การเพาะพันธุ์หญ้าใบมะกรูดในห้องปฏิบัติการ โดยการใช้ต้นเดี่ยว

Laboratory Culture of *Halophila ovalis* Using Single Shoot Method

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บทคัดย่อ

การปลูกหญ้าทะเลโดยการใช้ต้นเดี่ยว มีการนำไปใช้กันอย่างแพร่หลายในการปลูกหญ้าทะเลเพื่อการฟื้นฟู ข้อดีของการใช้ต้นเดี่ยวคือ มีผลกระทบต่อพื้นที่แหล่งหญ้าทะเลน้อย และใช้จำนวนต้นในการปลูกน้อย อย่างไรก็ดี ความสำเร็จที่ยั่งยืนของการปลูกหญ้าทะเลเพื่อการฟื้นฟูต้องการการเตรียมพันธุ์กล้าหญ้าทะเลที่ดี และมีความเหมาะสมในการนำไปปลูกในพื้นที่ธรรมชาติ หญ้าใบมะกรูด (*H. ovalis*) เป็นหญ้าทะเลที่มีขนาดเล็ก มีลำต้นทอดยาวและแตกแขนงอยู่ใต้ดิน ตลอดจนมีช่วงปล้องยาว จึงเหมาะแก่การเตรียมต้นกล้าเป็นต้นเดี่ยว การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อเปรียบเทียบการเจริญเติบโตของหญ้าใบมะกรูดที่อยู่ภายใต้วิธีการเตรียมที่แตกต่างกัน ทดลองปลูกโดยการใช้ต้นเดี่ยวของหญ้าใบมะกรูดในดินโคลนปนทรายภายในถ้วยกระดาษและแผ่นกระสอบป่านในเรือนเพาะพันธุ์หญ้าทะเล ผลการศึกษาเมื่อสิ้นสุดการทดลองแสดงให้เห็นว่าอัตราการเจริญเติบโตและอัตราร้อยละการอยู่รอดของหญ้าใบมะกรูดที่เติบโตในถ้วยกระดาษและแผ่นกระสอบป่านมีความแตกต่างอย่างมีนัยสำคัญ (p <0.05) โดยอัตราการเจริญเติบโตและอัตราร้อยละการอยู่รอดของการเพาะพันธุ์หญ้าใบมะกรูดในถ้วยกระดาษเท่ากับ 0.10 ± 0.002 ต้นต่อวัน และ 73 ± 5.59% ตามลำดับการเพาะพันธุ์หญ้าใบมะกรูดด้วยแผ่นกระสอบป่าน พบว่ามีอัตราการเติบโต (0.07 ± 0.001 ต้นต่อวัน) และอัตราร้อยละการอยู่รอด (63 ± 8.58%) ซึ่งต่ำกว่าหญ้าใบมะกรูดที่เติบโตในถ้วยกระดาษ อัตราการตาย อัตรางอกใหม่และอัตรางอกใหม่สุทธิ ไม่มีความแตกต่างอย่างมีนัยสำคัญ การปลูกหญ้าใบมะกรูดในถ้วยกระดาษซึ่งบรรจุดินโคลน

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ปนทราย มีอัตราร้อยละการอยู่รอดสูงและอัตราการตายต่ำ เนื่องจากธรรมชาติของหญ้าทะเลต้องการพื้นที่สำหรับการเจริญเติบโตขยายรากเหง้าฝังลงในดิน

คำสำคัญ: การเตรียมพันธุ์กล้า, หญ้าใบมะกรูด, วิธีการใช้ต้นเดี่ยว, การฟื้นฟูหญ้าทะเล

ABSTRACT

Seagrass planting using the single shoot method was applied in the large-scale of seagrass restoration project. The benefits of using the single shoot method are to minimize the impacts to donor site and the number of shoots. However, the sustainable achievement of seagrass planting for restoration requires a good preparation and should be convenience when taking to plantin natural habitat. To develop the suitable technique for planting small seagrass like *Halophila ovalis*, the presented study aimed in order to compare the growth performance of sibling under the difference preparation methods. The experiment was conducted by planting a single shoot of *H. ovalis* in sand-muddy soil within a paper-cup and hessian mat outdoor laboratory which natural light, temperature and sea water were used. Significant differences in growth rate and survival percentage between growing in paper-cup and hessian mat were found at the end of experiment (p<0.050). The growth rate and survival percentage of sibling culture in paper-cup were 0.10±0.002 shoot.day^{-1} and 73±5.59%, respectively. The sibling culturing on hessian mat showed lower growth rate (0.07±0.001 shoot.day^{-1}) and survival percentage was 63±8.58%. No significant differences were found on recruitment rate, mortality rate and net recruitment. The survival percentage higher and lower mortality rate for sibling planting in paper-cup possibly due to the nature of seagrass needs extensive space for rhizome network buried into sediment were found.

