Mangrove endophytic fungi from Sarawak:

Isolation of secondary metabolites and optimisation of growth conditions

by

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Abstract

Endophytic fungi are a group of organisms unique in the sense that they live virtually their entire life cycles within the tissues of a host; they have been known to confer beneficial properties to the plants they inhabit in terms of protection from predation and tolerance against abiotic stress. For all their unique properties, much work lies ahead in understanding these innocuous organisms. Mangrove endophytic fungi are the more interesting group within endophytes, this as they have adapted to not only the host plant but the extreme environment those mangrove plants are constantly subjected to. There has been limited work in Malaysia regarding mangrove endophytic fungi, with more focus being given to fungi which are associated to medicinal plants. Nevertheless, significant work has been done by countries which border the South China Sea. These works were reviewed in a bid to understand trends in mangrove endophytic fungi research on a regional scale. During the course of this research mangrove endophytic fungi were subjected to large scale fermentation to isolate natural products, as well as growth optimisation studies to discern the best growth medium for isolation and cultivation of these extreme-tolerant microbes. The well-known antioxidant trans-ferulic acid was successfully isolated from a batch of fermentation products from *Phyllosticta*-related strain of fungi. It appears to be the first known instance of finding this metabolite with the fungal genus tested. Growth optimisation studies revealed that the growth requirements of each strain of fungi significantly differs from each other. Salinity, nitrogenous compounds, pH, are all important factors to consider when optimising growth of mangrove endophytic fungi. Though the strains tested were limited in number and diversity, it is clear that follow-up studies will be required to reveal more about the growth behaviour of these poorly-studied life forms.

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Declaration

I hereby declare that my thesis titled "*Mangrove endophytic fungi from Sarawak: Isolation of secondary metabolites and optimisation of growth conditions*" is original and contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome; to the best of candidate‟s knowledge contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, discloses the relative contributions of the respective workers or authors.

(Yao Long Lew) Date: 27th August 2015

Publications Arising from this Thesis

The work described in this thesis has been submitted as described in the following:

Yao Long Lew, Huiqin Chen, Peter Proksch, and Moritz Müller „Isolation and characterisation of trans-ferulic acid from mangrove-derived endophytic fungi *Guignardia mangiferae*" (Submitted to Ocean Science Journal, Manuscript ID: OSJO-D-14-00069)

Early work has been presented in the following conference and contributed to the content presented in Chapters 4 and 5 of this thesis:

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Yao Long Lew, Angelica Tan, Changi Wong, Edwin Sia, Aazani Mujahid, Elena Ivanova, and Moritz Müller „Optimisation of growth media for cultivation of mangrove endophytic fungi from Kuching, Sarawak" (To be submitted to Mycoscience)

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1. Introduction

1.1 Mangroves

1.1.1 What are mangroves? Mangrove is the collective term used to describe a group of plants which inhabit the region where the land meets the sea (Kathiresan and Bingham 2001). Much research focus has been given to a few more dominant mangrove species including *Avicennia marina*, *Bruguiera gymnorrhiza*, *Ceriops tagal*, *Heritiera littoralis*, *Rhizophora mucronata*, *Sonneratia alba*, and *Xylocarpus granatum* (Polidoro et al. 2014). Mangrove wetlands typically occupy the area between mean sea level and the highest point of spring tide (Smith 1992). Mangrove trees exert an important stabilizing influence, protecting the soil and land from erosion by waves and tides (Furkawa and Wolanski 1996). They are also living habitats to many aquatic fauna, providing safety and nutrients to juveniles of many species, vertebrates and invertebrates alike (Kathiresan and Bingham 2001); a non-exhaustive list of mangrove-dependent fauna is given in **Table 1**. In addition to being a crucial component for a stable coastal ecosystem, there have been several reports regarding its importance in nutrient cycling, sediment accumulation, and carbon storage capacity. (Ong 1993; Hyde and Lee 1995; Alongi et al. 2005; Kristensen 2008)

Table 1: List of mangrove-associated fauna which critically depend on mangrove trees for one or more stages in their life cycle.

Organisms	Representative genera/species	References
Fish	*Sardinella melanura, Macrones gulio*	Chong et al. 1990
Crabs	*Heloecius cordiformis*	Maitland 1990
Birds	*Vireo pallens*	Parkes 1990
Molluscs	*Enigmonia aenigmetica*	Sigurdsson and Sundari 1990
Zooplankton	*Tintinopsis* sp.	Godhantaraman 1994
Mammals	*Aonyx cinerea*	Sivasothi and Burhanuddin 1994
Reptiles	*Crocodylus porosus*	Stuebing et al. 1994
Prawns	*Metapenaeus monoceras, Penaeus indicus*	Rajendran 1997

1.1.2 Distribution of mangroves. Mangroves forests occupy close to 75% of the world‟s coastline between 25°N and 25°S (Day et al. 1987), favouring tropical to subtropical climates. The determining factor of whether an area suitable for mangroves will actually be colonized is the mean surface water temperature as mangroves generally do not flourish when mean temperatures fall below 20°C (Duke et al. 1998). While temperature may be the critical factor for determining geo-spatial distribution of mangrove swamps, species diversity in any given colonized site may vary significantly based on numerous factors (Ball 1998). Occupying and thriving within a narrow geographical niche requires specific adaptations to the environmental conditions it entails; even minute changes can have profound effect on the distribution of the various species which make up a mangrove swamp. These conditions include and are not limited to: fluctuating temperatures and salinity, ever-changing water levels, soil type and chemistry, etc. Categorizing of mangroves is therefore a complicated task, with Woodroffe (1992) suggesting that distinctions can be made by factoring oceanic and estuarine factors, e.g. river-dominated, tide-dominated, wave-dominated, etc.

In a report by UNEP-WCMC (2001), the region of the world with the highest diversity of mangroves can be found within the coastal areas of Malaysia, Indonesia, and Papua New Guinea. The coastal region of these countries with high mangrove diversity shows significant overlap with the area of the world with highest diversity of coral reefs, also known as the Coral Triangle. The overlapping regions can be found in **Figure 1**. This may hint at mangrove diversity and distribution as important contributing factors for greater diversity of corals. The stabilizing presence of mangroves must not be overlooked; as mangrove macroflora provides significant physical buffering against natural and anthropogenic runoffs, coastal sedimentation rates are significantly lowered (Mazda et al. 1997). This means less turbid water flowing into the sea from terrestrial sources and better water quality for corals and coral-associated organisms to thrive in.

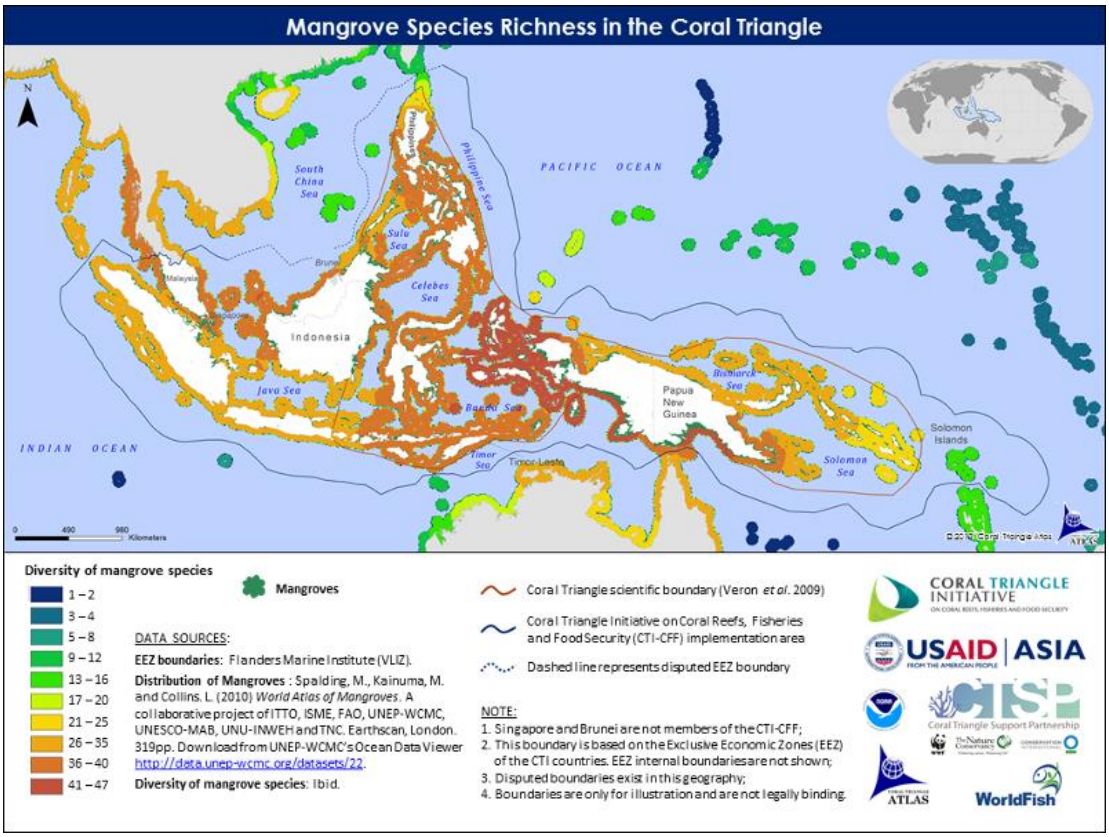


Figure 1: Overlap of regions with high mangrove diversity and the area covered by the Coral Triangle Initiative.

1.1.3 Adaptations of mangroves. Mangrove trees are highly adapted to life in their extreme environment. They take root and colonise an ecological niche which many other species of flora and fauna find inhospitable (Fromard et al. 1998). Being the group of flora which make up primary forests in coastal areas, mangroves are particularly resilient towards abiotic stresses arising from the interphase interactions between land and sea. Refer to **Table 2** (below) for a summary of mangal adaptations to abiotic stresses.

Mangroves are well known for their halophilic nature, their most effective adaptations to saline environments include: excretion of accumulated salts via glands on leaves (Scholander et al. 1962), over-accumulation of salts in mature leaves followed by leaf shedding (Cram et al. 2002). The use of osmolytes both organic (Yasumoto et al. 1999) and inorganic (Hwang and Chen 1995) have also been reported. In most cases, uptake of salts by mangroves from surrounding sea water is inevitable and often at higher rates than

terrestrial plants (Zhu 2003); nevertheless, a mildly effective salt exclusion mechanism is often in place to maintain normal cellular osmotic pressures and functions of cytoplasmic proteins. It is through these osmolytes – small molecular weight molecules or ions – that the difference between internal and external osmotic pressures can be regulated (Yancey 2001)

Table 2: A brief summary of major abiotic stresses experienced by mangroves and the different evolutionary adaptations to survive.

Abiotic stresses	Adaptations	Mangroves	References
Salinity & water stress	Salt excretion	*Aegialitis* sp.	Scholander et al. 1962
	Leaf fall	*Bruguiera cyclindrica*	Cram et al. 2002
	Osmolytes	*Kandelia candel*	Hwang and Chen 1995
		Sonneratia alba	Yasumoto et al. 1999
Tidal inundations & Unstable substratum	Stilt roots	*Rhizophora* sp.	Kathiresan and Bingham 2001
	Prop roots	*Rhizophora gymnorrhiza*	Kitaya et al. 2002
	Propagule	*Ceriops tagal*	McGuinness 1997
Anaerobic sediments	Pneumatophores	*Avicennia marinara*	Dahdouh-Guebas et al. 2004
	Knee roots	*Bruguiera* sp.	Ellison 1998
	Lenticels	*Avicennia marinara*	Hovenden and Allaway 1994

The rise and fall of tides occur twice a day, and this tidal inundation of mangroves has also led to crucial adaptations for support of the trees themselves. Prop roots (Kitaya et al. 2002) and stilt roots (Kathiresan and Bingham 2001) are adaptations which enable mangroves to remain rooted firmly in the face of disruptive forces of waves as well as loose and unstable soil it grows on. Prop roots typically originate from low-lying branches of mangrove trees; grow extending downwards, and upon nearing the water surface, fan out to create support platforms for the main plant. These prop roots have dual function as breathing roots as well as providing support for the tree, anchoring down even the branches onto soil (Ennos 2000). It was also noted that these roots do not perform absorptive functions unlike true roots. Stilt roots are rather similar to prop roots, with the distinction of being roots originating from buds around the lower regions of main tree trunk and extending in a curved manner outwards and eventually achieving a foothold on the surrounding soils (Ennos 2000). Both prop and stilt roots give the mangrove trees greater stability by expanding the area of the

base of the trees. Many mangrove species are reproductively adapted to life in the rushing waves and shifting sands by adopting a viviparous lifestyle – their seedlings germinate while still attached to parent plant (McGuiness 1997). These semi-developed young plants will only detach from parents when conditions are positive; often dropping onto the water surface during high tides to be carried away. The germinated seedling has the ability to carry out photosynthesis throughout its journey before attachment onto suitable substratum. This ensures the seedlings have maximum chance of survival by being able to take root quickly once it has reached a favourable site to grow; if germination were to happen only after arriving at final location, the halfway germinated plant could easily be swept away by waves, thus unable to begin its life cycle as a new tree (Wee et al. 2014).

As a result of constant tidal floods, the soils which mangroves grow on experience oxygen deprivation as complete water saturation of soil pores gives rise to anoxic conditions (McKee 1993). Mangrove roots, which make up much of the under-soil biomass, are subjected to soil phytotoxins which include reduced forms of iron and manganese (Jones and Etherington 1970), and several types of organic acids (Sanderson and Armstrong 1980). Disimilatory sulphate reducing bacteria in mangrove soils also reduce sulphates in marine waters, resulting in production of H_2S, a known inhibitor of eukaryotic mitochondrial enzyme cytochrome oxidase (Allam and Hollis 1972). In order to cope with the implications of flooded soils, mangroves have evolved certain physical structures which allow for adequate oxygenation and aeration of roots which are covered by anoxic soil and sediments (Furukawa and Wolanski 1996). Almost all mangrove trees have some form of breathing roots or similar organs. The most visually distinct of them are pneumatophores (Dahdouh-Guebas et al. 2004); these extensions of underground roots which grow upwards at frequent intervals, emerging from anoxic soil layers to allow for transport of atmospheric gases to the underground roots below. Pneumatophores are functionally similar to snorkels and they allow for adequate oxygenation of roots, preventing suffocation under oxygen-poor conditions. Likewise, knee roots are another class of adventitious roots which serve the same purpose of providing oxygen to mangrove tree roots (Ellison 1998). Instead of specialised structures, trees which evolved knee roots simply have horizontal roots which form aerial loops at intervals. These loops which the root makes generally have a knee-like shape, hence the name. Lenticels often present themselves as horizontal cuts on surfaces of

mangrove plants, but they serve as direct air channels for plant tissue within (Hovenden and Allaway 1994). Commonly found on pneumatophores, prop roots, knee roots, and other breathing structures; lenticels differ from stomata by allowing for more direct and efficient air exchange between inner air space of mangrove with external atmosphere.

1.1.4. Economic importance and threats to mangroves. Mangrove trees play a significant role for local communities; traditionally a source of wood for construction of houses and fences, as well as firewood for cooking and heating. Exploitation of mangrove timber often yield other products such as charcoal, tannins, resins, boats, fish traps, medicine, alcohol, etc. The wetland areas around mangroves are frequently subjected to aquaculture practices on small scale floating cages for fish to larger scale cultivation of shrimps in brackish water (Kathiresan and Bingham 2001)

Anthropogenic activities in and around mangrove areas pose significant risk towards the destruction of mangrove ecosystem, leading to irreversible loss of biodiversity (Dahdouh-Guebas and Koedam 2008). The destruction of mangrove forests for aquaculture and timber are most significant. Further loss of mangrove areas worldwide has been predicted, given the need for coastal space to carry out fish and shellfish farming is not expected to decrease for at least another ten years (Alongi 2002).

The importance of mangrove forests as zones of high biodiversity cannot be overstated (Ellison et al. 1999). Although the coverage along coastal regions can sometimes be narrow, mangrove forests play an important role to provide habitat for many faunal species (recall Table 1 earlier), thereby creating a large degree of interconnectedness between mangroves and adjacent habitats (Nagelkerken 2008). Many studies have been carried out on the microecosystems of sediment stabilised by aerial root structures (Holguin et al. 2001), decaying mangrove plant material (Alias et al. 2010), and the spaces within living mangrove trees (Sebastianes et al. 2013).

1.2 Mangrove endophytic fungi

1.2.1 Fungi. All life forms can be taxonomically assigned into one of three great Domains of Life – Bacteria, Archaea, or Eukarya. The individual domains distinguish themselves from each other in terms of biological and chemical characteristics. Bacteria are prokaryotic (they lack nuclear envelope) unicellular organisms which have cell walls made of peptidoglycan; organisms in the domain Eukarya, as the name suggests, are eukaryotic (they possess nuclear envelope) and can be both unicellular and multicellular life forms; the Archaea domain is similar to that of bacteria in terms of the members all being prokaryotic unicellular organisms, but they are distinctly different in terms of cellular biochemistry and primordial evolutionary history.

The Kingdom Fungi falls under the Eukarya domain. Fungi are known to flourish in a wide range of conditions – there are thousands of known terrestrial fungi which grow almost everywhere where moisture and moderate temperatures can be found (-), marine fungi well-suited to aquatic life (Sivakumar 2013), and a number unique extreme environment fungi capable of surviving harsh conditions such as high salinity, scorching heat, immense pressures, and ionizing radiation (Magan 2007).

Mycology, derived from the Greek words *mykes* (meaning "mushroom") and *logos* (meaning "discourse") is the term coined to describe the scientific study of fungi. Most mycologists tend to focus their endeavours on the physical characteristics, diversity, and potential uses of fungi (Kendrick 2011). Fungi morphology can be studied via macroscopic and microscopic methods; many fungi form large structures highly identifiable and easily studied using the naked eye when grown on certain surfaces or substrata (Hukka and Viitanen 1999), but a microscopic view is often crucial to provide conclusive morphological data regarding fungal hyphae structures (Trinci 1969). And though their sessile lifestyle may lead us to believe that they are plant-like organisms, they are actually more genetically related to animals than higher plants (Adl et al. 2012).

