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Effect of high temperature stress on changes in morphology, anatomy and chlorophyll content in tropical seagrass *Thalassia hemprichii*

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Abstract. This study aimed to discover the response of *Thalassia hemprichii* due to high temperature stress treatment (40.5°C). Morphological and anatomical changes of leaf, rhizome and root, and chlorophylls content were investigated. The results showed that morphological changes were organ damage, changes of colors and texture. Anatomical changes were indicated by changes in leaf thickness, size of epidermis and mesophyll tissues in leaf; size of pith, cortex and aerenchyma tissues in rhizome; pith and aerenchyma tissues of root. While the cortex and endodermis tissues of the root did not show any changes. Chlorophyll a, chlorophyll b and total chlorophyll contents significantly declined due to heat stress.

Key Words: heat stress, seagrass, leaf, rhizome, root, chlorophyll.

Introduction. Rising temperature, due to global warming, has not only affected on terrestrial organisms but also on coastal organisms. Heat stress because of high temperatures, could affect whether plant morphology, anatomy, physiology, photosynthetic capability, and genetic expression of plants (Chen et al 2014). Moreover, various physiological changes occur in plants such as scorching of leaves and stems, leaf abscission and senescence, shoot and root growth inhibition (Bita & Gerats 2013; Teixeira et al 2013). Heat stress also affects the photosynthesis, respiration, water relations and membrane stability, and modulates levels of hormones, and primary and secondary metabolites (Hemantaranjan et al 2014).

Seagrasses are key-species in coastal ecosystems, as important primary producers providing food, nursery and shelter for many marine species (Orth et al 2006). However, declining seagrass ecosystem has been reported throughout the both northern and southern hemispheres (Waycott et al 2009). Collier & Waycott (2014) reported that high temperatures during low tide could decline the rate of growth and even induce seagrass mortality. During tidal exposure, intertidal seagrass is susceptible to extreme radiation doses, desiccation, thermal stress and excess light leading to organ damage (Campbell et al 2006). To date, study about seagrass response under heat stress is still limited on temperate seagrass such as *Zostera marina* and *Posidonia oceanica* (Bergmann et al 2010; Marin-Guirao et al 2016). Only few studies have addressed to tropical seagrass.

Thalassia hemprichii (Ehrenb.) Aschers. is among the most widely-distributed seagrass species in the SE Asian region, especially in Indonesian waters (Nienhuis et al 1989; Aswandy & Azkab 2000). It means that *T. hemprichii* is a seagrass endemic to tropical waters so it may be used as an ideal model that could describe the ecological conditions of tropical coastal waters. Nugraha et al (2017) revealed that water temperature in Seribu Islands, where *T. hemprichii* inhabited, reached 34°C. Massa et al

(2009) also reported that the temperature of the shallow water during daylight low tide could reach 38°C. Collier & Waycott (2014) reported the worst record of shallow water temperature. It was 43°C. Nevertheless, the optimal temperature for seagrass to photosynthesis and growth ranges from approximately 30 to 35°C (Pedersen et al 2016). Extreme events occurring during low tide have been linked to seagrass loss (Massa et al 2009; Rasheed & Unsworth 2011).

Study about morphological and anatomical changes become important since limited details and plants with high temperatures stress symptoms are generally similar to those under drought stress. Thus, this study was aimed to determine morphological and anatomical changes of *T. hemprichii* under heat stress. Chlorophylls content also were observed to describe photosynthesis response during heat stress.

Material and Method. The study was conducted from February to May 2018 at Department of Biology, Faculty of Sciences and Technology, Airlangga University. The experiment was performed in hydroponic culture system under aquarium conditions based on adopted methods of Purnama et al (2015). Samples of *T. hemprichii* (10-15 cm from base to tip of leaf) were obtained from the north coast of Lamongan (6°52'40.8"S; 112°12'50.5"E). The experimental design consisted of two groups (heat and normal) replicated ten times in a split plot design. The whole cores including seagrass and rhizosphere sediment were collected to ensure that the samples were complete and undamaged. Then the intact plants were cultured in aerated aquarium (60 x 30 x 40 cm^3) filled with 100 L of seawater (salinity 30-33‰) from Kenjeran Beach, Surabaya. Plants of equal size were placed in each of the 2 aquaria. A total of 60 samples of *T. hemprichii* were divided into 2 treatment groups: heat (treatment with thermal stress) and normal (control at ambient temperature). Before heat treatment, seagrass samples were acclimatized for one week in laboratory conditions (light intensity of 250 μmol m^{-2} s^{-1} at temperature of 26±2°C). The temperature in the heat stress treatment aquaria was slowly increased from 28 to 40.5°C (increased 1.5°C d^{-1} over 8 d). The heat source was obtained from the water heater connected to the digital Thermostat STC 100 to maintain temperature. Seagrass in aquaria without thermal stress kept with each set of experiment as control.

