizer, Plant spacing, *Silybum marianum*, Silymarin.

INTRODUCTION

Liver disease is one of the most common causes of death and the mortality rates continue to increase. People who suffered chronic liver diseases reach 884 million worldwide the mortality rate of up to 2 million death per year (Marcellin and Kutala, 2018). Liver disease death in Indonesia reached 55,860 or 3.51% of total deaths (WHO, 2018). According to Indonesia's national-representative community-based survey (RISKESDAS 2018) held by the Ministry of Health of the Republic of Indonesia, the hepatitis prevalency in Indonesia increased double from 2013 to 2018 (NIHRD, 2018). Liver disease has become one of the health major problems in the world. Unfortunately, in current pharma industries, there are limited traditional medicines to treat chronic liver disease so far (Anonim, 2019) accordingly, there is a need for a new drug.

Silymarin (SM), a natural active compound from the plant of *Silybum marianum* (L.) Gaertn, has gained an enormous interest as liver disorder remediation. SM is a lipophilic extract from the seeds of the plant and is composed of a mixture of flavonolignans: silibinin, silychristin, silydianin and isosilybinin (Vaknin *et al*., 2008). It has been long used as traditional herbal remedies for almost 2000 years to treat a range of liver and gallbladder disorders and to protect liver damage due to many xenobiotic substances. SM has tissue regenerative properties and shows a strong antioxidant activity which is useful as a hepato-, neuro-, nephro- and cardio-protective (Surai, 2015). Pharmacological applications *S. marinum* include anti-inflammatory, anticancer, antidiabetic, hypocholesterolemic, antitoxic and drug diseases and anti-mushroom poisoning (Qavami *et al*., 2013), photoprotective, antidepressant, antihypertensive, antimetastatic and predominantly hepatoprotective (Camini and Costa, 2020).

S. marianum with common name 'milk thistle' originated from the Middle East, the Arabian peninsula, the Indian subcontinent, North Africa and parts of Europe (CABI, 2019). It has been introduced to many countries. However, except for internal factors such as genetic variation, the silymarin content of the seeds (achenes) of *S. marianum* is also determined by external factors such as geographic and climatic conditions. Plants grown in a subtropical climate found to have a higher silymarin level rather than grown in a temperate climate (Morazzoni and Bombardelli 1995). Under humid continental to sub-tropical climate, fertilizer

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and plant spacing affected the seed yield and silymarin complex content of the *S. marianum*. Silymarin content was influenced by the application of FF combined with plant hormone (Rum *et al*., 2005; Geneva *et al*., 2008a; Geneva *et al*., 2008b).

Medicinal Plant and Traditional Medicine Research and Development Centre (MPTMRDC) has been nursing *S. marianum* in the research station area mainly for collection, preservation and public education purposes. Similar to many other introduced plants, there is scarce information about technical methods for cultivating this plant in the tropical region. The fact that liver diseases have become global health problems and silymarin possess a high potential to overcome those diseases. Otherwise, the cultivation of *S. marianum* in the tropical region, especially in Indonesia, is still scarcely conducted, larger-scale cultivation would initiate the provision of affordable raw material medicine for all people. The present study aimed to obtain a cultivation technique in maximizing the silymarin production of *S. marianum* by using foliar fertilizer (FF) applied to different planting periods and varying planting densities per unit area.

MATERIALS AND METHODS

S. marianum seedlings provided by MPTMRDC Tawangmangu. Silymarin standard purchased from Sigma-Aldrich. TLC plate GF254, ethanol, methanol, chloroform, acetone, formic acid were obtained from Merck. Gandasil B as a FF contains: 6.0% N, 20.0% P_2O_5, 30.0% K_2O, Mn, B, Cu, Co, Zn, aneurine, lactoflavin and nicotinic acid amine purchased from P.T. Kalatham Co. All chemicals used were analytical grade.