1.2.2 Endophytic fungi. Endophytic fungi are a special group of fungi which spend virtually their entire natural life cycle within the living tissue of host plant. These fungi typically co-exist with the host without harming it, though there are an increasing number of reports (Arnold et al. 2003; Schardl and Leuchtmann 2005) detailing beneficial effects of

endophytic fungi on the host plant. We would like to believe that endophytic fungi will always confer protection and other benefits to the host plant; this is however, subject to much debate (Hyde and Soytong 2008). The relationship between endophytes and their hosts are poorly understood; an endophytic fungi strain can present itself as a pathogen under normal circumstances; however it contributes to plant defence against damage by herbivores by rendering the plant less desirable as a food source (Rodriguez and Redman 2008).

1.2.3 Bioactive secondary metabolites from fungi and endophytic fungi. The fungi are a class of organisms found to be prolific in synthesis of secondary metabolites, the most famous and one of the earliest examples being penicillin synthesised by the common mould *Penicillium notatum* (Fleming 1929); the organism itself used the secondary metabolite to inhibit growth of competing bacteria found naturally in its living environment. Humans have used the isolated drug to treat a plethora of bacterial infections (Bigger et al. 1944; Ercoli et al. 1945). The compound penicillin was discovered close to a century ago, and yet it and its derivatives remain highly used as a first line antibiotic against many common bacterial infections today.

Endophytic fungi too have been known to produce bioactive compounds (Radić and Štrukelj 2012). The multi-billion dollar cancer drug paclitaxel, or Taxol®, was found to be a product of the bark of Pacific yew tree, *Taxus brevifolia* (Wani et al. 1971). The problem with taxol was that treatment for every one patient would require the felling of around twelve trees; this presents a significant problem of limited natural resources. Alternatives were sought after in order to meet demands, and one was found under contentious circumstances to be *T. brevifolia*-associated endophytic fungus *Taxomyces andreanae* (Stierle et al. 1993). In the years to come, there have been numerous reports of endophytic fungi which also produce the same compound, but the relatively low yields have not allowed for commercialisation of those endophytic fungi (Strobel et al. 2004). Whether paclitaxel is truly a metabolite of higher plants or a product of endophytic fungi synthesis remains uncertain; as it is almost impossible to derive a pure plant specimen devoid of endophytes, and then cultivating such a sample in a fungi-free environment.

1.2.4 Mangrove endophytic fungi. Mangrove endophytic fungi are simply the fungi which reside within mangrove plants (Petrini 1991). What sets them apart from other endophytic fungal strains is their ability to tolerate the various stresses experienced by their host plant. Though a commensal relationship is possible, it is more likely that the interaction between endophytic fungi and host is symbiotic, conferring to the host plant a certain degree of protection from the physicochemical conditions experienced, other grazing fauna, and possibly pathogens of terrestrial and marine origin (Hanada et al. 2010). Many mangrove endophytes are not host-specific, meaning any one fungus isolated can easily be found in many mangrove trees of different species. In a study by Pang et al (2008) the most dominant fungi from a sample size of four individual adult trees of one species were cosmopolitan in nature and commonly isolated as endophytes of other plants. It was noted that fungi assemblages differ significantly according to the parts of host plant isolated from; with wood and bark tissue yielding a greater number of isolates as compared to leaf tissue samples.

1.2.5 Biotechnology of mangrove endophytic fungi.

Fungi are a group of microorganisms long adapted for use in many areas; fast-growing fungi are a means to generate large amounts of biomass (Gessner 1997) in a controlled manner. Mangrove endophytic fungi are long thought to be uniquely suited for industry. As they are halotolerant and proliferate in alkaline marine conditions, mangrove endophytic fungi are uniquely suited as a source of enzymes which are able to tolerate non-standard conditions (Thatoi et al. 2013). The South China Sea region is rich with mangrove biodiversity (Field et al. 1998), and by extension, brimming with biotechnological potential in terms of its mangrove endophytes. Greater collaboration amongst working groups of various fields would yield greater advances in biotechnology of mangrove endophytic fungi.

1.3 South China Sea

1.3.1 Geographical region. The South China Sea (SCS) region is, at the time of writing, an area whereby small islands with strategic geography are the subject of on-going political disputes. For the purpose of this review, the area defined as SCS was objectively determined based on oceanographic and geographical considerations (**Figure 2**).

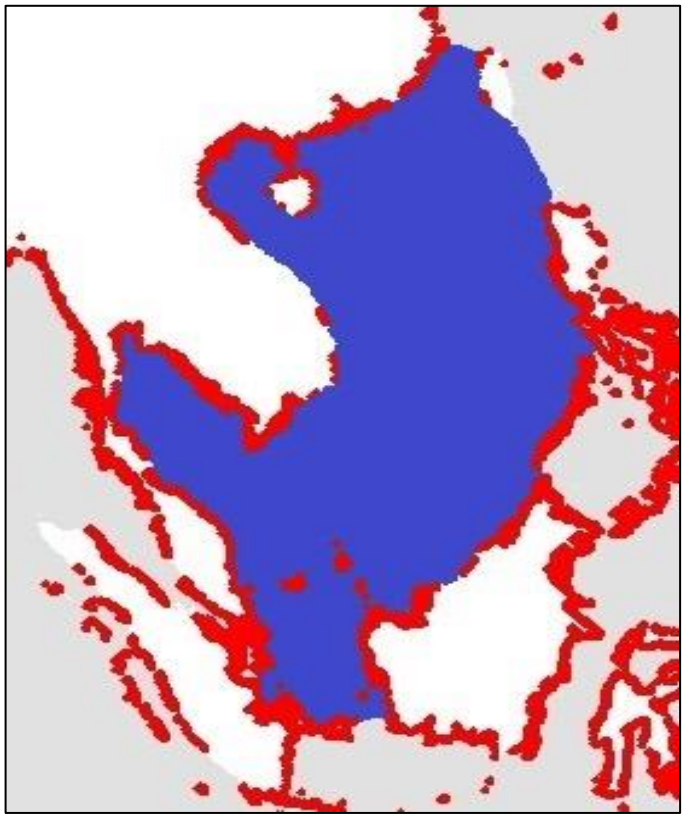


Figure 2: Partial map of Southeast Asia; Blue: Area of South China Sea region considered in this study; Red: Distribution of mangrove forests, derived from earth observation satellite imagery (Giri et al. 2011).

1.3.2 Mangroves in South China Sea region. The mangroves are the predominant flora type found along the coastal regions of the South China Sea; warm seawater throughout the year provides a major environmental factor for the mangrove ecosystem to thrive in. The area covered by mangroves in coastlines bordering the South China Sea was approximately 150,000 square kilometres (UNEP, 2004) but it seems likely that the actual current area might be smaller due to clearing of mangrove forests for aquaculture, timber and urban development over the years (Valiela et al. 2001).

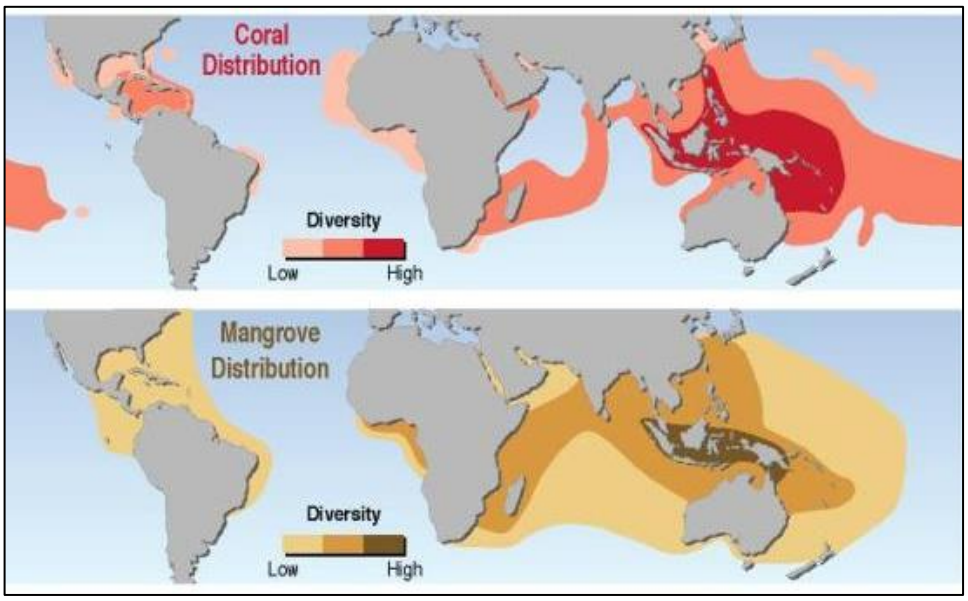


Figure 3: Correlation between areas of high diversity of corals with mangroves (UNEP-WCMC 2001). (Cartographer credit: Philippe Rekacewicz)

1.3.3 Mangrove plant diversity. The South China Sea region has the highest diversity of mangrove tree species in the world, with up to 46 species recorded from this region (UNEP. 2004). Considering an estimated 110 unique species of mangroves exist worldwide, approximately half the world‟s total mangrove biodiversity is contained within this region alone. High floral biodiversity of mangroves leads to high biodiversity of fauna as well. The actual spatial impacts remains unknown as coral diversity has been found to correlate with diversity of mangroves of the region (**Figure 3**). Animals which depend on mangrove forests for food, protection, and reproductive purposes thrive in the richness of mangrove species. Some 67 species of macro-crustaceans, 103 species of fish, and 98 species of indigenous birds were reported from mangrove swamps and forests in China, Vietnam and Thailand respectively. The mangrove forests of Borneo is host to the endemic species *Nasalis larvatus*, commonly known as the proboscis monkey; this unique mammal is found nowhere else and has been known to predate upon mangrove propagules (Meijaard and Nijman 2000). Coincidentally, the areas which mangroves were found to be high diversity overlapped significantly with areas of high coral biodiversity (Burke et al. 2001). This correlation may be explained by the role which mangrove swamps play as a safe haven for the young of fish and other marine life.

1.4 Mangrove endophytic research in the South China Sea region

1.4.1 Current literature. The works considered in this work were all found using Google Scholar. We consider all works being carried out by any institution worldwide with the criteria that the fungal material used has to originate from the South China Sea region. Scientific publications regarding mangrove endophytic fungi were then classified according to country of origin of mangrove endophytic fungi, as well as to the type research done, shown in **Table 3** below.

1.4.2 Baby steps. The earliest paper which deals with endophytic fungi in South China Sea region was published by Hyde and Alias (1999) whereby one of two fungi isolated from the mangrove palm *Nypa fructicans* was believed to be endophytic in nature. In the following year, a subsequent publication (Hyde and Alias 2000) provided a hint at the endophytic-saprobic dual nature of the mangrove endophytic fungi *Neolinocarpon nypicolum*. This double life of endophytic fungi was later discussed by Redman et al (2001) and further deliberated in greater detail by Rodriguez and Redman (2008); the interactions host and endophytic fungi is based upon a continuum Schardl and Leuchtmann (2005), a fragile balance which can easily tip in favour of parasitism against the host. Endophytic fungi form strong evolutionary attachments to their host, conferring the plant with various benefits which help it survive biotic and abiotic stresses (Arnold et al. 2003; Waller et al. 2005). It is important to note that some endophytic fungi may turn pathogenic if the host plant is unable to cope with stress experienced even with the help of endophytic fungi; the desire for its own survival outweighed dependency on host plants. After all, it is the very essence of evolution to be able to survive when conditions are not favourable, and that includes being able to thrive and make full use of a host which is dying or dead (Promputtha et al. 2007). More work is needed to truly understand the mangrove endophytes of the South China Sea region.

Table 3: List of publications pertaining to endophytic fungi research within the South China Sea region grouped according to fungi country of origin and alphabetically arranged according to authors' name.

Country	**Research topic/area**	**Strains of interest**	**Reference**
China	Natural products	N.G.	Chen et al. 2003
China	Natural products	*Penicillium thomi*	Chen et al. 2007
China	Taxonomy	*Fusarium proliferatum*	Cheng et al. 2008
China	Natural products	*Aspergillus terreus*	Deng et al. 2013
China	Natural products	*Corynespora cassiicola*	Ebrahim et al. 2012a
China	Natural products	*Bionectria ochroleuca*	Ebrahim et al. 2012b
China	Natural products	N.G.	Guo et al. 2007a
China	Natural products	*Paecilomyces* sp.	Guo et al. 2007b
China	Natural products	*Penicillium* sp.	Han et al. 2009
China	Natural products	*Alternaria* sp.	Huang et al. 2011a
China	Natural products	*Penicillium chermesinum*	Huang et al. 2011b
China	Natural products	*Phomopsis* sp.	Huang et al. 2008
China	Natural products	*Phomopsis* sp.	Huang et al. 2009a
China	Natural products	N.G.	Huang et al. 2007
China	Natural products	*Phomopsis* sp.	Huang et al. 2010
China	Natural products	*Phomopsis* sp.	Huang et al. 2009b
China	Natural products	*Penicillium* sp.	Jin et al. 2013
China	Natural products	*Alternaria* sp.	Kjer et al. 2009
China	Natural products	*Eurotium rubrum*	Li et al. 2008
China	Natural products	N.G.	Li et al. 2004
China	Natural products	*Penicillium* sp.	Li et al. 2014
China	Natural products	N.G.	Li et al. 2010
China	Natural products	*Xylaria* sp.	Lin et al. 2001a
China	Natural products	*Xylaria* sp.	Lin et al. 2001b
China	Natural products	*Penicillium* sp.	Lin et al. 2008
China	Natural products	*Talaromyces* sp.	Liu et al. 2009
China	Natural products	N.G.	Mei et al. 2012
China	Diversity	*Phomopsis* sp., *Pestalotiopsis* sp., *Guignardia* sp., *Xylaria* sp.	Pang et al. 2008
China	Natural products	*Pestalotiopsis virgatula*	Rönsberg et. 2013
China	Natural products	N.G.	Shao et al. 2007
China	Natural products	*Penicillium* sp.	Shao et al. 2010

China	Natural products	*Xylaria* sp.	Song et al. 2012
China	Natural products	N.G.	Tao et al. 2010
China	Natural products	*Phomopsis* sp.	Tao et al. 2008
China	Natural products	*Sporothrix* sp.	Wen et al. 2009
China	Natural products	*Paecilomyces* sp.	Wen et al. 2010
China	Natural products	*Paecilomyces* sp.	Wen et al. 2008
China	Diversity	*Glomerella* sp., *Phomopsis* sp., *Mycosphaerella* sp., *Fusarium* sp.	Xing et al. 2011
China	Natural products	*Pestalotiopsis* sp.	Xu et al. 2011
China	Natural products	*Pestalotiopsis* sp.	Xu et al. 2009
China	Natural products	*Phomopsis* sp.	Yang et al. 2010
China	Natural products	N.G.	Zeng et al. 2012
China	Natural products	*Halorosellinia* sp., *Guignardia* sp.	Zhang et al. 2010
Thailand	Natural products	*Xylaria* sp., *Cladosporium* sp.	Chaeprasert et al. 2010
Thailand	Natural products	*Aigialus parvus*	Isaka et al. 2002
Thailand	Natural products	*Phomopsis* sp.	Klaiklay et al. 2012a
Thailand	Natural products	*Pestalotiopsis* sp.	Klaiklay et al. 2012b
Thailand	Natural products	*Rhytidhysteron* sp.	Pudhom et al. 2014
Thailand	Natural products	*Phomopsis* sp.	Rukachaisirikul et al. 2008
Thailand	Natural products	*Pestalotiopsis* spp.	Rukachaisirikul et al. 2012
Thailand	Natural products	*Guignardia bidwellii*	Sommart et al. 2012
Malaysia	Taxonomy	*Neolinocarpon nypicola*	Hyde and Alias 1999
Malaysia	Biodiversity	*Neolinocarpon nypicola*	Hyde and Alias 2000
Malaysia	Bioremediation	*Culvalaria* sp., *Neosartorya* sp.	Onn et al. (2012a)
Malaysia	Natural products	*Guignardia* sp., *Neosartorya* sp.	Onn et al. (2012b)
Vietnam	Natural products	*Acremonium strictum*	Hammerschmidt et al. 2014
Indonesia	Natural products	*Penicillium* sp., *Fusarium* sp.	Prihanto et al. 2011

Note:

1. "N.G." denotes "Not Given", indicative that an endophytic fungus was isolated but the identity remained unknown.
2. In cases where more than one strain of fungi was studied, only up to four most significant strains were included.

1.4.3 Expanding field. As our knowledge of mangrove endophytic fungi deepens, the research carried out on it also intensified. Among the fifty five papers assessed in this review (**Table 3**), half that number (28) was published within the last five years (2010-2014); the remainder 27 papers were published between 1999 to 2009, a span of ten years. Though the list is by means exhaustive, the trend is obvious that research in mangrove endophytic fungi is gaining ground. Mangrove endophytic fungi is commonly categorised under the general terms marine fungi or marine organisms (Martins et al. 2014), these organisms have been widely researched as the new frontier of natural products discovery (Wang et al. 2013). Many papers have been published detailing the *in vitro* efficiency of secondary metabolites from marine organisms against a variety of illnesses, e.g. cancer (Deng et al. 2013), malaria (Isaka et al. 2002), methicillin-resistant *Staphylococcus aureus* (Mei et al. 2012), and candidiasis (Huang et al. 2008). With greater concerted efforts between mycologists and pharmaceutical chemists, we are optimistic that medically important discoveries are just around the corner.

1.4.4 Rampant China. At first glance, it is obvious that a high volume of research was carried out on mangrove endophytic fungi originating from China, either by Chinese scientists themselves or otherwise. This trend may be seen as a result of demands inevitably placed upon researchers to make full use of natural resources for national interest. Given that there has been no evidence which pointed to mangrove endophytic fungi as a major area of research, the efforts carried out was more likely by-products of knowledge expansion in the fungi science. In 2009, the China Fungal Genome Initiative Symposium was attended by close to 100 scientists based locally and abroad (An et al. 2010), the multi-disciplinary approach yielded various exchanges regarding research in biological interactions, natural products, phytopathology, fungi *in vivo* models, food mycology and fungal genomics. Also contributing to this is intrinsic curiosity of the Chinese people towards fungi (Kavaler 1965) and a need to better the country with applied research into economically important fungi in the areas of fungal pathogens (Skamnioti and Gurr 2009), bio-control fungi (Butt et al. 2001), edible fungi (Stamets 2000), and medicinal fungi (Zhou et al. 2009).