Key words: sibling preparation, *Halophila ovalis*, single shoot method, seagrass restoration

INTRODUCTION

Seagrasses are marine flowering plants important and ecosystem services to coastal marine environments, habitat and nursery for many marine species (Orth *et al*., 2006; Pratep *et al*., 2010; Short *et al*., 2007). Seagrasses is also an importance food source of many endangered species such as sea turtle and dugong (Nakaoka and Aioi, 1999; Nakanishi *et al*., 2006; Adulyanukosol and Poovachiranon, 2006). Kirkman and Kirkman (2002) had reported that the losses of seagrass in Thailand were around 20-30%. The major losing was a result of anthropogenic disturbances, such ascoastal development, over fishing and aquacultures were negatively affecting seagrass beds (Satumanatpan and Plathong, 2003).

As seagrass area is declining worldwide (Kamil *et al*., 2013),therefore many countries are considering to plant for restoration project. Seagrass planting method have often used to increase habitat and restoration (Calumpong and Fonseca, 2001; Fonseca *et al*., 1998). However, technique of planting is one of successful factor (Bastyan and Cambridge, 2008; Calumpong and Fonseca, 2001; Fonseca *et al*., 1998; Hamminga and Duarte, 2000; Katwijk and Hermus, 2000). In addition the planting method such as shoot with sediment intact method, sediment free and seeding method are limitation to specific of species.

Single shoot is one of sediment free method, include shoot along with a length of rhizome. Single shoot is a simpler technique for seagrass restoration, either anchored or unanchored shoot planted. The shoots could be planted either in single or in group (Davis and Short, 1997). The large-scale restoration projects using single shoot method for *Zostera marina* and *Posidonia australis* showed high survival and growth rate (Orth *et al*., 1999; Bastyan and Cambridge, 2008). Therefore, the selection of species is considered factor which have influence to project success (Fonesca *et al*., 1998; Hemminga and Duarte, 2000). The physiological aspects and life histories of seagrass are needed to consider before make a planting project. Some species such *Thalassis testudinum* and *Enhalus acoroides* are slow-growing, slow coverage rate and climax species. Their expansion was 0.5 m yr^{-1} and 1.5 m $yr^{-1}$for such *T. testudinum* and *E. acoroides*, respectively (Fonesca *et al*., 1998; Hemminga and Duarte, 2000). In comparison *Halophila ovalis* is a short of life-span seagrass and has fast horizontal rhizome growth rate. Their expansion was more than 5 m yr^{-1}. It is the pioneer species and has a single internode between shoot. The space length of rhizome is close together along the rhizome axis (Fonseca *et al*., 1998; Hamminga and Duarte, 2000; Calumpong and Fonseca, 2001). The new shoot by rhizome plantings is considered a reliable meadow restoration technique, there is accelerate vegetation expansion and establish technique (Balestri and Lardicci, 2006). Therefore *H. ovalis* may be had potential for a single shoot method developing. However, their single shoot is small and separate shoot. The preparation should be ensured that the shoot is healthy when planted; the shoots have intact meristems enhancing their expansion; they have enough shoots to facilitate growth and minimization of stress (Fonseca *et al*., 1998). Transplantation can be rendered almost totally ineffective if meristematic regions of these plants are damaged or non efficient and quality in a plant to initiate growth. The planting without sibling preparation is common facing on death of function organ and loss of avoid respiratory (Hamminga and Duarte, 2000). That will be made to ensure the presence of growing rhizome apical meristem in individual planting (Fonseca *et al*., 1998).

This study was aimed to compare the growth performance in term of growth rate, recruitment rate, mortality rate, net recruitment (R_{net}) and survival percentage of *H. ovalis* in laboratory using the single shoot method.