This experiment was conducted in 2019 from the period month of January to December that arranged on an RCBD consisted of nine package treatments with three replications. The treatments were the plant spacing, *i.e*: S1= 140 × 70, S2= 140 × 100 and S3= 140 × 140 cm; Foliar fertilizer (FF) dosage, *i.e*: D1= 6, D2= 4, D3= 2 mg per plant and the planting period, *i.e*: P1= February, P2= May and P3= August.

The field experiment was conducted at the Tlogodingo Research Station of MPTMRDC at 7°40′10.10″S 111°10′ 37.99″ E, the altitude of 1,750 m above sea level (msl) and the soil type: Andosol. After weed clearing, the soil was tilled by a depth of 15-30 cm then divided into three blocks, each with a size of 30 × 20 m^2. Every block consisted of nine plots with a size of 9 × 6 m^2 each, while the number of plants per plot was 63, 49 and 28 for S1, S2 and S3, respectively. FF was administrated three times; the first was at the time when the plant nearly entering the maximum vegetative growth stage (about 3 months after planting). The second and the third implementation were done at a consecutive 10 days interval after the first administration by spraying to the plant canopy. The observed parameter included plant growth and development (*i.e.*: plant height, leaves length, branch amount, stem diameter) and plant production (*i.e.*: plant fresh and dry weight, achenes fresh and dry weight and achenes silymarin content). Harvesting carried out when the achenes already mature, indicated by the capitulum nearly dry and the color turned brown. Capitula were picked up and dried under 50°C in the oven. Achenes were collected, dried and powdered before further analysis.

Silymarin content was determined using thin-layer chromatography (TLC). The silymarin standard was dissolved with ethanol in various concentrations to generate a standard curve. A volume of 5 µL of standard and sample were plotted on the TLC GF254 plate using Linomat 5 (Camag). The plate was then eluted in a saturated development chamber contained chloroform: acetone: formic acid (9: 2: 1) for ± 45 minutes. Quantitative densitometry analysis performed at λ 290 nm. The silymarin content in the extract was read by TLC-densitometer determined by interpolating the spotting area to the standard silymarin regression curve equation. The silymarin content was calculated using the equation:

$$\text{Silymarin content (\%)} = \frac{S \times V1}{V2} \div W \times 100$$

Where,
S = Amount of silymarin in a sample from the standard curve.
V1 = Volume plotted sample (µL) on the TLC plate.
V2 = Extract total volume (µL).
W= Achene powder weight (g).

The statistical analyses were performed using SPSS v.16. Data presented by mean±SD of each plant of every block treatment which compared using Duncan's multiple range test (DMRT) p=0.05 if the treatments gave significant effect base on analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The experiment was conducted on the west slope of the Lawu mountains. An initial soil analysis resulted that the andosol soil has clay loam texture, an acidic pH (pH 5.69), moderate C-organic content (2.15%). N-total, P-available, K-exchangeable and CEC, which were 0.47%, 9.65 ppm, 0.38 me % and 22.03 me %, respectively, whereas the base saturation was very low category (10.43%). Base on that result indicated that the soil needed additional fertilizer to achieve higher land productivity.

Plant height of *S. marianum* was significantly influenced by the planting period. The highest of the plant hight was obtained from planting in May, whereas planting in February gave the lowest height. Sunlight is crucial in plant growth and development. In mountainous tropical regions, the length of sunlight (daylight) is generally shorter during the rainy season (October-April) compare to that dry season (April-October). In the first three months, the growth of *S. marianum* was dominant for the vegetative phase, therefore it required higher intensity of sunlight for fulfilling a firm and robust growth. The plant density governed the intensity of the sunlight received by individual plants as a direct effect of spacing. The shorter the plant spacing, the higher the density of plant population, which enforces competition for light and many other environmental factors. The increase in light competition would stimulate apical dominance and inflict plant etiolation. Furthermore, it also influences the morphological and physiological modification that showed by the reduction of the length leaves and the number of the lateral branches (Table 1). All applied treatments on *S. marianum* affected the number of lateral branches formation. According to Qavami *et al*. (2013), its production number depend on plant density and climatic condition.