1.4.5 Natural products focus. Unsurprisingly, there is a significant focus on natural products research on mangrove endophytic fungi (Joel and Bhimba 2012). Significant sums are poured into natural products research by biotechnology and pharmacology industries in order to fund high impact research (Gunatilaka 2006). Isolation of natural products remains highly relevant, especially in an age of drug resistant bacteria and increasing incidences of chronic illnesses (cancer, hypertension, etc). Given the recent success rate of marine drugs in entering the latter stages of drug testing, the monetary amounts will only increase. If however, the general public becomes more aware of other potential benefits of mangrove endophytic fungi, the research direction may change to be more inclusive of other aspects.

1.4.6 Strain prevalence. In our assessment of prevalence of mangrove endophytic fungi, we take into account each time a genus was significantly mentioned in a publication as one instance; with no more than four instances allowed per paper, taking into account fungi diversity studies. We have determined that the most prevalent fungal endophytes were *Phomopsis* sp., *Penicillum* sp., and *Pestalotiopsis* sp.; these three genera together comprised of 23 distinct instances of 57 cases mangrove endophytic fungi studied. The number one prevalent fungus in our survey was *Phomopsis* sp., mentioned in 10 different papers covering isolation of natural products and fungi diversity. This particular genus, more commonly found as a plant pathogen, is well known for its widespread distribution and diverse interactions with various hosts (Udayanga et al. 2011). Thus it is unsurprising that many works on mangrove endophytic fungi have yielded *Phomopsis* sp. as a prolific producer of bioactive compounds. The two-fold reasoning behind this is that bioactive metabolites are useful as bio-protective agents when the fungus exist as a mutualistic endophyte, yet can also play significant part as a pathogen during the infection and colonisation of stressed or senescent host plants.

1.4.7 Etymology. Caution was taken each time the general term “marine fungi” was used to describe any mangrove associated fungi as it is important that the fungi strains discussed here be of endophytic in nature. Many fungi isolated when surface sterilisation was insufficient may have erroneously been identified as endophytes, but were actually exophytes, epiphytes, or saprophytes. Some publications were not included as ambiguity arose when the term “mangrove fungi” was used, or when fungal isolation protocols used were not compatible for obtaining true endophytic fungi of mangroves. Any descriptions given regarding plant samples used for isolation of endophytic fungi was also examined to exclude diseased plant tissue as that may indicate a parasitic strain of fungi.

1.4.8 Future research. Mangrove endophytic fungi are important microbes to be considered in further mycology research within the South China Sea region. There are so many things we do not yet know regarding mangrove endophytic fungi. In trying to learn more about them, the most obvious route is to know who they are; therefore further research is required to assess the spread of major fungal species across individual mangrove plant species – this can be done using traditional culturing methods or via environmental genomics. It will be interesting to discover more about presence of endophytic fungi and its implications for host plants, factoring the possibility of endophytes turning pathogenic when a host plant is under stress.

The development of new culturing techniques promises greater biodiversity of mangrove endophytic fungi being assessed. Non-standard media are to be used, which then allows for isolation of slower growing and fastidious endophytes. The majority of known endophytic fungi from mangroves to this day have been results of isolation using standard laboratory media. It will not be possible for certain strains to be isolated as the conditions on a petri dish will be too different from the environment within host plant.

1.5 Aims and Objectives of thesis

This thesis aims to explore deeper into the subject matter of mangrove endophytic fungi originating from the Santubong wetlands of Kuching, Sarawak. As it is a follow up to an earlier study by Onn et al. (2013), the methodology used in this body of work was devised to expand on the knowledge of selected fungal strains, covering isolation and identification of specific fungal metabolites, as well as characterization of fungi growth under a range of pH, salinity, and nutrient conditions. By the end of this thesis, it is hoped that a little more of mangrove endophytic fungi can be understood, and also justify greater attention in this niche topic for bioprospecting and biodiversity studies.

The objective of this research work includes:

1. Evaluation of the current research status of mangrove endophytic strains isolated from the South China Sea region. (Addressed in **1. Introduction**)
2. To isolate major compounds produced by one strain of mangrove endophytic fungi. Identification of compounds isolated via analytical HPLC, NMR, and LC-MS. (Addressed in **4. Isolation of trans-ferulic acid from mangrove-derived endophytic fungi *Phyllosticta capitalensis***)
3. Design a suitable experimental set-up in order to assess optimal growth medium for three selected mangrove endophytic fungi. Alteration of standard growth medium to mimic the fungi‟s natural environment in hopes of enhanced growth rate. (Addressed in **5. Optimisation of growth media for cultivation of mangrove endophytic fungi from Kuching, Sarawak**)
4. To make recommendations for future work on mangrove endophytic fungi in terms of isolation medium in order to better assess the diversity of fungi hidden within the tissues of any given sample. (Addressed in **6. Conclusions**)

2. Materials and Methods

2.1. Sample collection

All mangrove plant samples were collected by Onn May Ling from Kampung Pasir Pandak (**Figure 4**) during a collection trip in 2010. Plant material from mangrove trees were directly stored in bags and kept away from light at 4°C until isolation of fungi was carried out.

Figure 4: Sampling site of plant material – Kampung Pasir Pandak, situated to the north of Kuching city Sarawak, Malaysia. Inset: Exact location of collection site. (GPS coordinates: N01° 42'02.8" E110° 18'44.1")

2.2. Fungal material

2.2.1 Endophytic fungi isolation protocol. The externals surfaces of the plant samples were washed with clean running water, patted dry with sterile cotton cloth, then surface sterilised by immersion in EtOH (70%) for 90-120 s. The sterilisation process was stopped via immersion into sterile autoclaved water, followed by drying with a fresh piece of sterile cotton cloth. The plant samples were then dragged across the surface of a petri dish containing isolation medium (YGCA) as negative control for isolation of fungi. Following that, those surface-sterile pieces of plant samples were cut using sterile scalpel and placed in several other petri dishes containing isolation medium with the freshly cut edges being in direct contact with the agar surface. Incubation was carried out under static conditions at room temperature in the absence of sunlight. Within four days, fungal growth can be observed. Individual strains were isolated by transferring of actively growing hyphal tips onto freshly prepared cultivation medium (PDA).

2.2.2 Purification and cultivation of endophytic fungi. Purification was done by repeated transfer of actively growing fungal material until the growing colony was uniformed. Contaminating fungi and bacteria were removed at each step by aseptic transfers of biomass of fungi of interest using flame-sterilised curved iron needle fixed onto loop handle.

2.2.3 Long term storage of pure fungal strains. Long term storage of fungi was achieved by growing the pure fungal strains on long term storage medium (MexA agar) for several days. Freshly formed mycelia were then removed as agar plugs using cut pieces of sterile plastic straw. Several plugs were taken per plate into a single piece of straw, and then the straw containing fungal material was placed in a sterile Eppendorf tube (1.5 mL). The tubes containing fungal biomass were then sequentially frozen: first at 4°C (2 hr), then at -20°C (2 hr), and lastly kept away at -80°C until further use is required. Resuscitation of frozen cultures can be carried out by thawing the frozen culture at 37°C, then squeezing out one plug from straw into freshly prepared fungi growth medium, preferably in a liquid form, such as potato dextrose broth or malt extract broth.

2.2.4 Composition of media. The following are a list of media used during the course of initial endophytic fungi isolation up to large scale fermentation of fungal strains.

The medium used for isolation of endophytic fungi from mangrove plants was Yeast Extract Glucose Chloramphenicol agar (YGCA; **Table 4**). Yeast extract is a rich source of amino acids, minerals, and other growth factors; glucose is a carbon source which most fungi can utilise; chloramphenicol inhibits growth of contaminating bacteria residing in plant tissue.

Table 4: Composition of YGCA.

Yeast extract	5.0 g
D-glucose	20.0 g
Chloramphenicol	0.1 g
Bacto agar	15.0 g
Demineralised water	1000 mL
Final pH at 25°C	6.6 ± 0.2

The medium used for purification and short term storage of fungal strain was Potato Dextrose agar (PDA; **Table 5**). Potato infusion was derived from boiling of potatoes; upon discard of solid pieces, the water used was evaporated to give a nutrient-rich powder. Dextrose is a rich carbon source for fungi.

Table 5: Composition of PDA.

Potato infusion	4.0 g
Dextrose	20.0 g
Bacto agar	15.0 g
Demineralised water	1000 mL
Final pH at 25°C	5.6 ± 0.2

The medium used for long term storage of fungal strains was the MexA medium (**Table 6**), a fungal growth medium which combines yeast extract as nitrogen source and malt extract

for carbon source. Glycerin or glycerol was added as a cryoprotectant of fungal cells. When subjected to -80°C, the cytoplasmic fluids will not freeze and form cell-rupturing crystals.

Table 6: Composition of MexA medium/agar.

Yeast extract	0.1 g
Malt extract	20.0 g
Glycerin	50.0 g
Bacto agar	13.0 g
Demineralised water	1000 mL

The medium used for large scale solid phase fermentation of fungal strains was salt supplemented solid rice medium (**Table 7**). The rice grains used were high quality commercial rice, acting as a source of nutrients for the mass growth of fungi. Artificial sea salt was added to induce a saline environment, which may aid in the growth of mangrove endophytic fungi.

Table 7: Composition of salt supplemented rice medium.

Rice (polished, white)	100 g
Artificial sea salt	3.0 g
Demineralised water	110 mL
Prepared in 1000 mL Erlenmeyer flask	

In preparation of all the above media, a sterilisation step was carried out by autoclaving at 121°C for 15 minutes. The agar-based media were cooled to 60°C before pouring onto sterile petri dishes; storage of prepared plates was at 4°C, up to seven days until use. Solid rice medium was allowed to cool to room temperature after autoclaving and was used within 48 hours.

2.3. Identification of microbes

The identities of the fungal strains isolated were determined using molecular method of Internal Transcribed Spacer – Polymerase Chain Reaction (ITS-PCR). The ITS region in the eukaryotic genome codes for a portion of non-functional rRNA; as its names suggested, this DNA sequence will be transcribed into RNA form, then excised and removed after its role as a genetic spacer has been fulfilled. This region is targeted for fungi identification and taxonomy as it exhibits high degree of variation even among closely related species. DNA extraction was carried out using Precellys 24 cell homogenizer according to manufacturer"s protocol. Fungal cultures (approx. 500 mg) were transferred to CK28 tubes containing big ceramic beads and topped up with Tris-HCl buffer pH 8.0 (1000 µL). After cell lysis was done, cellular debris and precipitates were removed via centrifugation. Successful isolation of genomic DNA was verified using agarose gel electrophoresis.

Amplification of fungi ITS region was carried out using primer pairs ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), as previously described by White et al. (1990). ITS-PCR was carried out in a final volume of 25 µL containing 20 ng template DNA, 10 µM each of forward and reverse primers (ITS1 and ITS4), 100 µM of each dNTPs, 1.5 mM $MgCl_2$ and 0.5 U RedTaq DNA polymerase, as required by RedTaq Master Mix protocol (Invitrogen). Amplification reactions were performed in a thermocycler (Eppendorf) programmed as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 35 sec (denaturation), 52 °C for 1 min (annealing), 72 °C for 1 min (extension), concluding with a final extension at 72 °C for 5 min. The PCR products were held at 4°C to prevent denaturation in ambient temperature. PCR product (4.0 µL) were checked via electrophoresis using a 1.0 % (w/v) agarose gel in 1X TAE buffer, stained using ethidium bromide, and photographed under ultraviolet (UV) light with a Gel Doc visualisation system (GelDoc X, Biorad). PCR products of the correct size (approx. 500 bp) were then purified using commercial kit (Invitrogen). Purified PCR products were sent for Sanger sequencing at Beijing Genomic Institute, along with ITS1 primer. The returned sequences were analysed using the Basic Local Alignment Search Tool, Chromas 2.22, and MEGA version 5. **Figure 5** below provides an overview of the analytical steps involved in the molecular identification of fungal isolates.

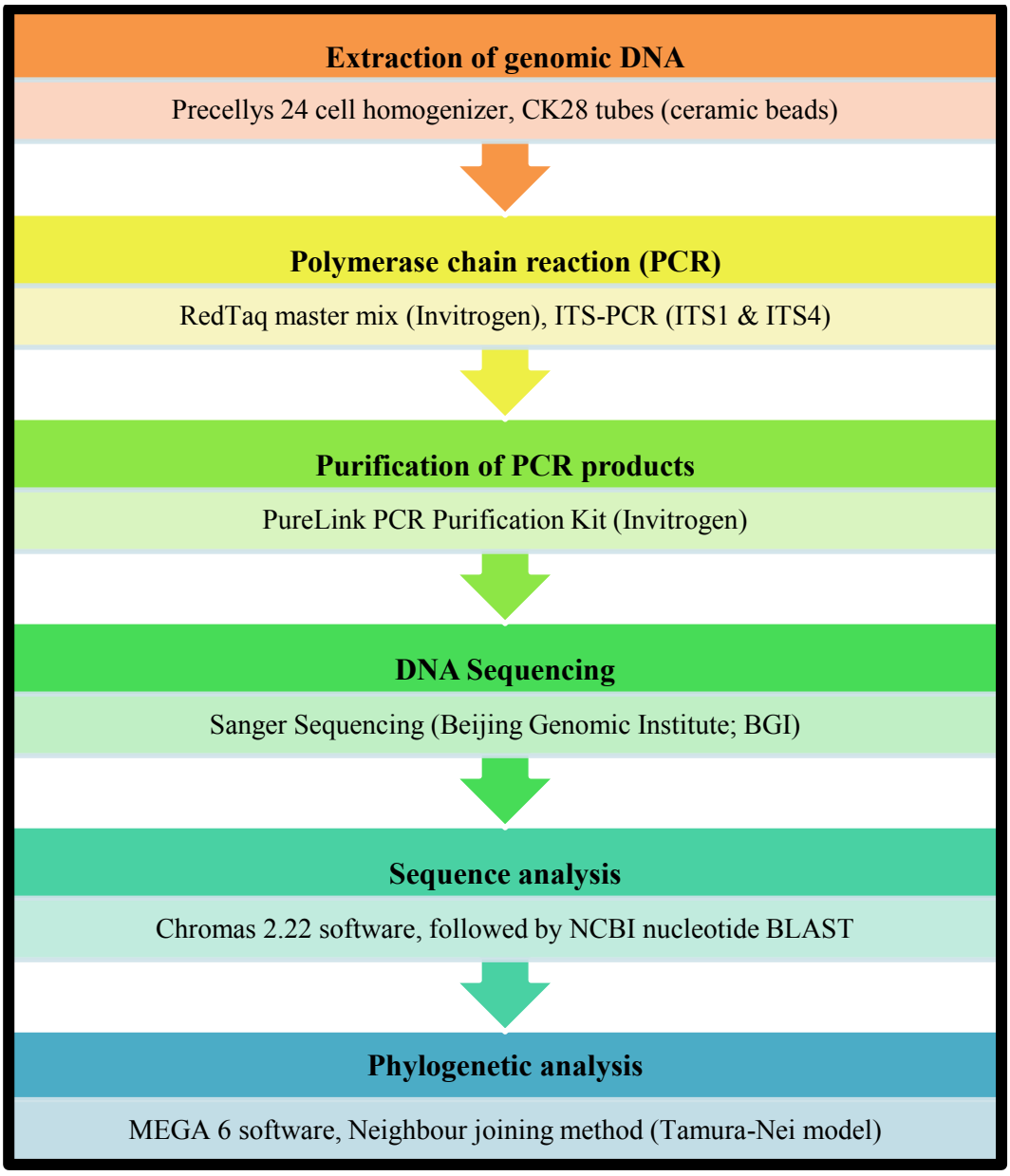


Figure 5: Flow diagram of methods used during molecular identification of fungal strains.

2.4. Fermentation and extraction of fungal cultures

2.4.1 Cultivation for secondary metabolites. Solid phase fermentation of purified fungal strain was carried out using salt-supplemented cooked rice medium. The necessary amount of biomass for inoculation was obtained by first growing the purified fungal strain in several petri dishes containing PDA until mycelia has covered almost the entire agar surface. The agar, along with actively growing fungus, was then cut into small pieces, then inoculated into culture flasks containing growth medium. A total of fifteen inoculated experimental flasks were prepared, and two more were left uninoculated to act as negative controls for incubation step. The flasks were incubated under static conditions at room temperature (25°C) and in the absence of sunlight for two weeks, or until the fungi completely cover the external surfaces of rice. The fermentation was brought to end by addition of 250 mL ethyl acetate (EtOAc) into the culture flask, sufficient to fully submerge the rice, and left standing overnight.

2.4.2 Extraction of fungi grown on solid rice medium. The culture medium was cut into small pieces and shaken (8 hr) fully immersed in EtOAc to allow for exhaustive extraction. The contents were filtered through a Buchner funnel and extracted for a further two times with fresh EtOAc until exhaustion. The EtOAc phases were then combined and washed with 300 mL demineralized water to remove sugars and starch. Negative control was prepared by solvent extraction of uninoculated rice which was autoclaved and left standing for the same amount of time.

The extract was evaporated to dryness at 40°C under reduced pressure and partitioned between 90% methanol (MeOH) and *n*-hexane to obtain two fractions, one highly polar and one less polar. After further evaporation, the two obtained fractions as well as the crude extract were submitted to thin layer chromatography and analytical high pressure liquid chromatography (HPLC).

2.5. Isolation of natural products

2.5.1 Thin layer chromatography (TLC). Thin layer chromatography provides a cheap, fast, and reliable method to qualitatively monitor the isolation and purification of secondary metabolites. The sample drawn to perform this test is minimal; hence it can be applied to extracts or fractions of small volumes without a need for pre-dilutions. The results obtained from analytical TLC allows for selection of appropriate conditions for column chromatography; most importantly stationary phase, solvents, and flow rate.

Throughout the course of this study, analytical TLC was carried out using pre-coated TLC plates (silica gel 60 F_{254}, layer thickness 0.2 mm). As the primary focus was on isolation of polar compounds, the solvent system used was a mixture of dichloromethane (DCM) and methanol. DCM:MeOH at ratios of 50:50, 40:60, 25:75, and 10:90 (V/V).

Band separation on TLC is indicative of separation efficiency between compounds present in sample. Separation was initially visualised under a standard UV lamp at 254 and 366 nm. Following that, a second visualisation step was carried out by first lightly spraying the TLC plates with anisaldehyde/sulphuric acid reagent, then heating the plates to 110°C. This allows for development of colours on the TLC plate, ranging from slight yellow to deep purple-brown; care was taken to ensure excessive heating was avoided lest the coloured areas all turn charcoal black. The composition of anisaldehyde/sulphuric acid spray reagent used is given in **Table 8**.