Sample leaf, rhizome and root were collected in last day from both conditions, cleaned from epiphytes and blotted dry using tissue paper. Morphological changes were investigated by describing the appearance, color, texture and presence of organ damage. Anatomical measurements were performed on the fifth fully expanded leaves (numbered from the center), rhizomes and longest roots (measured from rhizome). Samples were prepared for a standard paraffin sectioning according to adapted methods of Purnobasuki et al (2017). They were fixed and preserved in FAA (70% ethanol, 10% formalin, 5% acetic acid - 90:5:5). The air in the tissue was evacuated by oil rotary vacuum pump (Model SW-20, Sato Vacuum Machinery, Japan) for several times. Samples were dehydrated in an ethanol-xylene series (50, 70, 85, 95 and 100% ethanol, ethanol-xylene 1:1, xylene 100%) and embedded in paraplast plus (Oxford Labs, USA) in 59°C. Sections were cut longitudinally at 8-12 μm thick by rotary microtome HM 350 Microm (Heidelberg Germany), stained in Safranin O-Fast Green and permanently mount. Finally, observation work was done on a light microscope Mikroskop CX.20. Microscopic images were captured by Optilab camera using Optilab Viewer 2.2. Anatomical measurements were conducted by measuring epidermis, mesophyll and leaves thickness; stele diameter, cortex and aerenchyma thickness of rhizomes; and stele diameter, cortex, endodermis and aerenchyma thickness of roots used Image Raster 3.0.

Chlorophyll content was obtained by rinsed in 85% acetone solution, which is based on Lichtenthaler (1987), and measuring its absorbance using S-22 UV/Vis spectrophotometer (Boeco, Germany) at λ = 663 nm and λ = 645 nm. Chlorophyll a, chlorophyll b and total chlorophyll content were determined as mg L^{-1}. The data were statistically analyzed using SPSS ver. 22. The significance of differences between variables at $p < 0.01$ or $p < 0.05$ was checked with using Independent Samples t-Test.

Results. Morphological comparison of intact individual of *T. hemprichii* between heat treatment and normal group was shown on Figure 1. Normal individual had a brighter color and compact and firm texture, whether in leaf, rhizome and root. The opposite, heat-treated individu had a darker color and fragile texture. Both leaves and roots were easily detached from their rhizome.

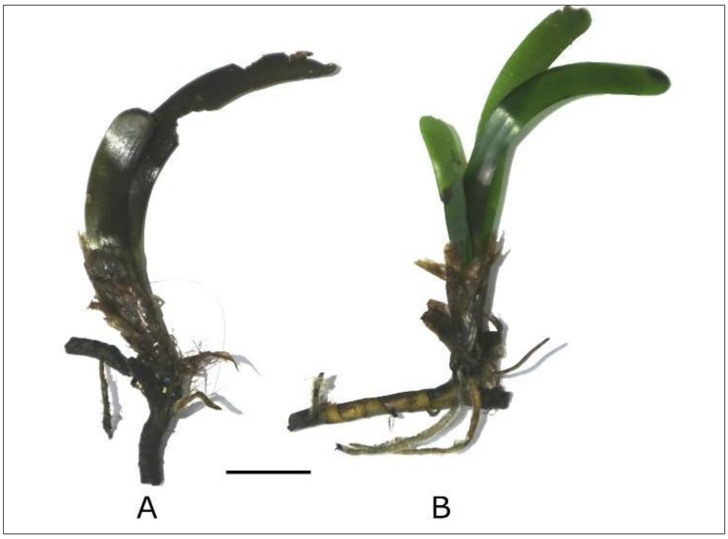

Figure 1. Comparison of the morphology of two intact individuals of *T. hemprichii.*
A. Individual of heat-treated group; B. Individual of control group. Bar scale: 2 cm.