Stem diameter is not only useful for determining drought stress conditions but also important for monitoring plant nutrition status and whole-plant functioning and growth (Swaef *et al*., 2015). Application FF and plant spacing have influenced on stem diameter of *S. marianum* in the same mode as those impact of the number of branch formation. The increasing FF dose would reduce stem diameter, whereas the widening plant spacing enhanced stem diameter growth. Plant spacing, FF dosage and planting periods significantly influenced the length leaves. The longer spacing, the shorter the leave grows. The higher the FF dosage, the shorter the length leaves. The changes morphological traits were pronounced by the interaction of both external and internal factors.

The wider plant spacing yielded to the higher the fresh weight of leaves, stems and seed (achenes) due to reducing interplant competition for water, light and nutrients (Table 2). The larger the plant spacing, the higher the intensity of sunlight received which plants could take advantage of for better growth and development. Planting in August gave the highest stem FW but it resulted in other lowest parameters such as leaves FW, seed fresh and dry weight. Better seed quality is obtained from *S. marianum* planted in May than those in other planting periods which showed in higher 1,000 seed DW. Sunlight that affects the growth and development of plants consists of three important factors, namely quality, duration of exposure and intensity. Plant in May received more intent and longer duration exposure to sunlight during both generative and vegetative phase. Even though planting with larger plant spacing resulted in better growth and higher yields, but too large spacing would yield the reduction of product per unit area.

FF influencing the number of capitulum which correlated to the number of branches since flower heads emerge and develop at the apex of the stems. The more branches formed, the more capitulum potentially produced. A higher number of capitulum was obtained from a lesser FF dosage regardless of whenever planting periods were undertaken (Fig 1). The highest capitulum yield was found from *S. marianum* planted in May, followed by that in February and that in August respectively. Whereas combination treatments of plant spacing 140 × 140 cm, FF dosage 2 per plant and planting period in May produced the highest capitulum number. Planting *S. marianum* in August bearing the lowest capitulum number indicated that the flowering stage was affected by climatic conditions, especially sunlight. In the

Table 1: The influence of plant spacing (S), foliar fertilizer dosage (D) and planting interval (P) on the growth of *Silybum marianum* (L.) Gaertn.

Treatment	Plant height (cm)	Lenght leaves (cm)	Number of branch	Stem diameter (cm)
S1D1P1	105.36± 7.10^{b}	54.57±4.56^{bc}	6.59±1.14^{c}	3.24±0.50^{c}
S2D2P1	88.35±14.04^{c}	55.56±5.18^{b}	6.80±0.70^{c}	3.68±0.23^{abc}
S3D3P1	87.95±11.10^{c}	61.95±2.58^{a}	9.70±0.68^{ab}	4.56±0.32^{a}
S1D1P2	148.81±2.47^{a}	49.80±2.64^{cd}	7.05±0.45^{c}	3.12±0.21^{c}
S2D2P2	146.76±4.66^{a}	51.76±3.17^{bcd}	8.10±0.96^{bc}	3.97±0.35^{abc}
S3D3P2	145.98±4.99^{a}	52.16±1.17^{bcd}	11.33±1.84^{a}	4.29±0.78^{ab}
S1D1P3	115.47±5.89^{b}	46.61±1.44^{d}	8.28±1.97^{bc}	3.51±0.78^{bc}
S2D2P3	114.57±3.01^{b}	46.94±0.53^{cd}	8.36±0.09^{bc}	3.61±0.36^{bc}
S3D3P3	112.96±2.39^{b}	52.66±2.32^{bc}	8.95±1.58^{bc}	3.81±0.37^{abc}

Note: Values in the same column followed by difference superscript letters indicate statistically different according to DMRT (P<0.05).

Table 2: The influence of plant spacing (S), foliar fertilizer dosage (D) and planting interval (P) on the fresh (FW) and dry weight (DW) of *Silybum marianum* L. Gaertn.