Table 8: Composition of anisaldehyde/sulphuric acid reagent.

Constituents	Volume (mL)
Methanol	85.0
Glacial acetic acid	10.0
Concentrated sulphuric acid	5.0
Anisaldehyde	0.5

UV-based visualisation at 254 nm is useful for detection of hetero atom-substituted benzene compounds, ketides, and aryl carbonyls compounds; second visualisation with anisaldehyde/sulphuric acid reagent allowed for detection of phenols, steroids, sugars, and terpenes. Compounds visualised can then be compared against each other as well as with

standard preparations of known compounds due to their specific retention factors. Retention factor, R_f, of a given compound is calculated as

$$R_f = \frac{\text{migration distance of compound}}{\text{migration distance of solvent front}}$$

Hence, the maximum R_f value for any given compound is 1, the compound being extremely similar polarity with the solvent system used and hence migrates together with the solvent away from the initial spotting site.

2.5.2 Vacuum liquid chromatography (VLC). The initial separation for large amounts of crude extracts (>1.0 g) can be accomplished by employing the "dry column" VLC method. Using this method, a large amount of extracts can be split to several simple fractions according to polarity; it can also be applied for large scale purification of organic synthesis products. The initial sample was fully adsorbed onto minimal amounts of silica gel using MeOH solvent to form a thick homogenous slurry. This was followed by a quick drying step using vacuum to regains powdery texture of silica gel. The VLC column was separately packed with fresh silica gel under applied vacuum into a hard cake. The sample-silica gel powder was then loaded onto the top of the packed column while still under pressure, and the top surface was covered with a protective layer of glass wool or cotton. The first non-polar solvent was then poured gently through the wool/cotton, coming into contact with the sample-silica layer, then proceeding to elute the compounds through the column. Successive fractions were collected via elution under stepwise increment of solvent polarity; the gradient system used was given in **Table 9**. Quick flow rate was achieved via continuous application of vacuum, and the column was allowed to run dry for each elution performed.

Table 9: Solvent system used for vacuum liquid chromatography.

Fractions	Volume of solvents used			
	n-hexane (mL)	EtOAc (mL)	DCM (mL)	MeOH (mL)
I	250		-	-
II	200	50	-	-
III	150	100	-	-
IV	100	150	-	-
V	50	200	-	-
VI	-	250	-	-
VII	-	-	250	
VIII	-	-	200	50
IX	-	-	150	100
X	-	-	100	150
XI	-	-	100	400
XII	-	-		500

2.5.3 Low pressure liquid chromatography (LPLC). Standard column chromatography was used to separate the compounds in simple fractions to give even simpler fractions and possible purified compounds. The appropriate stationary phase and mobile phase solvents were chosen based on TLC data, amount of mixture to be separated. In this study, size exclusion chromatography was the preferred method as it effectively eliminated interfering pigment molecules which are typically larger in size and takes longer time to pass through the column.

2.5.3.1 Size exclusion chromatography. Size exclusion chromatography involves separation of analytes based on their molecular sizes. The stationary phase comprises of porous beads (Sephadex LH-20) which acts as molecular sieves. Compounds with molecular diameter larger than the pore size will be excluded from the beads and be eluted first, whereas compounds with smaller molecular diameters will enter the pores of the beads and be retained within. The elution time of those compounds trapped by the beads is dependent on their ability to exit the pores once trapped. The mobile phase used in this study was MeOH (100%), though optimisation was carried out using MeOH-DCM mixtures of up to 1:1 (V/V) ratio; mixing in DCM changes the pore size and may give better resolution of compounds. Eluted fractions were collected via automated fraction collector and subsequently combined according to TLC results.

2.5.4 High pressure liquid chromatography (HPLC). HPLC is a chromatography technique which gives high resolution at a short amount of time (0.5 - 1.0 hour per sample). The fast separation is achieved by the mobile phase being forced through the stationary phase column by means of a mechanical pump. High resolution and separation is possible due to small particle size of reverse phase silica. The HPLC was coupled with a UV detector for monitoring the separation of eluted compounds.

2.5.4.1 Analytical high pressure liquid chromatography (HPLC). Analytical HPLC was used to monitor the purification steps at a more detailed level as compared to TLC. This method allows for identification of known compounds by comparison of UV spectrum of a given peak with the UV spectra library of previously discovered compounds. More importantly analytical HPLC greatly aids in determining the composition of fractions and purity of almost any sample. HPLC grade solvents were used as mobile phase, consisting of two solvents: (**Solvent A**) nanopure water (pH 2.0 by addition of phosphoric acid) and (**Solvent B**) MeOH. A flow rate of 1 mL/min was used, with the gradient system as given in **Table 10**.

Table 10: Gradient system used to perform analytical HPLC (Solvent A: analytical grade water, pH 2.0; Solvent B: analytical grade methanol)

Time (min)	**Solvent A (%)**	**Solvent B (%)**
0	90	10
5	90	10
35	0	100
45	0	100
46	90	10
60	90	10

2.6. Structure elucidation of the isolated fungal metabolites

2.6.1 Liquid chromatography mass spectrometry (LC-MS). The analytical method which combines HPLC with a mass spectroscopy analysis is termed liquid chromatography mass spectrometry, or LC-MS. This technique allows for fast resolution of components in a sample and detection of each UV-absorbent component, allowing for high throughput characterisation of components. The mass spectrometry carried out in this study was the electron spray ionization mass spectrometry (ESIMS) method. ESIMS involves the ionisation of sample as it passes through a high voltage metal capillary, followed by aerosolisation of the ionised components with nitrogen gas. The droplets of sample shall pass through a heated chamber and begin to evaporate, at which point the ions within each droplet will be ejected and be captured by the vacuum of the mass analyser. With this technique, information regarding the compounds analysed can be derived; the most significant are the mass (m) to charge (z) ratios (m/z), the molecular ion peaks of the positive and negative charged fragments ($[M+H]^+$ and $[M-H]^-$), and by extension, the molecular weights of pure compounds. Fragmentation data may also be obtained for more complicated structures. Chromatography grade solvents were used as mobile phase, consisting of two solvents: (**Solvent C**) 0.1% formic acid and (**Solvent D**) acetonitrile. A flow rate of 0.4 mL/min was used, with the gradient system as given in **Table 11**.

Table 11: Gradient system used to perform LC-MS (Solvent C: analytical grade 0.1% formic acid; Solvent D: analytical grade acetonitrile)

Time (min)	**Solvent C (%)**	**Solvent D (%)**
0	90	10
5	90	10
35	0	100
45	0	100
46	90	10
60	90	10

2.6.2 Nuclear magnetic resonance spectroscopy (NMR). This technique is based on the peculiar nuclei spin behaviour of atoms with odd numbered nucleons, e.g. commonly found isotopes ^{1}H and ^{13}C. A deuterated solvent was first used to dissolve the sample to remove solvent interference, a varying magnetic field over a small range was then applied onto the sample; resonance signals from the sample was observed. The protons attached to molecules experience a range of electronic environments due to shielding and deshielding effects of electrons around them. The result is an NMR spectrum unique to each molecule; the resonant frequencies of nuclei are represented by the chemical shifts (measured in ppm), and the coupling constants between adjacent nuclei (measured in Hertz, Hz) gives us an indication of the bonds present in the molecule. In this work, only one dimensional analysis (1D NMR) was performed. NMR spectrum was measured in Institut für Anorganische Chemie und Strukturaufklärung, Heinrich-Heine Universität, Düsseldorf with a Bruker ARX-500 NMR machine.

2.7 Media optimisation

During the earlier steps of isolation of secondary metabolites, two strains of the three strains studied grew too slow in rice medium to obtain any meaningful data; it took ten days for those strains to occupy half the area of 100 g cooked rice. The growth of true endophytes *in vitro* will differ from natural growth *in planta* as the physical and chemical conditions would have deviated significantly. During the course of bioprospecting for industrially important strains, many fungi would have been overlooked or uncultured simply due to oversight towards slow-growing fungi. This section attempts to develop a suitable media for upkeep of mangrove endophytic fungi strains with a view of inducing faster primary growth and initiation of secondary metabolism.

2.7.1 Standard media. For optimisation of growth medium, a baseline data set must be obtained. It was decided that fungi growth on standard cultivation medium be an adequate model for generation of such data points. This is in accordance with the objective of finding the best in vitro cultivation medium for slow growing mangrove endophytic fungi; any significant deviation of growth rates when compared to a standard profile will be clearly

seen. The standard medium used in the study was Potato Dextrose Agar (PDA) as mentioned in Table X.

2.7.2 Non-standard media. Several non-standard media were prepared for cultivation of endophytic fungi strains for optimisation studies. All of them with PDA as a basic growth medium with more additives included in a "stepwise" manner to more closely replicate the conditions of mangroves.

Potato Dextrose Yeast-supplemented Agar (PDYA) was prepared according to the given recipe in **Table 12**. The addition of yeast extract as an organic nitrogen source was targeted at fungi with fastidious nitrogen metabolism.

Table 12: Composition of PDYA.

Potato infusion	4.0 g
Dextrose	20.0 g
Bacto agar	15.0 g
Yeast extract	1.5 g
Demineralised water	1000 mL
Final pH at 25°C	5, 7, 9 ± 0.2

Potato Dextrose Yeast-supplemented Artificial Seawater Agar (PDYASA) was prepared according to the given recipe in **Table 13**. The addition of artificial sea salts was targeted at halophilic fungi which might grow better under saline medium. The percentage used (3.0 % sea salt) in this preparation was to simulate the average salinity of the ocean. The artificial sea salts used were commercially available salt water aquarium salts.

Table 13: Composition of PDYASA.

Potato infusion	4.0 g
Dextrose	20.0 g
Bacto agar	15.0 g
Yeast extract	1.5 g
Artificial sea salts	30.0 g
Demineralised water	1000 mL
Final pH at 25°C	5, 7, 9 ± 0.2

Potato Dextrose Yeast-supplemented Natural Seawater Agar (PDYNSA) was prepared according to the given recipe in **Table 14**. The addition of filtered seawater was to discern any potential differences of fungi growth when salinity of medium was derived naturally as compared to artificial saline. Seawater was collected from the same site in which the fungi was isolated from, and then left overnight for sediments to settle. The clear upper portion was then filtered through a 0.45 μm pore size membrane.

Table 14: Composition of PDYNSA.

Potato infusion	4.0 g
Dextrose	20.0 g
Bacto agar	15.0 g
Yeast extract	1.5 g
Filtered natural seawater	1000 mL
Final pH at 25°C	5, 7, 9 ± 0.2

Potato Dextrose Yeast-supplemented Mangrove Natural Seawater Agar (PDYMNSA) was prepared according to the given recipe in **Table 15**. The addition of mangrove plant material to the media containing ample nutrients, nitrogen, and naturally occurring salinity was hoped to exhaustively simulate the conditions of mangrove plant tissue. The medium was, in theory, the closest match to the conditions endophytic fungi would experience within the host plant.

Table 15: Composition of PDYMNSA.

Potato infusion	4.0 g
Dextrose	20.0 g
Bacto agar	15.0 g
Yeast extract	1.5 g
Finely grounded mangrove leaves	10.0 g
Filtered natural seawater	1000 mL
Final pH at 25°C	5, 7, 9 ± 0.2

2.7.3 Adjustment of pH of growth medium. While standard PDA was formulated for final pH 5.6, it was in light of most terrestrial fungi‟s preferences for slightly acidic environments. In this study, we were dealing with fungi of marine and endophytic origins; thus a range of pH was used in order to compare growth of fungi when subjected to low pH (5.0; favoured by terrestrial strains) and high pH (9.0; closer in value to the pH of the ocean). The pH of non-standard medium was adjusted via the addition of mostly NaOH and HCl, then checked using pH meter. After pH adjustment, the modified media were then subjected to autoclave at 121°C for 15 minutes.

2.8 Experimental set-up and inoculation

2.8.1 Plate preparation. Standard Petri dishes (disposable, plastic, 80 mm diameter) were used to grow the fungi. Into each dish, a fixed volume (10 mL) of molten agar was dispensed via sterile syringe, swirled, and then left to cool under UV. After the agar had solidified, the plates were labelled appropriately and stored for up to 3 days at room temperature pre-inoculation.

2.8.2 Inoculation method. The fungi strains chosen were first grown in standard PDA for one and half weeks, multiple plates were prepared to verify that the fungus growing on each plate is still in its logarithmic growth phase as well as uncontaminated. Using a modified single-spore method, adapted from Choi et al. (1999), a tiny piece of agar containing actively growing hyphal tips of fungi was carefully transferred onto fresh medium.

2.8.3 Experimental set up. Each strain was inoculated onto standard PDA and 4 different nutrient-modified medium, with each of the modified media prepared at 3 different pH. Hence, each fungal strain was subjected to 13 unique conditions, one of which forms the baseline data set. Quadruplicates were also performed to ensure reliability of measurements. All inoculations were performed within a 4-hour window to ensure age of inoculum is constant. **Figure 6** shows a visual guide to the inoculation steps carried out, whereby an actively growing axenic culture was inoculated onto standard and modified medium.

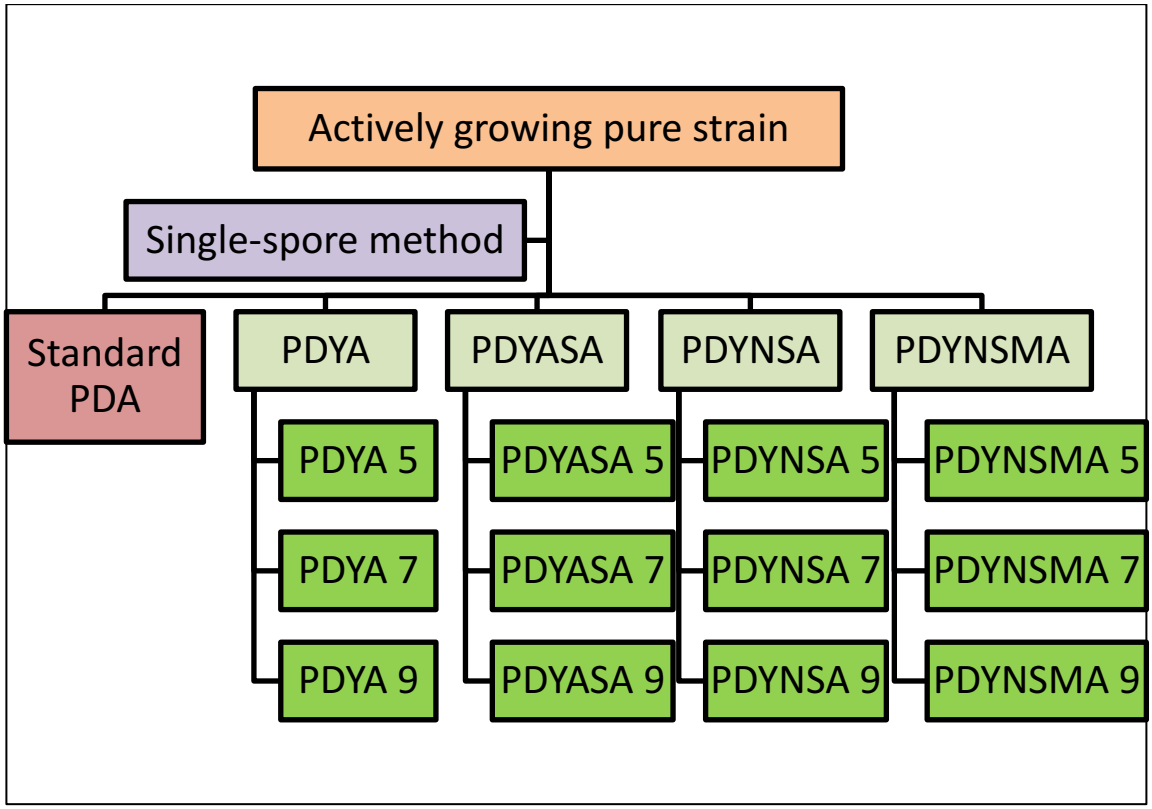


Figure 6: Experimental set up of media which was inoculated with fungi for growth optimisation study.

2.9 Measurement of growth rate

The gridlines used were drawn using fine tip permanent marker onto the surface of transparency film. The dimension of the smallest square was 1.0 mm × 1.0 mm, giving the smallest measurement taken to be 1.0 ± 0.3 mm^2. The error of 0.3 mm^2 was based on the concept of *reading error equals half the smallest division*; as the area measured was calculated from two "readings" of 1.0 mm, the error incurred was hence 0.5 × 0.5 mm =

0.25 mm = 0.3 mm (expressed to one significant figure). This source of error is important to note, but ultimately insignificant as the statistical deviations generated from each set of fungi growth area measurements far outweigh reading errors.

As growth of fungi from a single point on agar is never perfectly circular, measuring of the area occupied by mycelia is the best indicator to correlate with increase of fungal biomass. The usage of such a method is due to the need for quick and non-destructive method for assessing fungal growth on solid medium over a period of time. Spectroscopic analysis based on fungal turbidity and metabolites can easily be performed if growth medium was liquid; to undertake such measurements for fungi growing on solid substratum would require maceration or solvent extraction – both methods will lead to death of fungi culture.

3. Results and Discussion

3.1 Identification of organisms

3.1.1 Mangrove plant identification. The plants collected belonged to the taxonomical group *Avicennia* sp. based on comparison of the leaf and receptacle with a visual guide by Yong and Sheue (2014).

3.1.2 Endophytic fungi isolation. The standard procedure for isolation of endophytic fungi was followed. Negative control plates were prepared by streaking the outer surface of plant tissue onto YGCA medium. This ensures the fungi obtained was not already present on plant surface as saprobes and are true endophytes (Amadi 2005).

During the course of isolation and purification of fungi strains, a sectoring event was observed and resulted in the isolation of two distinct morphological types from previous subculture plates (**Figure 7**). The isolated morphological types were subcultured for a further three generations to assess their purity and stability. Molecular identification using ITS-PCR for both types was carried out to check for potential genetic mutation; this would provide some certainty regarding the genetic similarity between the two strains even though they may be phenotypically different.