Leaves of normal *T. hemprichii* were greenish color while leaves from heat-treated had brownish-green color. It is assumed that *T. hemprichii* leaves were experiencing chlorosis symptom. Leaves damage also shown at heat-treated *T. hemprichii*. Rhizomes of normal *T. hemprichii* were yellowish-green color as well as roots color (Figure 2). Otherwise, the color of rhizomes and roots of heat treatment was brownish black. It seemed that individual from heat treatment underwent decay.

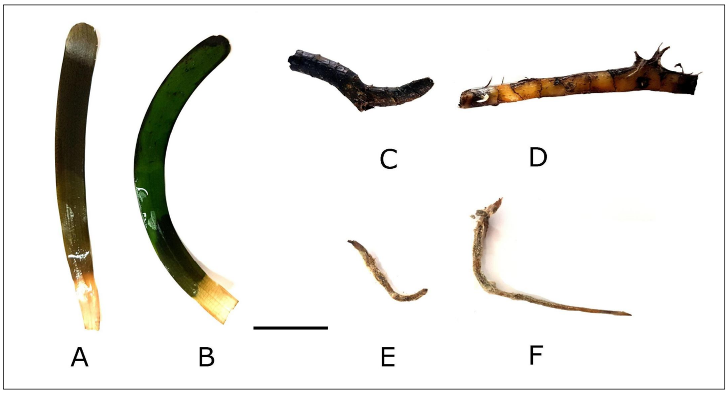

Figure 2. Leaf, rhizome and root morphology of *T. hempcihii*: A-C-E - organs of heat-treated individual; B-D-F - organs of control individual. Bar scale: 2 cm.

Chlorophylls content were measured from the leaf of *T. hemprichii*. These measurements include the content of chlorophyll a, b and total. Generally, the content of chlorophyll a was lower than chlorophyll b. The treated samples had less chlorophyll than the control (Table 1). All parameters were significantly different ($p < 0.01$).

Table 1

Chlorophyll content of *T. hemprichii* on each treatment

Chlorophyll type	*Treatments*	
	Heat	*Normal*
Chlorophyll a	2.93±0.20**	7.01±0.33
Chlorophyll b	2.21±0.15**	4.76±0.32
Total chlorophyll	5.14±0.35**	11.77±0.63

Values represent Mean±SE; ** significant in α: 0.01; n = 10.

Leaf of *T. hemprichii* was isolateral, the abaxial and adaxial sides have the same face. It had cuticle layer outside epidermis and absence of stomata. Epidermis cell in heat-treated leaf had irregular shape (Figure 3A) and its thickness was lower than control leaf (Table 2). In contrast to epidermis thickness, leaf thickness of heat-treated leaf was greater than control leaf. Magnitude of mesophyll thickness seemed to contribute to the thickness of the heat-treated leaf. All parameters of leaf size were significantly different ($p < 0.01$). Stele diameter and aerenchyma thickness of heat-treated rhizome were significantly lower than control rhizome ($p < 0.01$). However, cortex thickness was significantly greater ($p < 0.01$). Microscopic observation of the heat-treated roots showed the presence of various tissue damage (Figure 3E). Stele diameter of heat-treated roots was significantly lower than control root ($p < 0.01$). Whereas, aerenchyma thickness of heat-treated roots was significantly greater in $p < 0.05$ level. Nevertheless, both cortex and endodermis thickness did not show any difference in both groups.