Treatment	Leaves FW (g)	Stem FW (g)	Seed FW (g)	Seed DW (g)	1,000 seed DW (g)
S1D1P1	61.73±5.24^{b}	37.63±2.06^{d}	46.30±12.97^{de}	40.12±11.16^{de}	18.9±1.00^{b}
S2D2P1	83.33±27.78^{b}	63.42±11.83^{c}	50.00±5.56^{de}	42.96±3.57^{de}	17.8±0.68^{c}
S3D3P1	152.78±26.79^{a}	102.29±9.27^{b}	69.44±10.49^{cd}	63.89±10.48^{cd}	17.8±0.46^{c}
S1D1P2	59.26±11.11^{b}	40.65±9.64^{d}	92.47±17.73^{c}	87.65±16.80^{c}	24.4±0.47^{a}
S2D2P2	70.37± 8.49^{b}	44.68±3.68^{cd}	131.48±21.03^{b}	124.07±20.09^{b}	24.5±0.58^{a}
S3D3P2	83.78±8.34^{b}	36.96±8.16^{d}	186.11±28.36^{a}	177.78±28.36^{a}	23.9±0.59^{a}
S1D1P3	22.22±2.55^{c}	123.52±6.38^{ab}	33.39±4.37^{e}	28.55±4.48^{e}	12.7±0.92^{d}
S2D2P3	23.61±1.73^{c}	133.82±18.86^{a}	40.01±3.01^{e}	34.64±3.28^{e}	12.6±0.12^{d}
S3D3P3	24.72±2.10^{c}	120.37±7.28^{ab}	56.67±8.87^{de}	52.26±3.57^{de}	11.8±0.42^{d}

Note: Values in the same column followed by difference superscript letters indicate statistically difference according to DMRT (P<0.05).

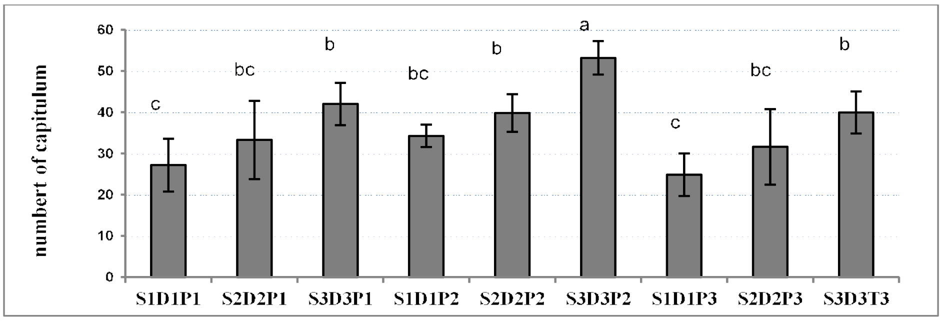

Fig 1: The influence of plant spacing (S), foliar fertilizer dosage (D) and planting interval (P) on the number of *Silybum marianum* (L.) Gaertn capitulum. Bar with different letters indicate statistically different according to DMRT (P<0.05).

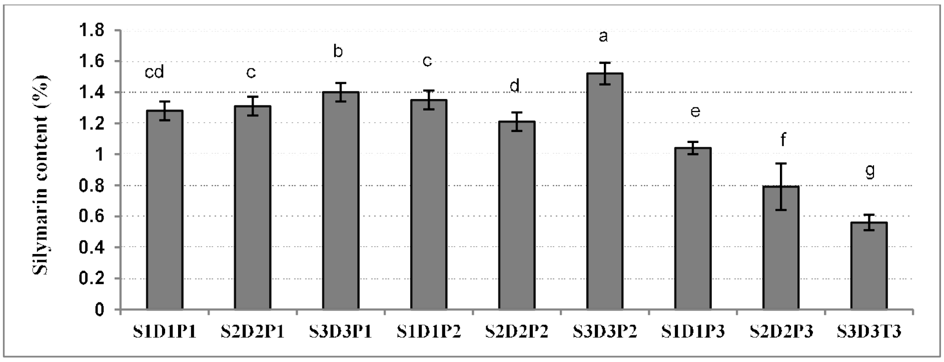

Fig 2: The influence of plant spacing (S), foliar fertilizer dosage (D) and planting interval (P) on silymarin content of *Silybum marianum* (L.) Gaertn achenes. Bar with difference letters indicate statistically difference according to DMRT (P<0.05).