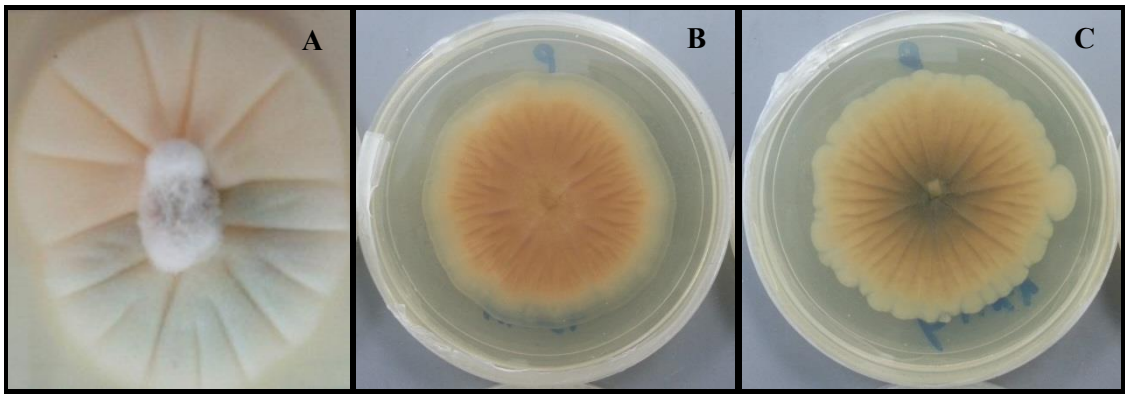


Figure 7: Observed sectoring in axenic culture, which led to the isolation of two relatively stable strains of fungi used in the study (A: initial sectoring giving two distinct morphotypes; B: YL1; C: YL2; reverse side shown for B and C).

3.1.3 Endophytic fungi identification. Identities of fungi were determined via Sanger sequencing using ITS1 forward primer. The sequences obtained were compared against other submitted sequences on the nucleotide collection database found on the NCBI BLAST site. Phylogenetic reconstruction was carried out on MEGA6.0 programme based on maximum likelihood method (Tamura-Nei model); the most probable identities of each fungal strain is given in **Table 16**.

Table 46: Identities of fungal strains used in this study based on molecular methods.

Strain code	Closest match on BLAST	Identities	E value	Taxonomical name
YL1	*Neosartorya hiratsukae* isolate UOA/HCPF 10765 [GQ461906]	547/547	0.0	*Neosartorya hiratsukae*
YL2	*Neosartorya hiratsukae* isolate CCF 4102 [FR837959]	547/547	0.0	*Neosartorya hiratsukae*
YL3	*Guignardia mangiferae* strain L2-1 [EU747726]	596/600	0.0	*Phyllosticta capitalensis*

3.1.3.1 ***Neosartorya hiratsukae.*** The identities of fungi strains YL1 and YL2 were determined using the returned ITS sequence from fungal 18S rDNA gene to be related to *Neosartorya hiratsukae*. This species was first described by Udagawa et al. (1991) based on an isolated fungal strain from pasteurised aloe juice. It is the teleomorphic form (sexual state) of the anamorphic fungi *Aspergillus hiratsukae* of the *Aspergillus* section *Fumigati*, a group of fungi renowned for its polyphasic tendencies (Samson et al. 2007). *N. hiratsukae* has a ubiquitous nature, with subsequent isolation from various sources such as soil, fruit juices, indoor air, and human tissue. This fungus is a known producer of avenaciolide, a bicyclic bis-butyrolactone antifungal compound. First isolated in 1963 by Brookes et al. from *Aspergillus avenaceus*, this lipophilic metabolite is also a known inhibitor of glutamate transport in mitochondria of rat liver cells (McGivan and Chappell 1970). Although *N. hiratsukae* was designated a Risk Group 1 organism (non-pathogenic), there have been reports of its ability to cause aspergillosis in immunocompromised individuals (Guarro et al. 2002; Arabatzis et al. 2011). There have been no publications describing *N. hiratsukae* as an endophyte of plants, however due to the polyphasic nature of fungi from *Aspergillus* section *Fumigati*; taxonomy of this particular strain might be limited as overly specific nomenclature could potentially be confusing.

3.1.3.2 ***Phyllosticta capitalensis.*** Fungus strain YL3 was identified as related to *Phyllosticta capitalensis*, a common species of fungus with a worldwide distribution. This fungal species was first described by Hennings (1908) based on an isolate from *Stanhopea* (*Orchidaceae*) from Brazil. *Phyllosticta capitalensis* is now the accepted name for all strains previously incorrectly referred to as the teleomorphic *Guignardia mangiferae* (Wikee et al. 2011), which was why the assigned taxonomical name for YL3 was not in exact agreement with BLAST results (**Table 46**). Phytopathogenic *Phyllosticta* species have long been associated with the Citrus Black Spot disease in a variety of host plants (Wulandari et al. 2010)). Not too long ago, Glienke et al. (2011) proposed that endophytic, non-pathogenic isolates occurring over a wide host range be more accurately referred to as *P. capitalensis*, pending further analysis to resolve complexity of morphological variations observed within this group of fungi. Although species of *Phyllosticta* (teleomorph *Guignardia*) have been known to cause leaf spot symptoms and fruit diseases across a wide range of commercial crops, there has not been any evidence of pathogenicity towards humans. The cosmopolitan nature of *P. capitalensis* has resulted in its isolation from mangroves as well as many other plant communities of different conditions (Suryanarayanan et al. 2004), indicative of a very adaptable fungal species and unsurprising candidate for further biotechnological research. A member of the *Phyllosticta* genus, *P. cirsii*, was found to be a candidate for weed biocontrol (Evidente et al. 2008); and it seems likely *P. capitalensis* could be identified as a source of bioactive compounds in the future.

3.1.4 Risk group of fungi. All fungal material was classified as Risk Group 1 organisms, meaning that they have no major pathogenicity towards humans. This allowed for large scale cultivation of fungi to be carried out without significant risk of exposure.

3.2 Analysis of fungal secondary metabolites

3.2.1 Extraction of fungi grown on solid rice medium. Negative control was prepared by direct EtOAc extraction of rice medium which was not inoculated with fungi. No growth was observed in control flasks throughout the two weeks of incubation carried out for test flasks. A total of 452 mg of extracts was obtained from 200 g of cooked rice medium and subjected to analytical HPLC. The chromatogram obtained is given in **Figure 8**; and

although all the peaks observed were very low, matching compounds isolated in the test samples were excluded from subsequent analysis so as to reject false positives.

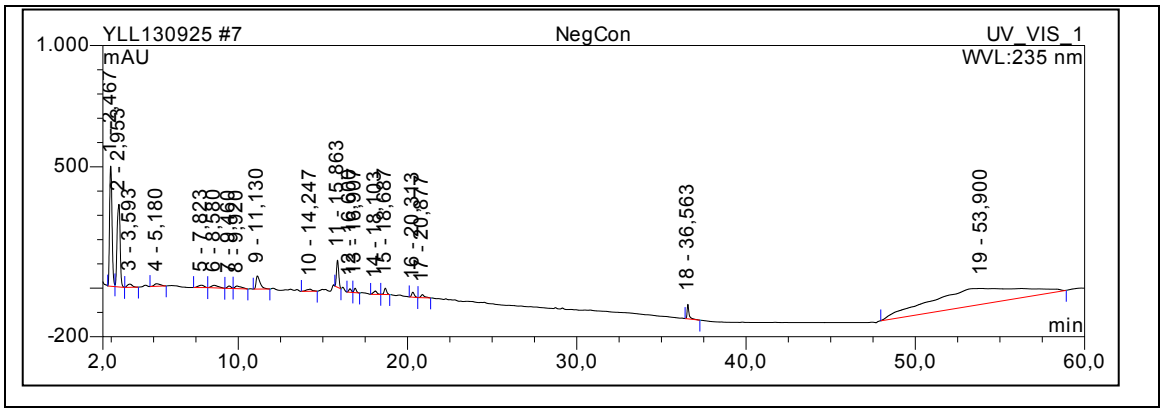


Figure 8: HPLC chromatogram obtained for extract (452 mg) obtained from negative control; taken at UV wavelength: 235 nm.

Extraction on test flasks was carried out using EtOAc to give an initial crude extract amount of 6937 mg. Following solvent partitioning using 90% MeOH and *n*-hexane, the polar fraction was found to weigh only 583 mg, whereas the non-polar fraction weighed 6215 mg. The HPLC chromatograms for UV_{235} visible compounds of the two fractions obtained are given in **Figure 9**, it is clear that a greater complexity of compounds exist in the lipophilic fraction in addition to being the heavier one.

The bulk of compounds present in crude extract were highly lipophilic, which made further work difficult as the apparatus available was suited for separation of polar compounds. Biologically active molecules are more likely to contain functional groups which interact with ligand binding sites; many such functional groups contain one or more oxygen and/or nitrogen atoms, which confer polarity to the molecule as a whole.

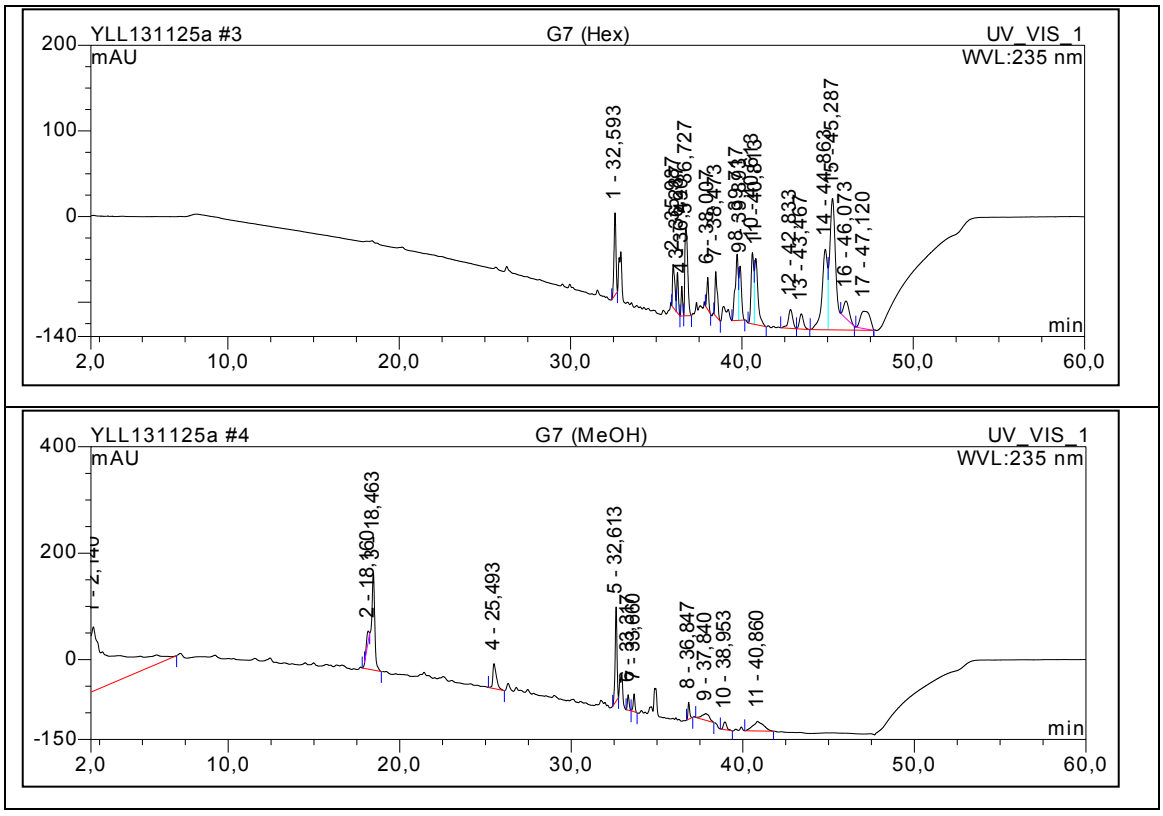


Figure 9: Comparison of HPLC chromatogram for n-hexane fraction (top, 6.215 g) and 90% MeOH fraction (bottom, 0.583 g); taken at UV wavelength 235 nm.

3.2.2 Vacuum liquid chromatography (VLC). Given the small amount collected, further work was still carried out on the 90% MeOH fraction in hope of isolating bioactive secondary metabolites. The hydrophilic fraction was subjected to VLC to yield thirteen fractions, as shown in **Table 17**. Each fraction was then subjected to analytical HPLC to screen for metabolites of unknown structure. The most promising fractions were G7M3 (62.73 mg) and G7M7 (185.35 mg), which were then further subjected to size exclusion chromatography.

Table 17: Fractions collected from VLC of hydrophilic portion of extract (583mg).

Fractions	Volume of solvents used				Weight of fraction (mg)	Compounds of note
	n-hexane (mL)	EtOAc (mL)	DCM (mL)	MeOH (mL)		
G7M1	250		-	-	0.55	-
G7M2	200	50	-	-	146.32	Aureonitol
G7M3	150	100	-	-	62.73	Ferulic acid
G7M4	100	150	-	-	32.70	
G7M5	50	200	-	-	29.51	
G7M6	-	250	-	-	40.47	Myrocin A
G7M7	-	-	250		185.35	*Unknown*
G7M8	-	-	200	50	9.56	
G7M9	-	-	150	100	8.75	
G7M10	-	-	100	150	3.43	
G7M11	-	-	100	400	3.22	
G7M12	-	-		500	1.54	
G7M13	*Note: 0.1% TFA was added			300	38.94	

3.2.3 Size exclusion chromatography. All samples obtained from sephadex column chromatography were pooled together based on observations of TLC spotting pattern. Eluents from G7M3 exhibited a simple pattern, thus pooled into three fractions; G7M7 yielded a total of ten distinct fractions.

Further work was carried out on selected fractions G7M3 and G7M7 as their weights were substantial and interesting peaks were observed. A summary of isolation and purification of metabolites from *Phyllosticta*-related strain YL3 is given in **Figure 10.**

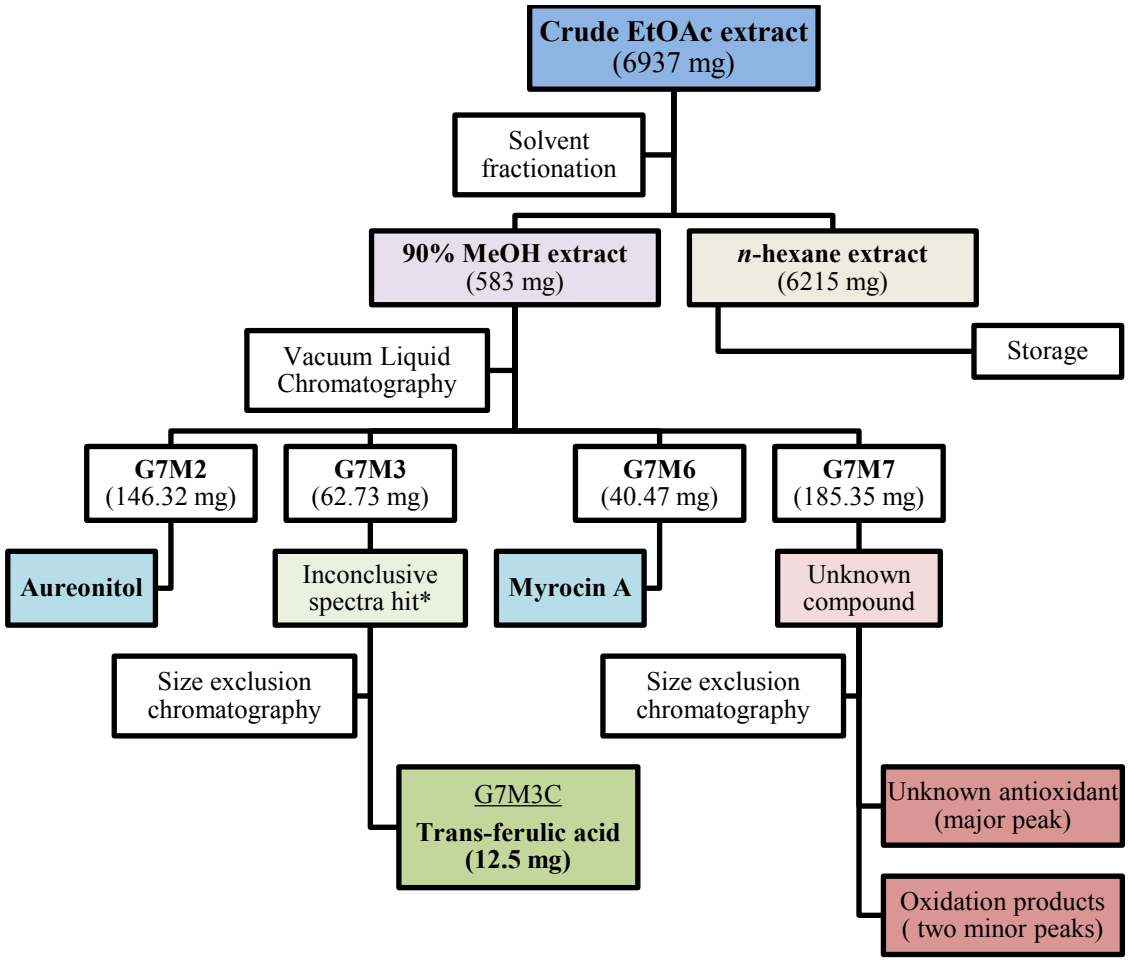


Figure 10: Secondary metabolites isolated from solid phase fermentation of YL3, a *Phyllosticta capitalensis* related species.

3.3 Compounds of interest

3.3.1 Trans-ferulic acid (G7M3C). The yellowish white powder afforded after evaporation of solvents from sample G7M3C was identified as ferulic acid; an overview of its chemical structure, HPLC chromatogram, and ESI-MS data is given in **Figure 11**.

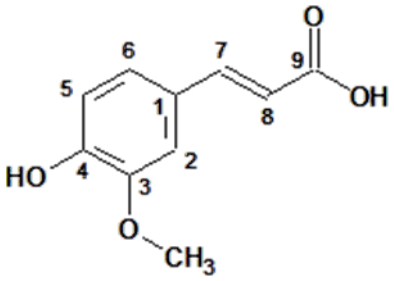
Synonyms	(E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid (IUPAC) 4-hydroxy-3-methoxycinnamic acid
Sample code	G7M3
Biological source	*Phyllosticta capitalensis*
Sample amount	12.5 mg
Physical description	Yellowish white, crystalline powder
Molecular formula	$C_{10}H_{10}O_4$
Molecular weight	194 g/mol
HPLC retention time	13.14 min (standard gradient)
Ferulic acid	

Figure 11: Summary of data obtained regarding trans-ferulic acid; inclusive of basic chemical information, exact chemical structure, HPLC chromatogram, and LCMS peaks.