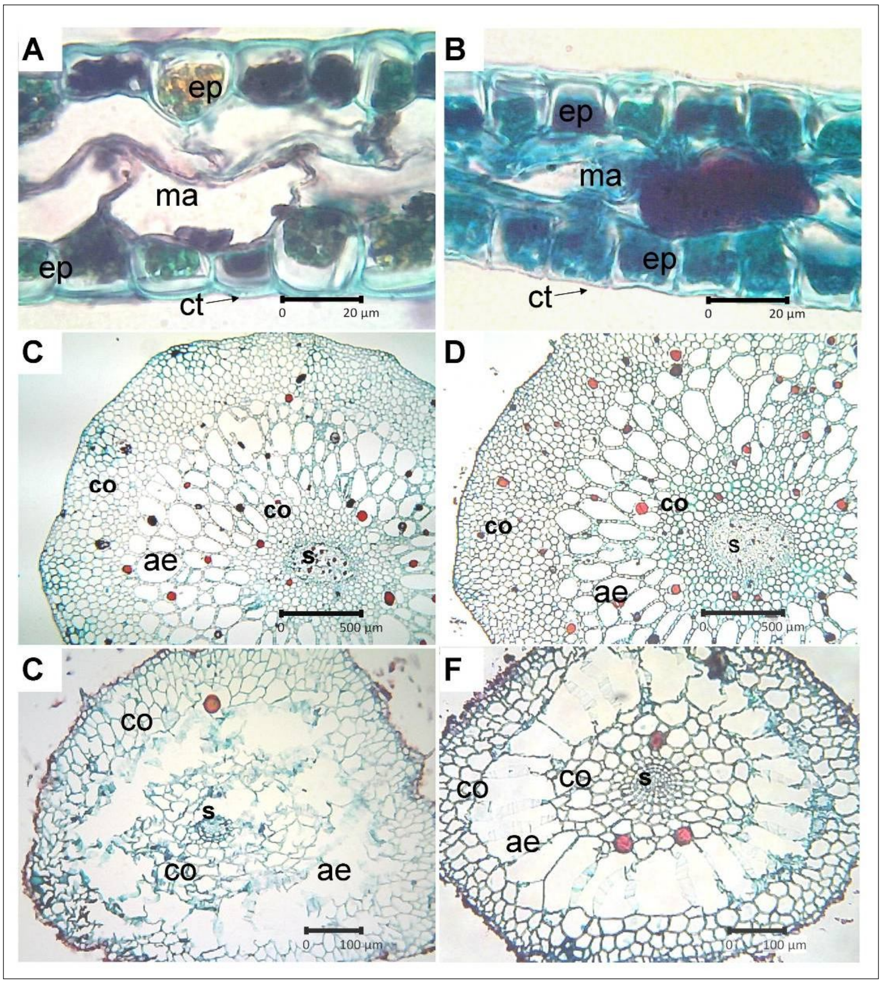

Figure 3. Changes in the anatomical stucture of leaves, rhizomes and roots.
A. heat-treated leaf; B. control leaf; C. heat-treated rhizome; D. control rhizome; E. heat-treated root; F. control root; ae. aerenchyma; co. cortex; ct. cuticle; ep. epidermis; ma. mesophyll aerenchymatous; s. stele.

Table 2

Anatomical changes in leaf, rhizome and root of *T. hemprichii* on each treatment

Organ	Tissues	Treatments (µm)	
		Heat	Normal
Leaf	Epidermis thickness	20.39±0.65**	23.47±0.41
	Mesophyll thickness	49.71±1.89**	30.48±2.08
	Leaf thickness	90.50±1.20 **	77.43±1.67
Rhizome	Stele diameter	352.81±18.00**	434.46±6.49
	Cortex thickness	851.22±35.79**	709.09±35.56
	Aerenchyma thickness	584.86±24.24**	925.02±26.91
Root	Stele diameter	63.41±1.73 **	76.26±1.88
	Cortex thickness	148.09±8.97	156.66±9.50
	Aerenchyma thickness	167.68±6.79*	148.65±7.25
	Endodermis thickness	10.29±0.40	10.80±0.49

Values represent mean±SE; * significant in p < 0.05; ** significant in p < 0.01; n = 20.

Discussion. Organ damage due to heat stress in *T. Hemprichii* has similiar condition to terestrial plants. Heat stress leads to scorching of organs, senescence, abscission, and inhibits shoot and root growth (Guilioni et al 1997; Ismail & Hall 1999; Vollenweider & Gunthardt-Goerg 2005). Plants exposed to high temperatures above the threshold value (5-10°C above normal temperature) will suffer irreversible damage to their function and development or metabolism cycles, which further lead to a decrease in growth (Porter 2005).

Heat stress on *T. hemprichii* not only caused irreversible damage of any organ and physiology interruption, but also leads to mortality. Leaf chlorosis was closely related to a significant decrease in chlorophyll content due to high temperatures. Siddiqui et al (2015) argued that the decrease in total chlorophyll content is the response of plants due to the heat stress. Declining of chlorophyll content was also reported by Shen et al (2017) in *Rhododendron* sp.