MATERIALS AND METHODS

Collection of adult and healthy plant

The sediment, substrate for experiment and the *H. ovalis* were sampling during low tide. Sediment, healthy adult plants with attached root and rhizome were shoveled from a small donor seagrass bed located at Boon-Kong Bay, Trang Province, southwestern Thailand (37 25′ 19.1″N, 122 05′ 06″W). *H. ovalis* were carefully cleaned using seawater. The intacted plants were kept wet while transported to laboratory and soak in seawater for 48 hrs., approximately. Sediments were sieved using a mesh size of 0.5 mm for sorting out the debris and worm.

Experimental designed

The experiment was designed to test the growth performance under different substrates, natural soil substrate and hessian mat substrate. The experiment was established in Seagrass Seeding Nursery, Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya. The single shoot, comprising rhizome, root and pair of leaves was cut from either expanding rhizome axis by scissors. Each treatment prepares for six replicate and ponds size 1x1x0.5 m. were used. Each pond were containing of 100 shoots of planted paper-cup and hessian mat. For the natural substrate experiment the sediment sieved 0.5 sizes were usedand put into the paper-cup. One single shoot was soaked into the paper-cup containing muddy sediment. For the hessian mat substrate (25x25 cm.), root and rhizome of single shoot was carefully attached by penetrating into hessian mat (Fig.1). Four of single shoot were carefully attached on hessian mat. Sea water, salinity of 29-31 psu was filled in the ponds reach 0.4 m. in depth.

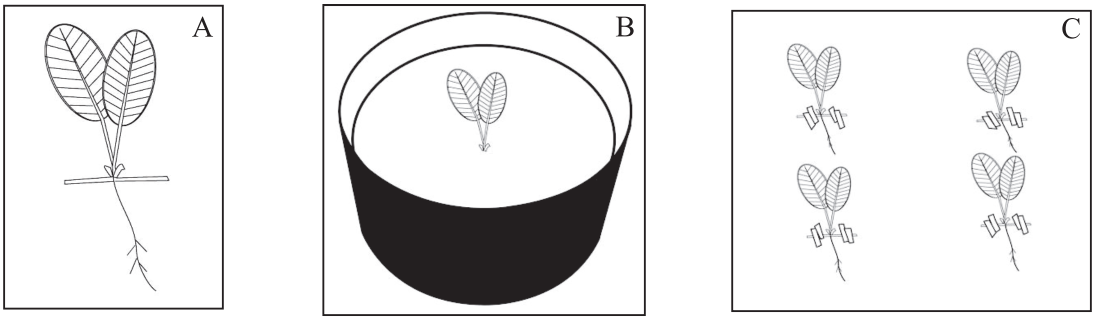

Figure 1 Preparation of single shoot and planting method: A; Single shoot. B; Planting method in paper- cup. C; Planting method in hessian mat.

Monitoring and Evaluation

The experiment was monitored for 36 days, during April to May 2013. All the single shoots were examined for the growth rate, recruitment rate, mortality rate, net recruitment (R_{net}) and survival percentage. The growth rate and recruitment rate were monitored in every 4 days by direct counted of the new shoot germinating from mother shoot. The mortality rate (M), recruitment net (R_{net}) and survival percentage were calculated using the exponential equations following (Daurte *et al*., 1994).

$$\text{Recruitment rate (R)} = \frac{(ln\ N_0 - ln\ (N_0 - N_{lost})}{t}$$

$$\text{Mortalityrate (M)} = \frac{(ln\ (N_0 + N_{new}) - ln\ N_0)}{t}$$

Net recruitment (R_{net}) = *R-M*

Survivalpercentage (%) = (Number of survival x 100)/Number of total (n=600)

Growth rate = Shoot production (shoot.day^{-1})

Where N_0 is the number of shoots present at beginning of each observation period, N_{new} is the number of new shoots; *t* is the duration of the observation period in days and N_{lost} is the number of previously shoot missing at the end of observation period.

Calculation of growth is base on the measurement of mature part (above) and the appropriate plastochrone interval. Count all of the new shoots produced. Shoot production (shoot day^{-1}) is the number of new shoots produced since rhizome tagging.

Salinity refractometer (ATAGO S/Mill-E), pH Meter Clean CS 1930 plane pH electrode were used in salinity pH and water temperature, respectively were measured weekly throughout the monitoring period.