Ferulic acid was one of the major polar metabolites produced by *Phyllosticta capitalensis*. Its UV maxima were found to be at 218, 292, and 322 nm (**Figure 11**). Pseudomolecular ions generated from positive and negative ionization by ESI-MS were detected at *m/z* 195.1

$[M+H]^+$ and *m/z* 193.4 $[M-H]^-$ respectively (**Figure 11**); the molecular weight was hence determined to be 194 amu. The NMR data (**Figure 12**) obtained was in general agreement with an earlier work by Yoshioka et al. (2004); the consistent differences in chemical shift values (δ_H) by approximately 0.2-0.3 ppm (**Table 18**) were most likely due to differences in instrumental calibration. Further detailed discussions regarding the isolation and potential roles of ferulic acid in mangrove endophytic fungi can be found in the following chapter.

Table 18: NMR data of trans-ferulic acid at 500 (^{1}H), measured in CD_3OD.

Proton	δ_H (*J* Hz) **Present study**	δ_H (*J* Hz) **Yoshioka et al. 2004**
2	7.21 d (1.9)	6.93 (2.1)
5	6.85 d (8.2)	6.67 (8.3)
6	7.10 dd (8.2, 1.9)	6.83 (8.3, 2.1)
7	7.64 d (15.9)	7.44 (15.6)
8	6.35 d (15.9)	6.15 (15.6)
3-OCH_3	3.93 s	3.64

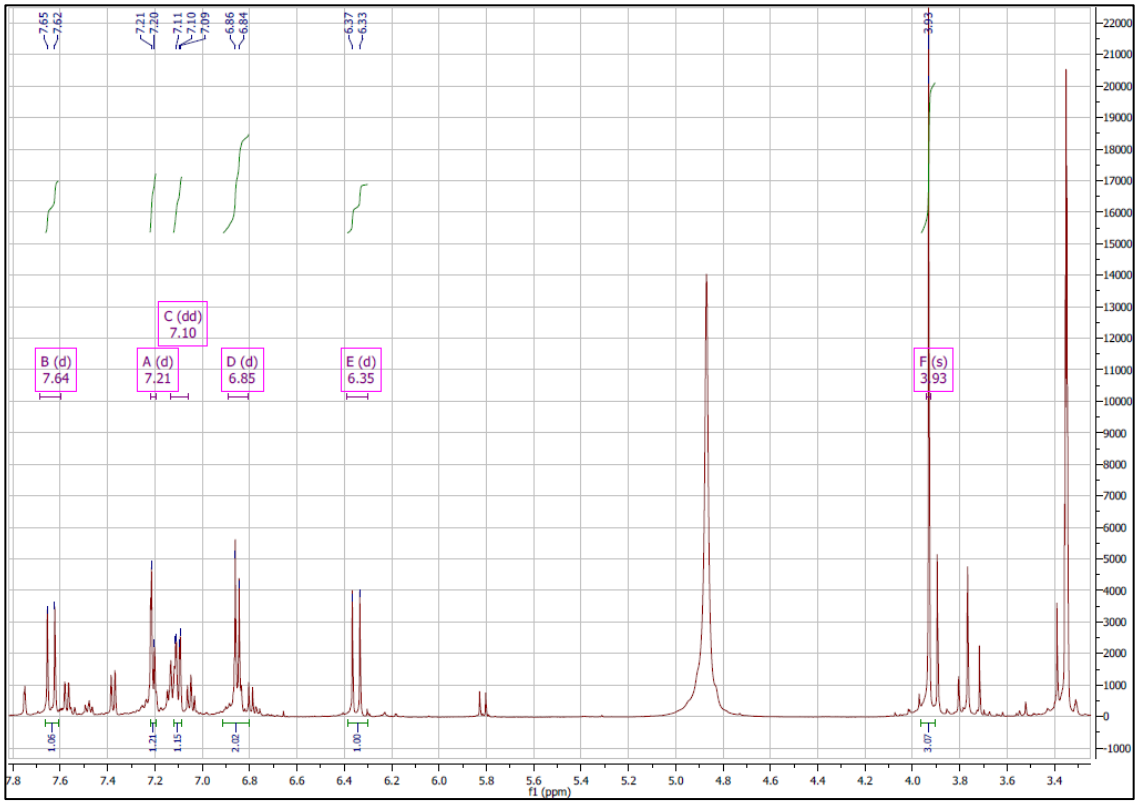


Figure 12: NMR spectrum for purified trans-ferulic acid.

3.3.2 Aureonitol (G7M2). The metabolite aureonitol (**Figure 13**) was originally isolated from the fungi *Chaetomium coarctatum* (Burrows 1967); known at that time only as a tetrahydrofuran derivative of unknown *cis-trans* configuration. It was later independently isolated from a plant of the sunflower family, *Helichrysum aureonitens* (Bohlmann and Ziesche 1979), with some progress made towards elucidating its structure and was given its current name based on the plant species. The absolute configuration of aureonitol was confirmed much later when Abraham and Arfmann (1992) successfully applied the Mosher method in determining the configuration of both the previously isolated compounds and found them to be the same. Aureonitol is not known for bioactivity, however a research done by Marwah et al. (2007) found that an epimer of aureonitol was capable of total broad spectrum inhibition of *Streptococcus pyogenes, Escherichia coli, Staphylococcus aureus, Salmonella choleraesuis,* and *Corynebacterium diphtheriae* at concentrations less than 35 mg/mL (35 ppt).

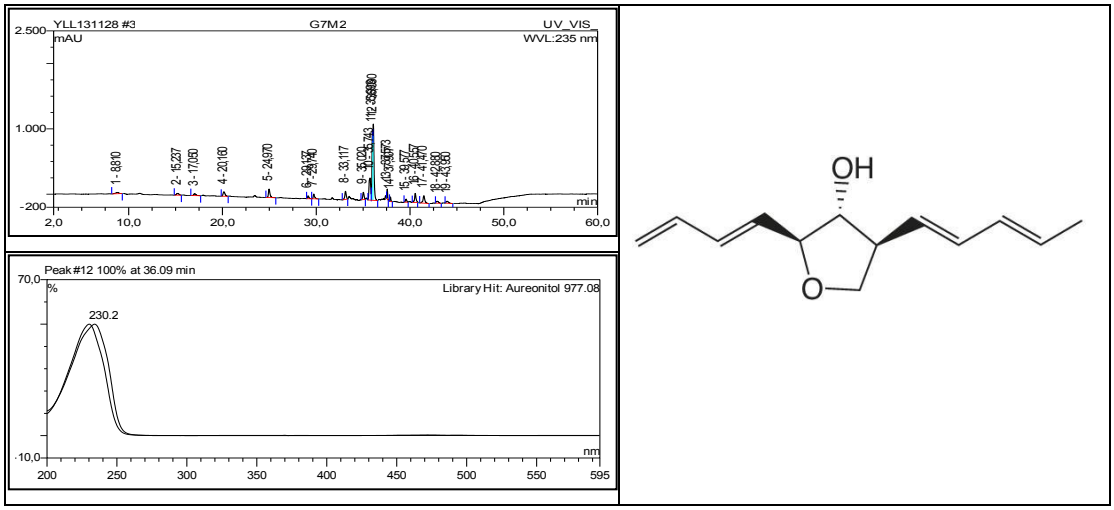


Figure 13: HPLC chromatogram of aureonitol (Peak #12), and its chemical structure.

It is not known whether the aureonitol identified using analytical HPLC in this study possesses the conformation of original aureonitol or its epimeric form, as it was found mixed with several compounds with similar polarity and UV spectra. This led us to believe that several different isomers of aureonitol were likely present in the sample. Further purification was not carried out due to the complex nature of the peak; separation using conventional methods will be difficult, whereas chiral chromatography was not readily available.

3.3.3 Myrocin A (G7M6). The metabolite Myrocin A was discovered by Klemke et al. (2004) from the marine endophytic fungus *Apiospora montagnei* from the inner tissue of the North Sea alga *Polysiphonia violacea.* Myrocin A (**Figure 14, left**) is a primarane type diterpene with no known bioactivity; it is however, closely related to Myrocin B (**Figure 14, right**), a diterpene with antibiotic properties isolated from the terrestrial phytopathogenic fungi *Myrothecium verrucaria* (Hsu et al. 1988). A broad range of bioactivity was reported for myrocin B, including antibiosis towards *Bacillus subtilis*, *Aspergillus niger*, and *Candida albicans*. This functional metabolite differed from myrocin A by having a four-ring structure formed by an intramolecular ester instead of a three-ring molecule with enone and carboxylic acid groups (**Figure 14**). Given that the compound myrocin A was isolated from *A. montagnei* without artificial induction, it is expected to play a role in the biochemistry of the fungus. Equipped with this information, the potential of myrocin A as a precursor molecule for structure activity studies should be explored.

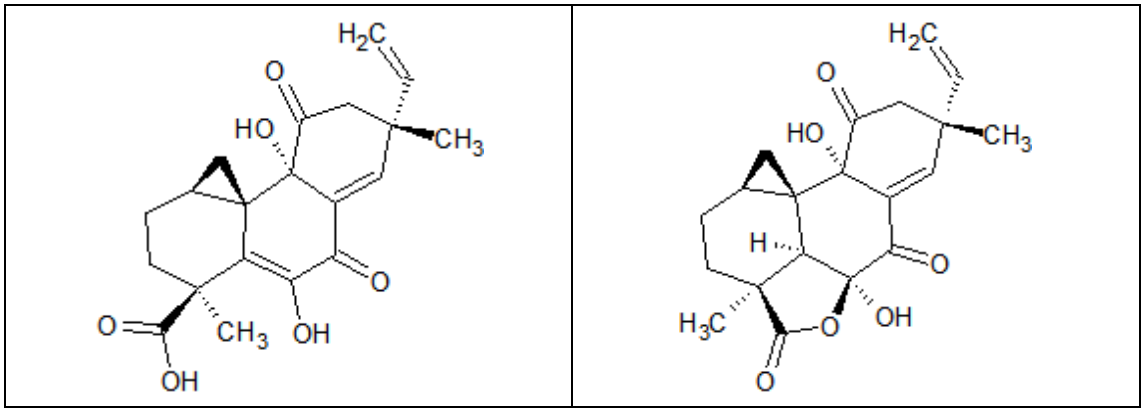


Figure 14: Molecular structures of Myrocin A (left) isolated from marine endophyte, and Myrocin B (right) isolated from terrestrial phytopathogen.

3.3.4 Unknown compounds from G7M7. An unknown compound was initially detected in sample G7M7 via analytical HPLC (**Figure 15**). Given that it appeared relatively pure, the fraction (185.35 mg) was subjected for removal of pigments via size exclusion chromatography. Stationary phase of Sephadex LH-20 and mobile phase of 100% MeOH was used, a total of 10 pooled fractions were collected based on distinct TLC spot profiles.

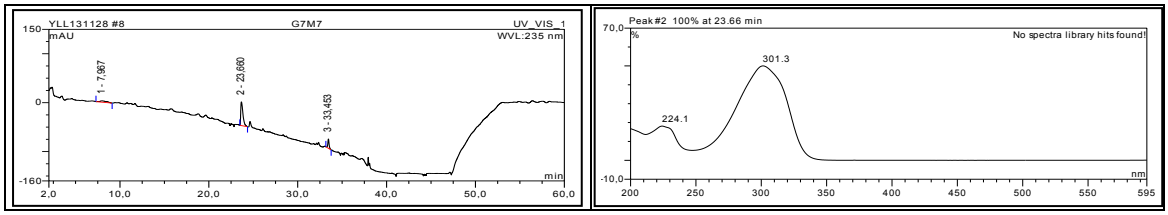


Figure 15: HPLC chromatogram (Peak #2) and UV spectrum obtained for unknown compound in G7M7.

Upon examination of the simple fractions obtained, the unknown compound was present in fraction G7M7C. However, the presence of two additional minor peaks flanking the original peak was also detected (**Figure 16**). The UV spectra of the minor peaks appear highly similar to that of the main peak; the UV maxima and general profile being just slightly off with to each other. This led to the conclusion that the unknown compound was most likely an antioxidant; whereby exposure to atmospheric oxygen during storage and chromatography procedures has led to the formation of two distinctively different oxidation products. The isolation and structural elucidation of this reactive species will prove difficult given that the quantity available is rather small.

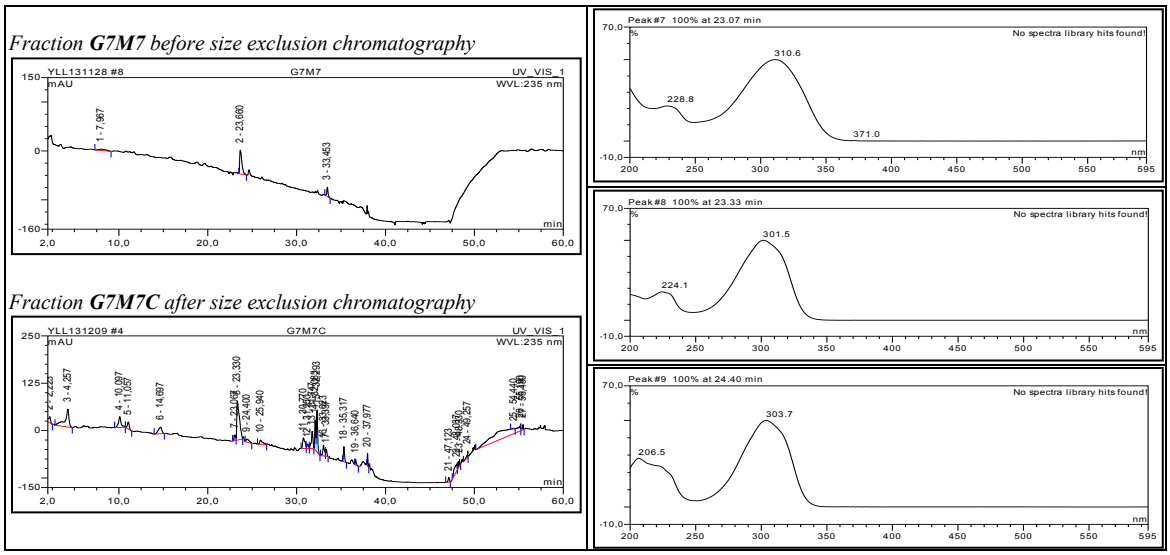


Figure 16: HPLC chromatograms before and after size-exclusion chromatography (left); the UV spectra of the major peak (Peak #8) and minor peaks (Peak #7 and #9) found after size exclusion chromatography (right).

3.4 Optimisation of growth of mangrove endophytic fungi

3.4.1 Growth measurements. Fungi were grown in quadruplicates. Mean surface area (MSA) was calculated by rejecting the least statistically significant reading and taking the mean of the remaining three readings. This was carried out for each of the three strains at grown in standard and modified growth medium. Measurements were taken every 2-3 days from Day 0 (MSA_0) until Day 21 (MSA_{21}), giving a total of nine data points for each of the 13 different medium used in this study.

Table 19: Mean surface area of strain YL1 on various growth media collected over 21 days (I: PDYA; II: PDYASA; III: PDYNSA; Numbers in brackets indicate pH of medium).

Day	Std. PDA	I(5)	I(7)	I(9)	II(5)	II(7)	II(9)	III(5)	III(7)	III(9)
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	19.2	16.3	14.0	15.0	5.3	10.8	3.8	8.7	4.2	3.7
4	88.0	105.0	111.7	110.0	65.3	71.5	35.2	56.3	49.8	83.3
6	180.0	173.3	156.7	138.3	91.2	110.0	53.8	77.4	123.3	106.7
9	323.3	336.7	305.0	315.0	296.7	275.0	170.0	348.3	240.0	231.7
11	361.7	443.3	385.0	356.7	353.3	356.7	231.7	366.7	346.7	331.7
13	455.0	501.7	433.3	456.7	431.7	456.7	268.3	531.7	466.7	365.0
15	505.0	551.7	538.3	528.3	501.7	515.0	301.7	720.0	591.7	506.7
18	643.3	675.0	563.3	663.3	636.7	608.3	485.0	1040.0	741.7	623.3
21	846.7	860.0	766.7	871.7	826.7	840.0	478.3	1466.7	975.0	836.7

Table 20: Mean surface area of strain YL2 on various growth media collected over 21 days (I: PDYA; II: PDYASA; III: PDYNSA; Numbers in brackets indicate pH of medium).

Day	Std. PDA	I(5)	I(7)	I(9)	II(5)	II(7)	II(9)	III(5)	III(7)	III(9)
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	8.2	14.8	9.0	5.7	7.8	12.2	4.3	2.2	5.3	4.3
4	62.2	94.7	69.5	68.0	49.8	58.8	33.0	29.3	52.2	46.3
6	114.5	118.3	90.3	85.2	81.7	86.2	44.3	76.0	83.3	70.2
9	181.7	198.3	165.0	158.3	246.7	201.7	105.0	121.7	191.7	186.7
11	196.7	246.7	191.7	206.7	418.3	290.0	110.0	161.7	233.3	225.0
13	340.0	250.0	226.7	198.3	638.3	375.0	135.0	238.3	325.0	228.3
15	371.7	308.3	255.0	235.0	830.0	455.0	153.3	306.7	356.7	270.0
18	468.3	381.7	290.0	271.7	1358.3	626.7	186.7	390.0	426.7	333.3
21	511.7	468.3	336.7	291.7	1775.0	1046.7	245.0	516.7	641.7	511.7

Table 21: Mean surface area of strain YL3 on various growth media collected over 21 days (I: PDYA; II: PDYASA; III: PDYNSA; IV: PDYMNSA; Numbers in brackets indicate pH of medium).