Decrease in chlorophyll content may be caused by the interference with chlorophyll biosynthesis. The interruption of the chlorophyll biosynthesis process occurs due to the inhibition of the electron transport process (Mohanty et al 1989) and inhibition activity of δ-aminolevulinic acid (ALA) synthase, ALA dehydratase, and porphobilinogenase (Tewari & Tripathy 1998). In addition, it was also affected by losing of cell membranes due to accumulation of reactive oxygen species (ROS) resulting in the destruction of thylakoid structures in chloroplasts (Karim et al 1997; Gupta et al 2013). Such effects on chlorophyll or photosynthetic apparatus were suggested to be associated with the ROS production (Camejo et al 2006; Guo et al 2006).

Reaction center of photosystem II (PSII) is highly thermolabile and its activity is briefly decreased even stopped under heat stress (Bukhov et al 1999; Camejo et al 2005). It may be due to the properties of thylakoid membranes where PSII is located. Heat stress may lead to the dissociation of oxygen evolving complex (OEC), resulting in an imbalance between the electron flow from OEC toward the acceptor side of PSII in the direction of PSI reaction center (De Ronde et al 2004). Heat stress causes dissociation of a manganese (Mn)-stabilizing 33-kDa protein at PSII reaction center complex followed by the release of Mn atoms (Yamane et al 1998). Heat stress may also impair other parts of the reaction center, e.g., the D1 and/or the D2 proteins (De Las Rivas & Barber 1997). This study suggested that measurement of chlorophyll content could be a reliable parameter to describe the chloroplast damage and the disturbance of photosynthesis due to high temperature stress.

The decrease in the thickness of the epidermis tissue and the increase in mesophyll tissue thickness and the thickness of the leaf occuring in *T. hemprichii* due to high temperature treatment were also reported in other plants. Similar responses are also reported in *Rhododendron* sp. (Shen et al 2017), *Brassica campestris* (Yuan et al 2017) and *Salvia splendens* (Natarajan & Kuehny 2008). According to Zhang et al

(2005), increased mesophyll thickness due to high temperature was resulted by increased permeability of the plasma membrane.

Anatomical observations of roots and rhizome of *T. hemprichii* were performed by measurement of cortical tissue, aerenchyma tissue and stele tissue. Aerenchyma is developed by aquatic plants along roots, rhizomes to leaves to adapt in conditions of minimal oxygen (Drew et al 2000). The aerenchyma tissue not only provides an internal pathway in oxygen transfer but also reduces the number of oxygen-consuming cells (Canny 1995). Furthermore, Drew et al (2000) also explained that the formation of aerenchyma tissue in the cortical area of root and rhizomes is closely related to programmed cell death (PCD) as a non-tolerant plant response to a stress. High temperatures could denature membrane proteins and increasing unsaturated fatty acids that make cell membranes more fluid (Savchenko et al 2002). Solute leakage occurs as a result of decreased integrity of cell membranes. This condition is commonly used as an indirect indicator of stress on various types of plants (Wahid et al 2007).

Aerenchyma pattern of *T. hemprichii* rhizome was honeycomb schizogeny aerenchyma. However, aerenchyma pattern in root was radial lysigenous. Jung et al (2008) described that those aerenchyma pattern also found in others submerged aquatic plant include family Hydrocaritaceae.

While observation stele tissue aimed to determine the response of vascular tissue due to exposure to high temperature stress. Tupan & Azrianingsih (2016) noted that vascular tissue of *T. hemprichii* was located in stele (central cylinder). During heat stress, in cortical and tracheary elements such as xylem, increased calcium ions and rupture of tonoplasts occur. High temperatures also lead in decreased cell size and an increase in the number of xylem vessels in stem and root of *Lotus creticus* (Bañon et al 2004). In this study, the formation of aerenchyma tissue was the adaptation of *T. hemprichii* as an aquatic plant. While the size change and the presence of damage in various cells and tissues suspected as a result *T. hemprichii* was not able to tolerate the high temperature stress that treated. Therefore, the plant went into death condition (PCD).

Conclusions. High temperature stress affects the morphological and anatomical changes of leaves, rhizomes and roots and decrease of chlorophyll content of *T. hemprichii*. These findings illustrate how *T. hemprichii* response to increase sea temperature due to global warming future. Early mitigation is required for handling and policy making on coastal waters management.