Statistical analysis

Prior to analysis, data were transform ($\sqrt{x+0.5}$) and examined for normality using Kolmogorov-Smirnov tests, then examined for homogeneity of variances using Levene's tests. Differences were considered significant at a probability level of $p<0.05$. The mean of all variables, were tested using Mann-Whitney U-test to determine if non-normal distribution data. Statistical analysis was performed using SPSS version 21for all variables, Growth rate, Recruitment rate, Mortality rate, Recruitmentnet and survival percentage were analysis using Test of Reliability, comparison of attributes in a sample (T-Test).

RESULTS AND DISCUSSION

The measured water quality parameters in the experiment such as water temperature, pH and water salinity were narrow fluctuation. Weekly of water temperatures in experiment was 27.93±0.89°C while the pH was 8.16±0.06. The salinity of sea water using for seeding culture was 29.01±1.07 psu.

The growth rate and recruitment rate of both treatments were showed descending in the initial period and declining in later. The growth rate (shoot.day^{-1}) of *H. ovalis* soaking in paper-cup and attaching on hessian mat were 0.10±0.02 and 0.07±0.01, respectively. The result showed that cultivating in paper-cup revealed the highest growth rate at the 16 days of observation, while planting on hessian mat showed the highest values at 24 days. The culturing both treatments were showed the trend of declining after 36 days (Fig.2). There was significant difference of growth rate between paper-cup and hessian mat (p<0.05).

Recruitment rate (day^{-1}) of *H. ovalis* soaking in paper-cup was 0.06±0.01 (day^{-1}), while recruitment rate of *H. ovalis* planted on hessian mat was 0.05±0.00 (day^{-1}). The result showed that growing in paper-cup revealed the highest recruitment rate at 16 and 24 days of observation while cultivated on hessian mat showed at 16 and 24 days (Fig.3). The recruitment rate observing in both treatments was showed the trend of declining after 36 days. There was no significant difference between paper-cup and hessian mat.

At the end of the experiment; the population dynamic of *H. ovalis* were assessed the recruitment rate, mortality rate, recruitment net and survival percentage of shoots in laboratory (Table 1 Fig. 4). The result showed that; survival percentage of seeding cultivate in paper-cup was showed 73±5.59% which higher than cultivating on hessian mat 63±8.58% (Fig. 4D) (p<0.05). The mortality rate (day^{-1}) of soaking in paper-cup was showed lower than on hessian mat.Their mortality rate was 0.007± 0.002 day^{-1} and 0.008±0.004 day^{-1}, respectively (Fig. 4B). There were no significant differences statistical. The recruitment net of paper-cup treatment was 0.06±0.01 day^{-1} which has higher than hessian mat treatment (0.05±0.02 day^{-1})

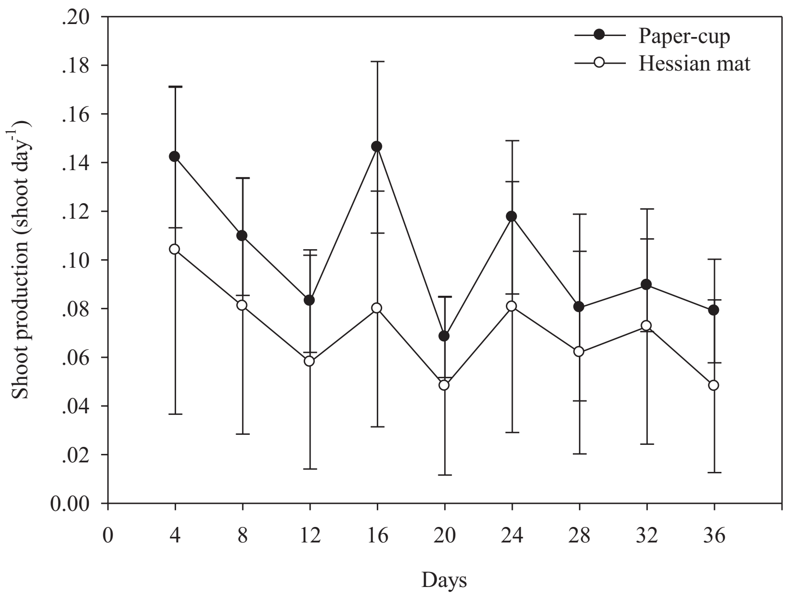

Figure 2 Shoot production (shoot.day^{-1}) of *H. ovalis* culture in laboratory.

(Fig. 4C). There was no significant difference between the treatments.