Day	Std PDA	I(5)	I(7)	I(9)	II(5)	II(7)	II(9)	III(5)	III(7)	III(9)	IV(5)	IV(7)
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	11.5	20.2	20.8	24.3	79.3	64.7	203.3	64.0	52.7	170.0	18.3	36.0
4	109.5	120.0	178.3	170.0	133.3	175.0	555.0	193.3	293.3	963.3	313.3	165.0
6	271.7	213.3	285.0	246.7	208.3	223.3	628.3	260.0	415.0	1286.7	461.7	251.7
9	618.3	705.0	910.0	805.0	310.0	571.7	1263.3	543.3	1050.0	2046.7	946.7	521.7
11	823.3	910.0	1386.7	1076.7	498.3	808.3	1408.3	745.0	1270.0	2483.3	1028.3	646.7
13	1098.3	1238.3	1773.3	1578.3	590.0	913.3	1571.7	941.7	1520.0	2896.7	1543.3	946.7
15	1435.0	1566.7	2381.7	1965.0	676.7	1226.7	1703.3	1245.0	1650.0	3138.3	2023.3	1323.3
18	1873.3	2206.7	2828.3	2431.0	813.3	1443.3	1880.0	1541.7	2045.0	3286.7	2158.3	1575.0
21	2445.0	3215.0	3563.3	3525.0	1203.3	1820.0	2061.7	2320.0	2763.3	3353.3	2798.3	2113.3

Processed MSA values are given in the **Tables 19, 20,** and **21** for strains YL1, YL2, and YL3 respectively. Raw data of MSA measurements are given in the attached Appendix section. The values were then selectively grouped and plotted as scatter plots, with only lines of significant data groups drawn and assigned error bars.

Figure 17 shows growth of strain YL1, grouped based on pH levels of medium. In **Figure 17A**, significantly high MSA_{21} on PDYNSA relative to MSA_{21} on PDA was recorded. **Figure 11** shows growth of strain YL2, grouped based on pH levels of medium. A distinct preference for PDYASA was clearly seen in **Figures 11A** and **11B**; although like its related strain YL1, the lower pH level of 5.0 was clearly favoured. When subjected to high pH levels of 9.0, it was noticed that some inhibition was quite noticeable (**Figures 10C** and **11C**). Yeast extract supplement alone did not give visible improvements in MSA_{21} values.

Figure 12 show growth of strain YL3, grouped based on media type. The reason for this is to better observe the significant influence of media composition on the growth of this strain. In **Figure 12A**, it can be seen that MSA_{21} of growth on PDYA was significantly higher than that of PDA regardless of pH levels. Growth was significantly enhanced by presence of yeast extract supplement irrespective of pH levels. From **Figures 12B** and **12C**, it was observed that the presence of salts in growth medium contributed to inhibition of growth. The attempt to induce increased growth by incorporation of mangrove plant material was not so successful, as shown in **Figure 12D** by the lack of large MSA values observed.

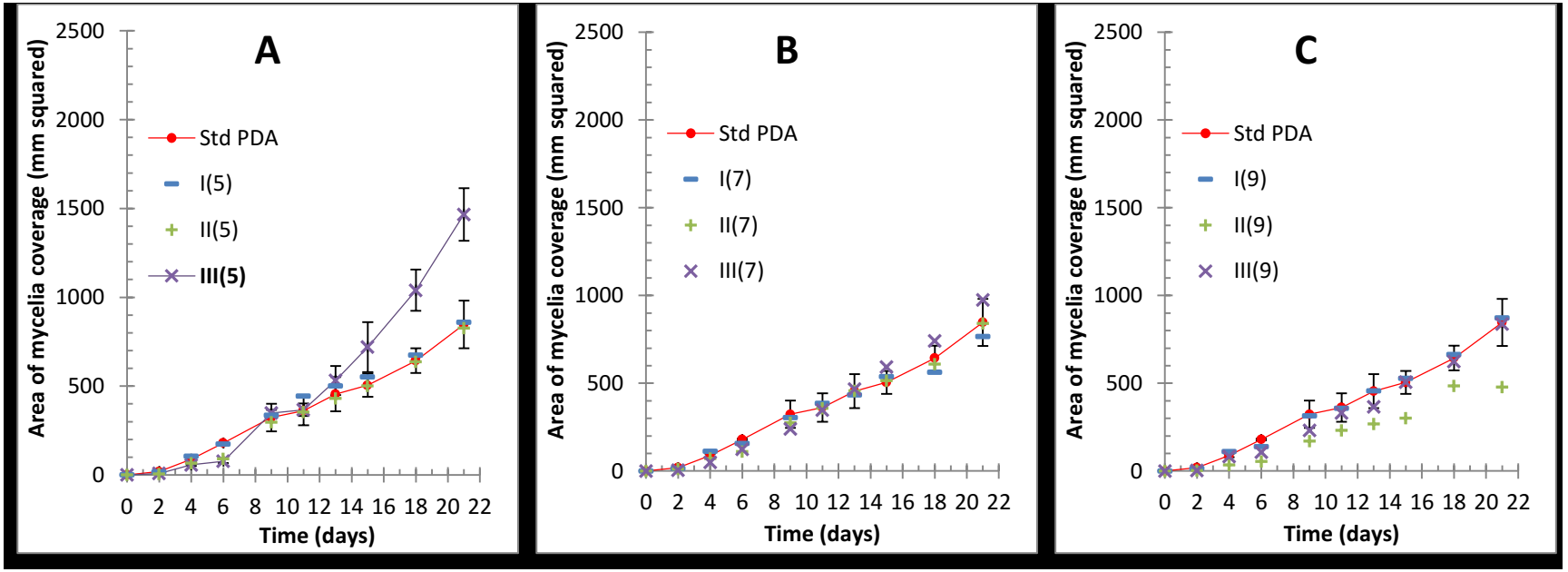


Figure 17: Growth curves for strain YL1 (A: growth at pH 5.0; B: growth at pH 7.0; C: growth at pH 9.0). Baseline data (growth on standard PDA) showed here as red line. (I: PDYA; II: PDYASA; III: PDYNSA).

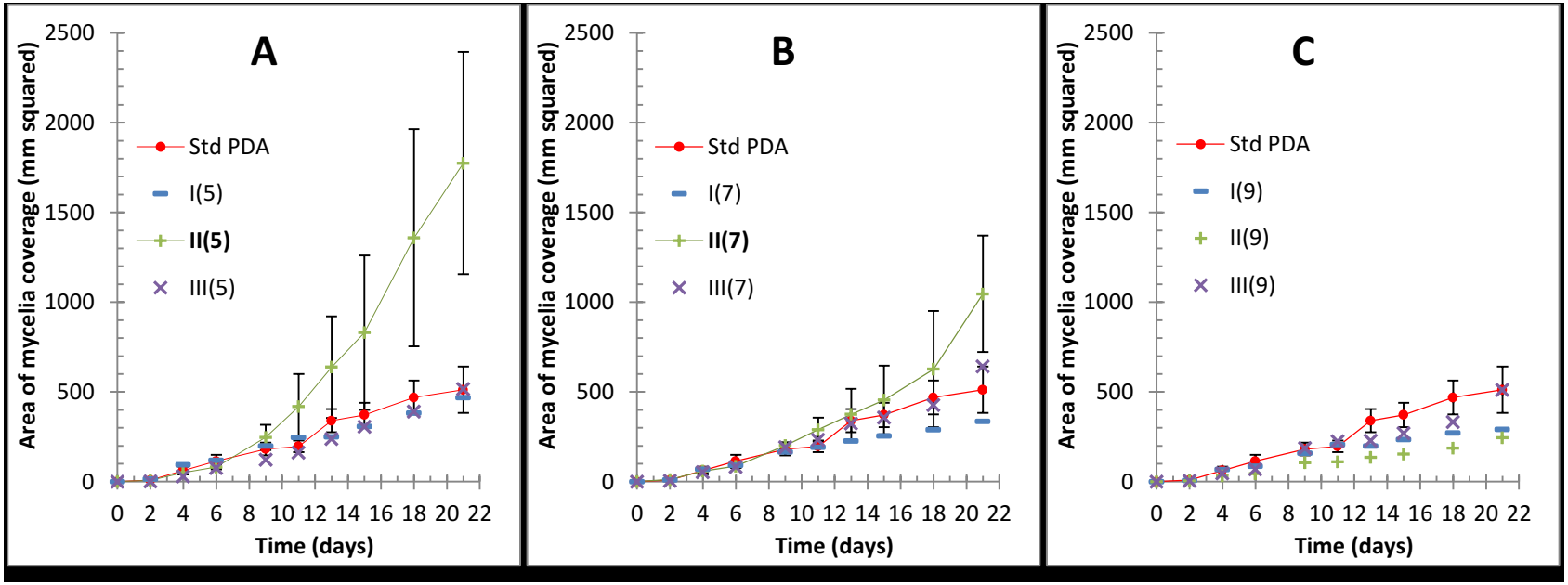


Figure 18: Growth curves for strain YL2 (A: growth at pH 5.0; B: growth at pH 7.0; C: growth at pH 9.0). Baseline data (growth on standard PDA) showed here as red line. (I: PDYA; II: PDYASA; III: PDYNSA).

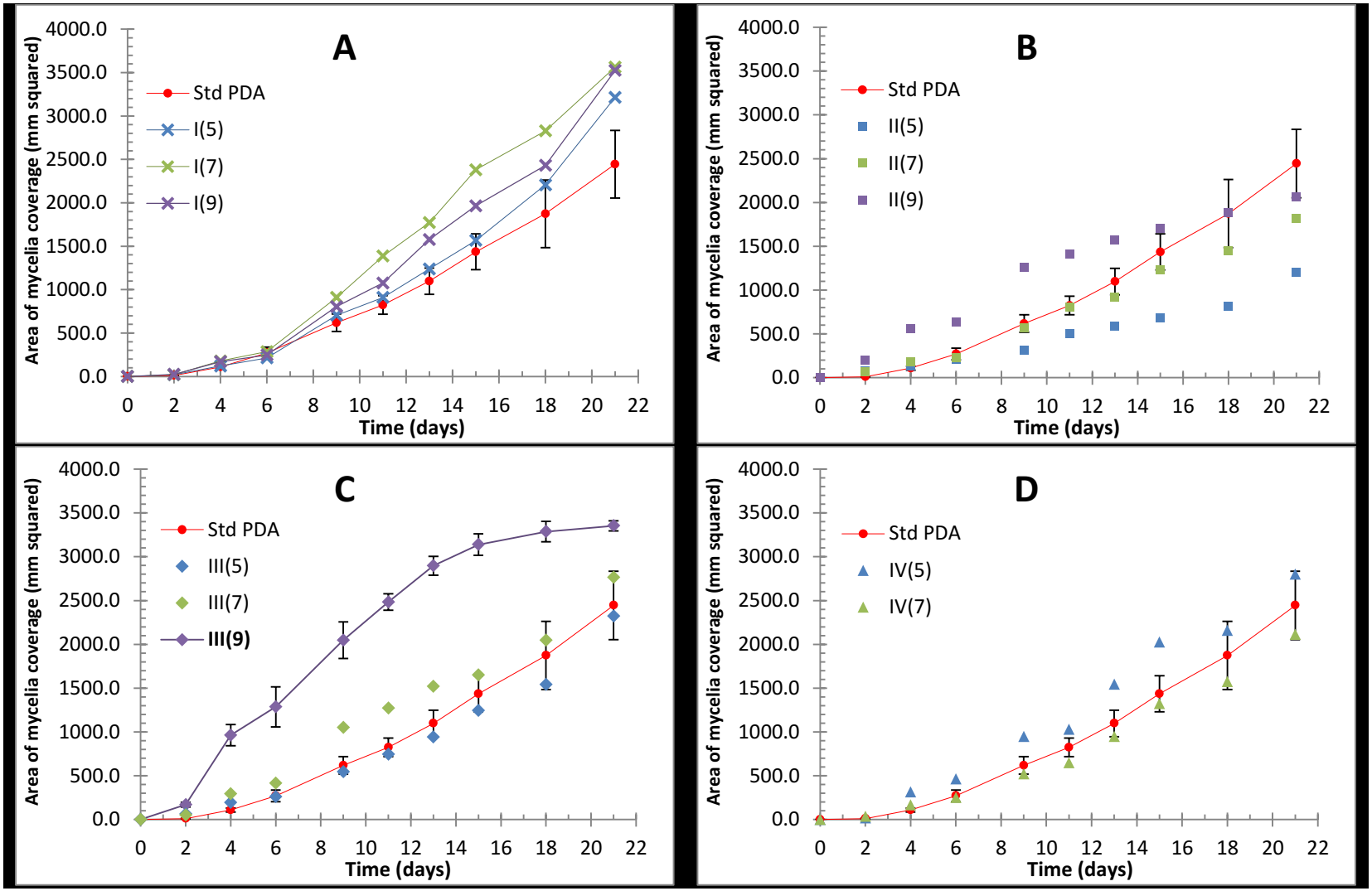


Figure 19: Growth curves for strain YL3 (A: growth in medium I; B: growth in medium II; C: growth in medium III; D: growth in medium IV). Baseline data (growth on standard PDA) showed here as red line. (I: PDYA; II: PDYASA; III: PDYNSA; IV: PDYMNSA).

3.4.2 Assumptions in method development. Key assumptions made during the course of this study are discussed in this section. It was understood that the chosen methodology comes with its inherent limitations but they appear to be not a great issue for consistency and reproducibility of experiment. ***Mycelia thickness.*** During this study, the thickness of mycelia remained relatively constant over time. The initial hypothesis was that MSA measurements were taken as an indicator of fungal growth which may be directly correlated to total fungal biomass. Trinci (1969) made a connection between radial growth rate and the lengthening of fungal hyphal tips, a similar trend was observed in this study but was not confirmed. ***Start of secondary metabolism.*** It was also assumed that all inoculated fungi will fully utilise the nutrients in growth medium before engaging in secondary metabolism. In order to ensure consistency, each petri dish was made using a fixed amount (10 mL) of medium to give a suitable substrate thickness. This standardised thickness of agar medium will theoretically allow for proper comparison of secondary metabolism amongst strains and growth conditions if present.

3.4.3 Trends observed. There were some general observations which can be made from the plots of MSA values in **Figures 17, 18**, and **19**. From Figure **17** and **18**, it is clear that the *Neosartorya*-related strains YL1 and YL2 favoured low pH ranges of 5.0 to 7.0. There were variations between the two strains in terms of the preferred medium for enhanced growth of fungal strains to yield increased MSA_{21} values. A distinct preference for salinity was observed for strains YL1 and YL2; *Phyllosticta*-related strain YL3 do not generally favour excess salts in growth medium (**Figures 19B, 19C**, and **19D**). These observations were within expectations, given that past studies have shown that salinity does exert a significant effect on the growth of fungi (Byrne and Jones 1975), especially the sodium ions which make up the bulk of cations in saline solutions (Jones and Jennings 1965).

4. Isolation of *trans*-ferulic acid from mangrove-derived endophytic fungi *Phyllosticta capitalensis*

4.0 Abstract

Mangrove wetlands form a unique ecosystem between land and sea; organisms which occupy this ecological niche have to tolerate temperature, salinity, and tidal fluxes on a daily basis. Endophytic fungi from mangrove are a distinct group as they have been found to produce a variety of secondary metabolites in response to biotic and abiotic stresses as well as being able to tolerate salinity levels beyond that of the typical fungi. A crude extract from mangrove-derived endophytic fungus *Phyllosticta capitalensis* (teleomorph *Guignardia mangiferae*) cultured on salt-supplemented rice medium was subjected to solvent partition, vacuum liquid chromatography, gel filtration chromatography, and semi-preparative HPLC. A phenolic phytochemical was isolated from the methanolic fraction of fungal extract and determined to be trans-ferulic acid via LC-MS and NMR. This is the first time in which ferulic acid was isolated from *Guignardia mangiferae*. Ferulic acid has been proven to possess significant antioxidant activity *in vitro* and *in vivo*, and is used widely as traditional medicine.

4.1 Introduction

Mangroves form the frontier between land and sea across a quarter of the world‟s coastline (Sridhar 2004). They are hardy plants which live in the extreme; they survive and thrive in fluctuating moisture conditions, and can tolerate a significantly large range of salinity from freshwater to sea brine (Shearer et al. 2007). Endophytes are organisms which grow within another host organism without causing apparent disease (Khan et al. 2010). The endophyte-host relationship can be commensal or mutualistic in nature, more often the latter as many endophytes actually confer certain survival advantages to the host (Maddau et al. 2009). Given that mangrove plants grow exclusively under conditions which are unfavourable to most organisms, the probability is high that mangrove endophytic fungi are equally unique in their own manner (Debbab et al. 2013; Li et al. 2009). Studies have shown that a

symbiotic relationship between mangrove host and fungal endophyte gives the trees an edge against other plants and animals via beneficial metabolites synthesized by the endophytes (Strobel and Daisy 2003).

Bioactive metabolites isolated from endophytes have the potential to be developed as a new class of antibiotics against various human pathogenic bacteria (Strobel et al. 2004; Montaser and Leusch 2011). With the rise of MRSA (Zetola et al. 2005), drug-resistant tuberculosis (World Health Organization 2010) and many other multi-drug resistant pathogenic bacteria strains, the need for novel and effective antibiotic treatments becomes ever more important for the health care sector.

This study is follow up to an earlier work by Onn et al. (2012b) who screened for antimicrobial properties of metabolites derived from liquid fermentation of *Phyllostica* sp. The same fungal strain was fermented in solid rice medium in hopes of a much larger yield of metabolic products, especially those with bioactivity.

4.2 Materials and methods

4.2.1 Fungal Material. The fungus *Phyllosticta capitalensis* was isolated from roots of a young mangrove plant identified to be *Avicennia* sp. The plant material was collected in 2010 as described by Onn (2013). The collected plant materials were cut into 1 cm fragments before being surface sterilized by immersing them sequentially in 70% ethanol for 3 minutes and 0.5% sodium hypochlorite for 1 min. Thereafter, the fragments were rinsed thoroughly with sterile distilled water and surface-dried before being placed onto Potato Dextrose Agar (PDA) (Difco). The plates were incubated at 28^{o}C for 1 week. After incubation period, hyphal tips of fungi growing out from the plant fragments were transferred to new PDA plates.