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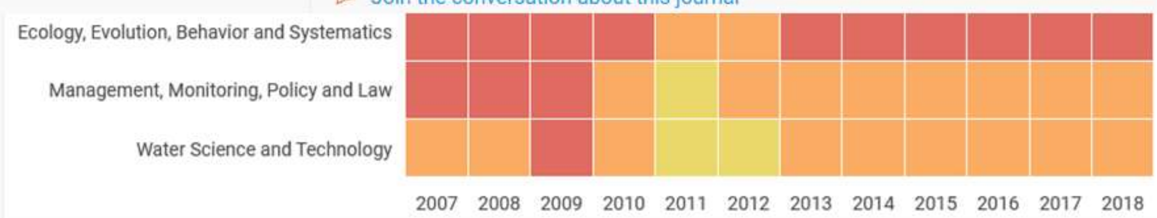

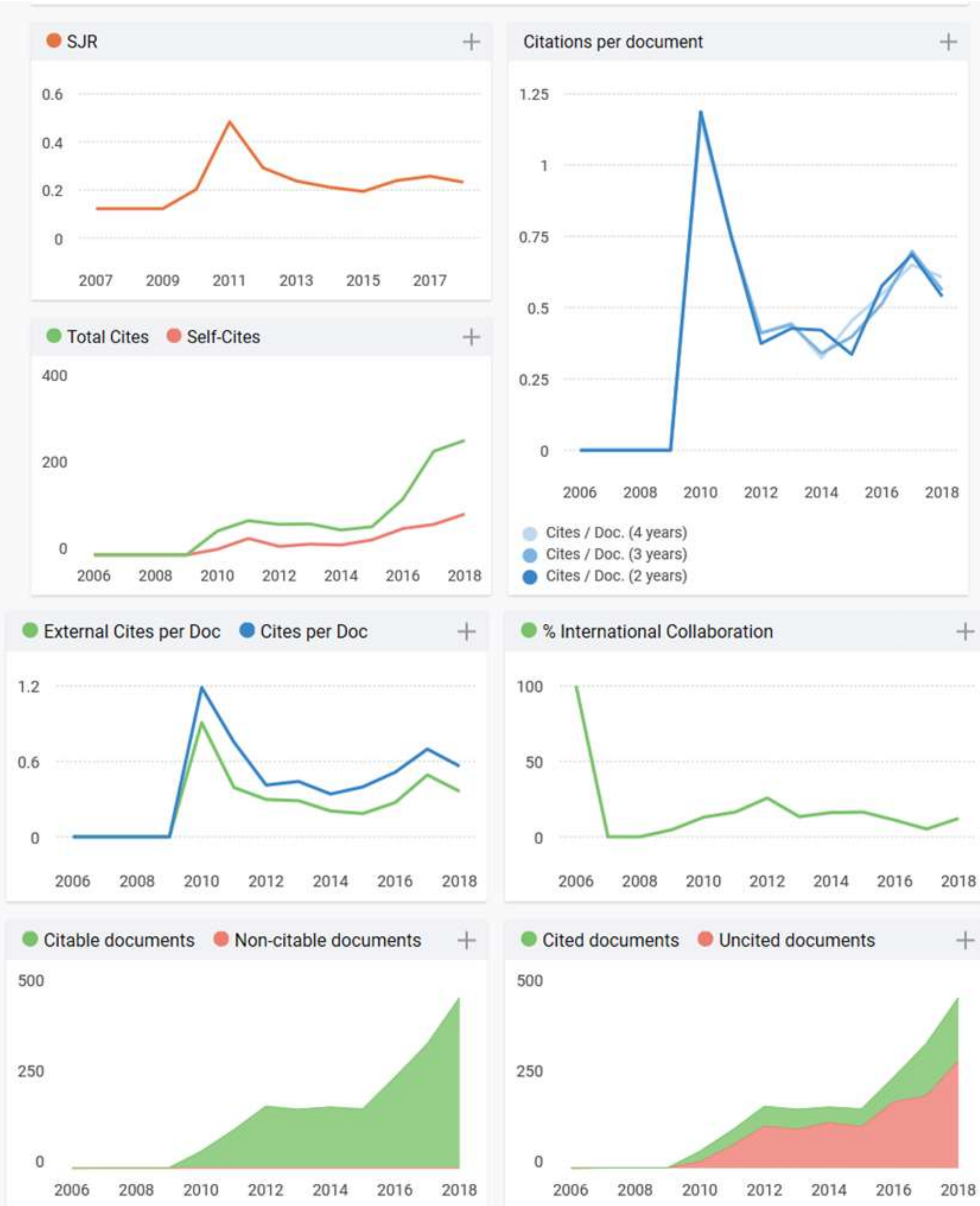
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