Results of presented study demonstrated that it is possible to plant *H. Ovalis* in to areas that were previously vegetated with seagrass. Healthy, adult plant and removal of rhizome are normally of single shoot method. Pre-planting and laboratory culture for planting are standard practices developed for reforestation in new area or seagrass bed should be well developed before they are planting in the field (Balestri and Lardicci, 2006).

The growth of new shoot in laboratory are similar to new shoot reported for *H. ovalis* elsewhere which the first week after planting, *H. ovalis* began to multiply propagation and produce new shoot that continue propagated (Bujang *et al*., 2008).

The growth rate of *H. ovalis* in laboratory was high in the initial month similar to growth rate reported for *Halodu leunivernis*, *H. pinifolia* and *Ruppia maritina* (Wagey, 2012). It can be distinguished in their adaptation to new environmental conditions (Meinesz *et al*., 1991).

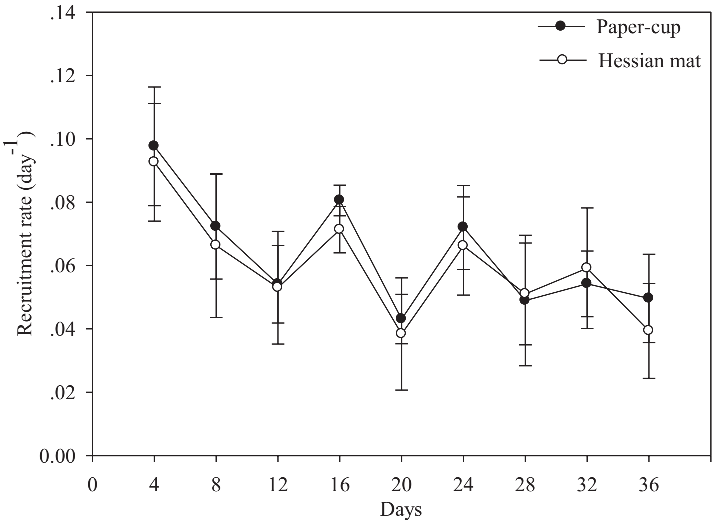

Figure 3 Recruitment rate (day^{-1}) of *H. ovalis* culture in laboratory.

Table 1 Growth performance parameter of H. ovalis were planted in laboratory by single Shoot technique

Substrates	Growth performance parameters				
	Growth Rate (shoot.day^{-1})	Recruitment Rate (day^{-1})	Mortality Rate (day^{-1})	Recruitment $_{net}$ (day^{-1})	Survival (%)
Paper cup	0.10±0.02*	0.06±0.01	0.007±0.002	0.06±0.01	73±5.586*
Hessian mat	0.07±0.01*	0.05±0.00	0.008±0.004	0.05±.0.02	63±8.579*