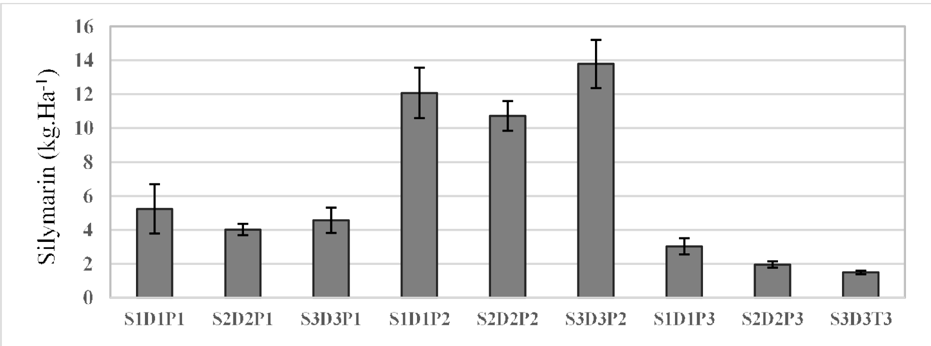

Fig 3: The estimation of silymarin production per Ha of *Silybum marianum* (L.) Gaertn. by cultivating treatment of plant spacing (S), foliar fertilizer dosage (D) and planting interval (P) under tropical climate.

subtropical region, the plant naturally fowers from April to May and the achene ripens in July (Das *et al*., 2008).

Silymarin content was measured from extracted achenes of the mature capitulum. A single capitulum can produce approximately 50-150 achenes. Generally, the content of the silymarin complex of *S. marianum* achenes is about 0.2-0.6%. However, the content could reach 2.0% from a breed cultivar plant (Habán *et al*., 2009). In the present study, the highest silymarin content was 1.52±0.07% that obtained from the combination treatment of plant spacing of 140 × 140 cm, FF 2 per plant which and planted in May, followed by the same treatment that planted in the February which gaining silymarin content of 1.40±0.06% (Fig 2). The highest silymarin estimated production per unit area, calculated by multiplying dry seed production per plant with the number of plants per Ha and silymarin content, was also obtained from that treatment combination, which was 13.79±1.42 kg.ha^{-1} (Fig 3). Interestingly planting in August regardless of any treatments, gave the lowest silymarin content. According to Andrzejewska and Sadowska (2007), the content of the silymarin complex is more correlated with the weather conditions during the vegetative period than other factors.

The environmental moisture highly affected silymarin accumulation *S. marianum*, not only excessive water soil, but also deficient moisture would also hamper the silymarin production. Planting in August resulted in the lowest silymarin content due to a higher precipitation rate during

November-December in most tropical regions at which time the plants entering fruit-bearing stages. According to Qavami *et al*. (2013) moderate irrigation level (60% field capacity) resulted in plants with the highest silymarin production.

CONCLUSION

S. marianum is one of the important medicinal plants in the world. The achenes (seeds) contain a high amount of a mixture of flavonolignans called silymarin. Silymarin showed a remarkable pharmacological activity that beneficial properties to maintain health, particularly liver and gallbladder disorder medication. The silymarin product accumulation in achenes is affected by plant variety nature, geographic and climatic conditions. Though this plant is native to the mediterranean region, it is potentially cultivated under tropical climates. Various treatments in the cultivation of *S. marianum*, including fertilizer, plant spacing and planting period affect both plant development and plant production. The field experiment at high altitude area (±1700 msl) on andosol vulcanic soil with combination treatments of planting space 140 × 140 cm, FF 2 mg per plant which planted in May resulted highest silymarin content (1.52±0.07%).

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Autors' contribution

Heru Sudrajad was responsible for the research design, methods design, collecting field experiment data, references collection, managing field experiment, manuscript drafting. Harto Widodo worked on data analysis, collecting data from a field experiment, manuscript drafting and finishing. Fauzi conducted data collection and laboratory analysis. Yuli Widiyastuti dealt with methods design and finishing manuscript.

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