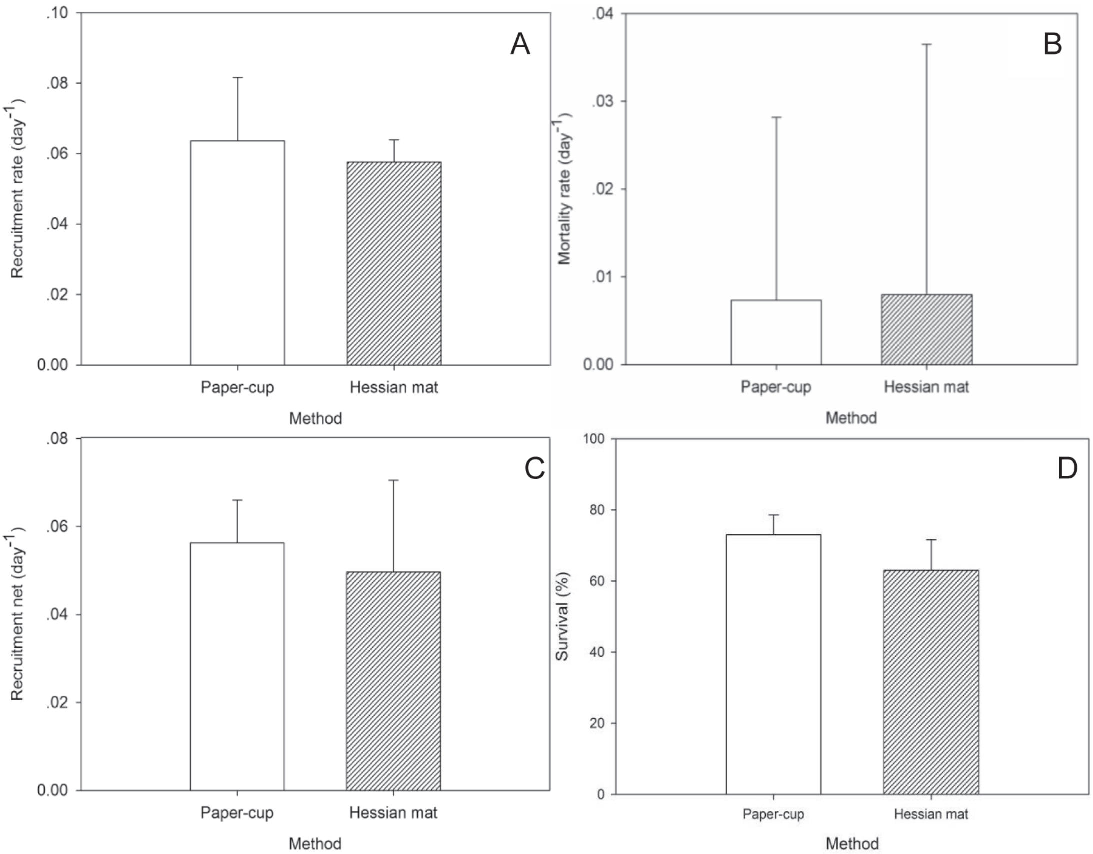

Figure 4 Evaluationsof *H. ovalis* culture in laboratory were conducted in 36 days of both methods; (A) Recruitment rate (day^{-1}), (B) Mortality rate (day^{-1}), (C) Recruitment net (R_{net}) and (D) Survival percentages of *H. ovalis* culture in laboratory.

The mortality rate of the shoot was highest in hessian mat. The dead of the shoots and the rhizome were observed which the scission zone infection. These may have been related to bacterial infection by manifested white gel coating over their apical meristem zone (Meinesz *et al*., 1991). The bacterial infections influenced the mortality rate which the mortality rate was high during the first few months (Meinesz *et al*., 1991) and mortality rate fell in later stages (Hillman *et a*., 1995; Meinesz *et al*., 1991).

Demographic estimates obtained in this study is expanded within the range of value is 1.4-1.5 day^{-1}, demonstrate that positive of R_{net}. Quantification of the shoot demographic parameter for detection of decline and can be assessed new shoot and reasonable time to preparation of seagrass for planting restoration. The high growth rate of *H. ovalis*is associated with a high mortality rate, although it is true that this pioneer species will form dense meadows much more quickly (Fonseca *et al*., 1998)

The leaf sizes of *H. ovalis* were different between planted in the paper-cup and hessian mat which the leaves of *H. ovalis* in paper-cup are bigger than hessian mat. This may have been related to difference in the substrate type (Hillman *et al*., 1995) and the source of nutrient . Sediment native into the paper-cup is a source of nutrient but hessian mat is not a source of nutrient while nutrients are essential for seagrass growth (Kilminster *et al*., 2006). This may have been nutrient availability may limit *H. ovalis* leaves size owing to leaves of *H. ovalis* is a part which up-take the nutrient from the sediment (Bujang *et al*., 2010). However *H. ovalis* is a canopy species they were competition for space and nutrient (Bujang *et al*., 2010).

CONCLUSIONS

The result demonstrates that planting using single shoot technique of *H. ovalis* is viable method and can be improve to restoration activity. The recruitment rate and mortality rate are influence to recruitment net. Recruitment and mortality are only indication of the population dynamics which undergoing at least transient decline demonstrated is negative net of population (R<M) and positive net change R>M indicate thickening stands of demonstrate recruitment net. Although recruitment net of both method were shown approximately. Therefore, the both methods are required a good site and efficiency for seagrass planting restoration.

However nature of seagrass need extensive rhizome network buried into sediment for propagation.Populations of *H. ovalis* require more energy for propagation as they were competition for space and nutrient.

ACKNOWLEDGMENT

I am grateful to Wanvisa Ponpai and Tosaporn Klomkleang for their field works.

I am grateful to Assoc Prof.Dr.Suwat Tanyarosand Dr.Woraporn Tarangkoon on their supports for my work

This research was supported by Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya.

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