4.2.2 Identification of fungal culture. The fungal strain (YL3) was identified using molecular method of amplification and sequencing of the internal transcribed spacer (ITS) region of fungal genomic DNA. Polymerase chain reaction (PCR) of the ITS region was performed with the primer pair ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) according to the method described by White et al. (1990). The PCR reaction mix was prepared according to the RedTaq MasterMix protocol

(Invitrogen) as follows: Template DNA (20.0 ng), forward primer ITS1 (10 μM), reverse primer ITS4 (10 μM), dNTP mix (100 μM), $MgCl_2$ (1.5 mM) and RedTaq DNA polymerase. The reaction mixture was made up to a final volume of 25.0 μL using dH_2O, with the mixing steps carried out on ice to prevent degradation of nucleic acids and enzymes. A negative control tube was prepared in the same way as stated above except for template DNA being substituted for dH_2O; positive control was not required as the amplification products were found to be a distinct bright band of the correct size when checked using agarose gel electrophoresis. The amplification reaction was carried out using a thermocycler (Eppendorf) programmed according to the following: initial denaturation at 94 °C for 1 min; followed by 35 cycles of denaturation at 94 °C for 35 sec, primer annealing at 52 °C for 1 min, and extension at 72 °C for 1 min (extension); a final extension step carried out at 72 °C for 5 min. PCR products were held at 4°C until collection.The PCR product was stored at -20°C until purification using commercial DNA purification kit (Thermo), and then sent to Beijing Genomic Institute for Sanger Chain Termination sequencing. Large scale growth of fungus strain was allowed after its identity as *Phyllosticta capitalensis* was determined from NCBI BLAST and cleared as Risk Group 1 (non-pathogenic fungi) in accordance to German Technical Rules for Biological Agents (TRBA 2002).

4.2.3 Cultivation. Large scale fermentation of fungi was carried out in fifteen Erlenmeyer flasks (1 L each) on solid rice medium. The media consist of rice (100 g), distilled water (110 mL), and sea salt (3.0 g) which was then autoclaved prior to use, following the general protocol described in (Kjer et al. 2010). The pure fungal strain was inoculated, followed by incubation under static conditions and daylight for 14 days, sufficient time for the mycelia to completely cover the surface of the rice and initiate secondary metabolism (**Figure 20**).

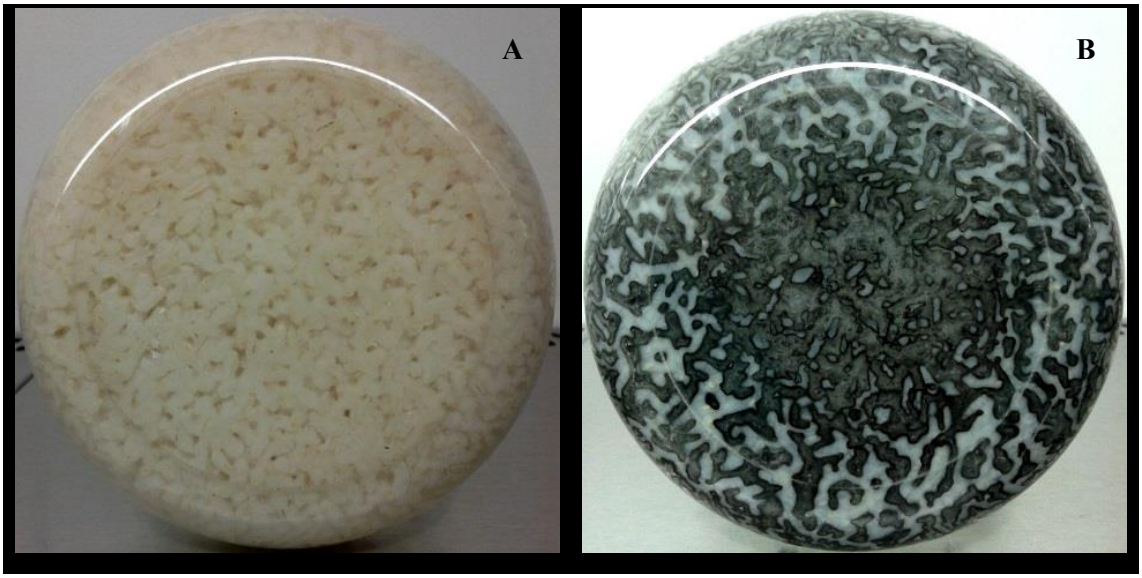


Figure 20: Results of solid phase fermentation of fungal strain YL3 after 14 days incubation. (A: Negative control flask containing uninoculated rice medium; B: Experimental flask which indicates fungal colonisation of entire external surface of cooked rice grains)

4.2.4 Extraction and isolation of secondary metabolites. The fungal cultures were extracted with analytical grade EtOAc on the final day of cultivation. Bulk extraction solvent EtOAc (approx. 300 mL) was poured into each flask and the rice inside was broken apart using a clean spatula. Flasks were then shaken for 6-8 hours and left to stand overnight. The clear solvent turned a golden yellow upon diffusion of fungal metabolites and pigments; the solvent extraction of negative control flask was carried out in the same manner, only yielding a very pale green-yellow hue. The content of each flask was passed through filter paper (11 μm) in order to remove particles of rice and mycelia; additional solvents were added (200 mL) to ensure exhaustive extraction of metabolites. The collected filtrate was then subjected to drying using rotary evaporator (Buchi). Negative control extract was subjected to HPLC analysis to be used as comparison with the extracts containing fungal metabolites.

A small portion of the dried crude extract (**G7**) was taken for HPLC analysis; the remainder (6.937 g) was partitioned between *n*-hexane and 90% MeOH (1:1) to give MeOH-soluble hydrophilic portion (**G7M**) and hexane-soluble lipophilic portion (**G7H**). The 90% MeOH-soluble portion (0.586 g) was further fractionated by vacuum liquid chromatography **(VLC)** over silica gel via a step gradient elution with *n*-hexane–EtOAc–CH_2Cl_2–MeOH solvent

system; this step yielded thirteen simple fractions. Fraction **G7M3** (62.73 mg) was subjected to size-exclusion chromatography over a Sephadex LH-20 column using MeOH as eluent; this separation yielded three fractions pooled from collected tubes of eluent. A relatively pure compound (12.5 mg) was identified based on TLC and HPLC, and then subjected to further structural elucidation.

4.2.5 Analytical separation. Thin layer chromatography **(TLC)** spot tests were performed on TLC plates pre-coated with silica Si 60 F_{254} (Merck, Germany) to detect compounds and fractionation efficiency; their UV absorbance at 254 and 366 nm were monitored by spraying the plates with anisaldehyde reagent. High Performance Liquid Chromatography **(HPLC)** analysis was used throughout the isolation of compounds up to the final verification of compound purity using a HPLC (Dionex P580) system coupled to PDA detector (UVD340S). Routine detection was carried out at 235, 254, 280, and 340 nm. The stationary phase was Eurospher-10 C_{18} (Knauer, Germany), the mobile phase consisted of MeOH and 0.02% H_3PO_4 in H_2O at the following elution gradient: 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100% MeOH.

4.2.6 Structure elucidation. Electrospray ionization mass spectroscopy **(ESI-MS)** was carried out using Finnigan LCQ Deca mass spectrometer at the same elution gradient as HPLC. Proton NMR **(^{1}H NMR)** spectra were recorded on Bruker ARX 500. Solvents were distilled prior to use, appropriate spectral grade solvents were used for spectroscopic measurements.

4.3. Results

4.3.1 Fungal identity. PCR product was checked using agarose gel electrophoresis (1.1%), and a bright single bright band was obtained around the 550-600 bp regions which corresponded well with expected band size of 500-650 bases (**Figure 21**). The negative control lane was devoid of clear bands with only a thin faint smear at the low bp region indicative of primer dimers.

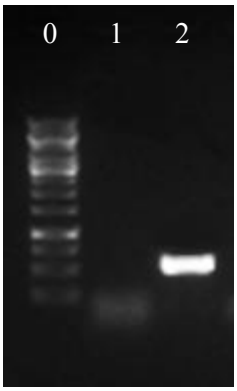


Figure 21: Gel electrophoresis of ITS-PCR product (Lane 0: 1kb DNA ladder; Lane 1: Negative control; Lane 2: ITS-PCR product for strain YL3)

Sequencing of PCR products was carried out via Sanger chain termination method with ITS1 forward primer. The returned sequence was submitted to BLAST and compared against other submitted sequence on the nucleotide collection database. Phylogeny of strain YL3 was determined using maximum likelihood method (Tamura-Nei model); phylogenetic tree (**Figure 22**) was generated with *Penicillium chrysogenum* added as an out-group.

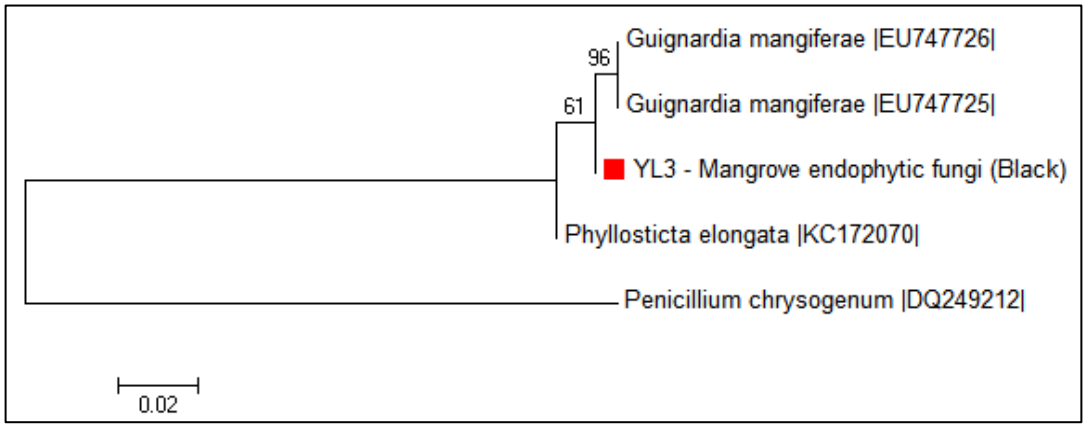
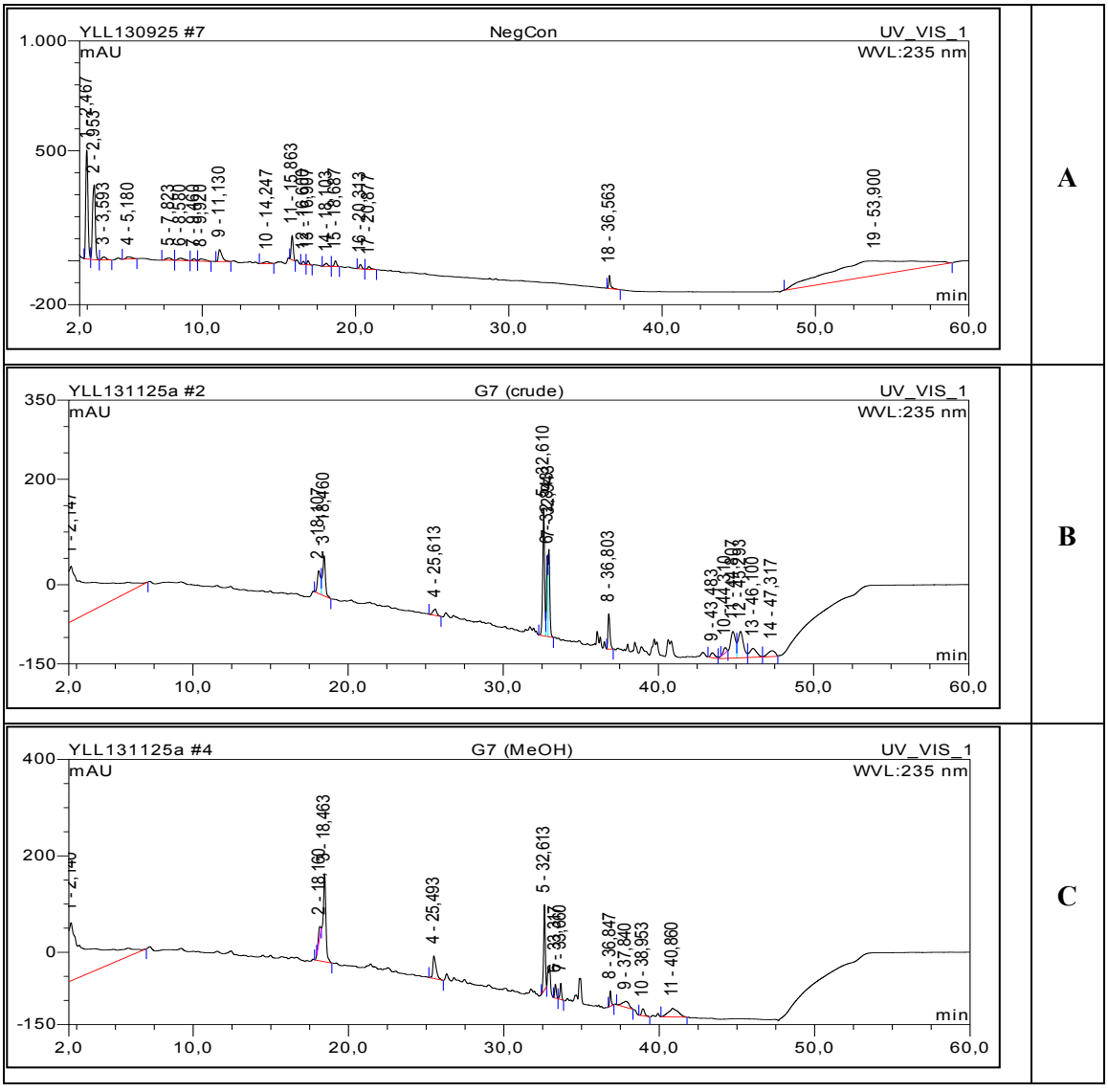


Figure 22: Phylogenetic tree constructed based on sequence of fungus strain used in this study and closest matches according to BLAST database.

4.3.2 Extraction of fungal metabolites. Extraction and subsequent purification of ferulic acid began with obtaining initial crude extracts; this was carried out by directly extracting metabolites from cultivation medium using EtOAc. Crude extracts obtained from uninoculated solid rice medium was used as negative control (452 mg; **Figure 23A**). Crude metabolite extracts (6.937 g) from fungal cultivation over a fortnight was obtained and

subjected to HPLC (**Figure 23B**). The large amount of non-polar compounds found, seen in chromatogram as peaks appearing beyond the 40th minute mark, prompted a separation step to remove lipophilic metabolites. Partitioning of crude extracts was carried out over a 90% MeOH:*n*-hexane (1:1) system, and the resultant phases were kept for further work up. The 90% MeOH portion (**Figure 23C**) was subjected to VLC to give thirteen simple fractions. The 3rd fraction, G7M3 was selected for its relative purity (**Figure 23D**) and was further subjected to size exclusion chromatography. The three fractions obtained gave one fraction with the majority of desired compound manifested as a single peak (**Figure 23E**).



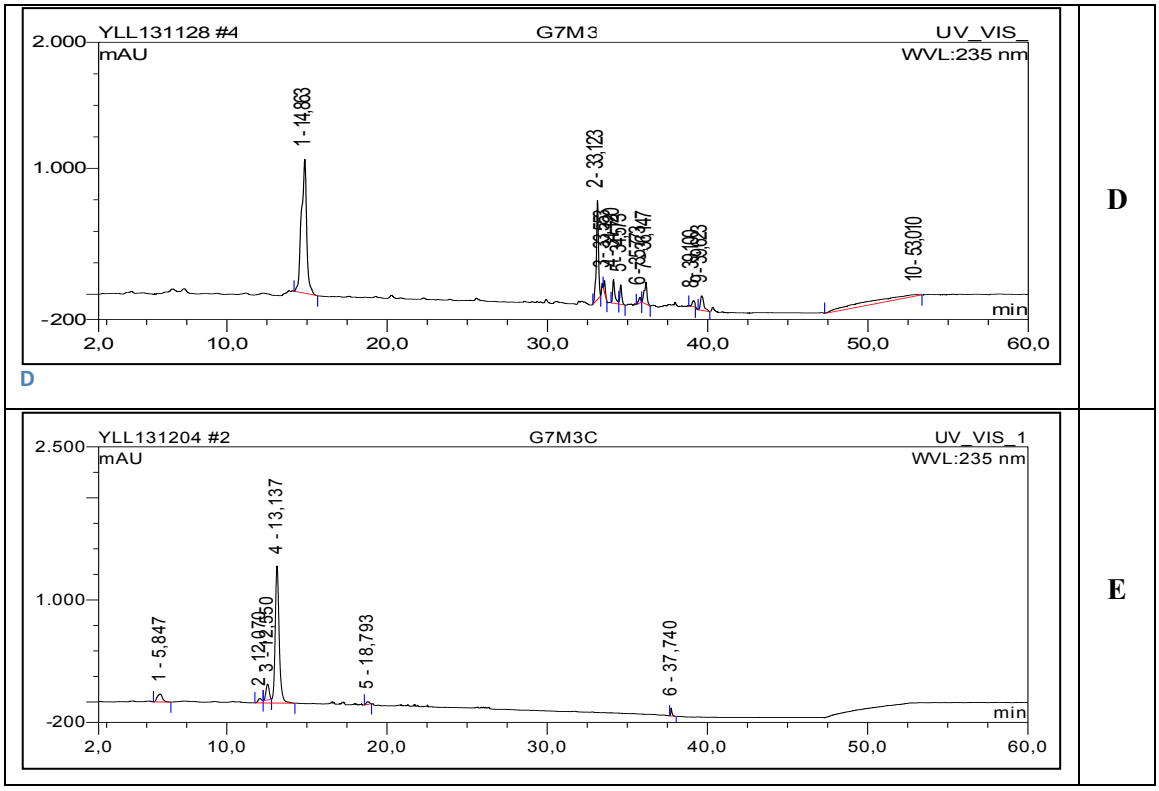


Figure 23: HPLC chromatograms taken at 235 nm indicating progressive purification of compounds.

4.3.3 Trans-ferulic acid. Obtained as a light yellow powder (12.5 g) upon drying *in vacuo*; ^{1}H NMR (500 MHz, CD_3OD): δ = 3.93 (s, 3H, 3-OCH_3), 6.35 (d, *J* = 15.9 Hz, 1H, 8-H), 6.85 (d, *J* = 8.2 Hz, 1H, 5-H), 7.10 (dd, *J* = 8.2, 1.9 Hz, 1H, 6-H), 7.21 (d, *J* = 1.9 Hz, 1H, 2-H), 7.64 (d, *J* = 15.9 Hz, 1H, 7-H); ESI-MS positive *m/z* 195.1 $[M+H]^+$, negative *m/z* 193.4 $[M-H]^-$ (calcd. For $C_{10}H_{10}O_4$, 194.0579). The values obtained from proton NMR were comparable with those in an earlier work by Yoshioka et al. (2004) in which ferulic acid was also isolated.

4.4. Discussion

Ferulic acid is a ubiquitous chemical compound associated with the cell wall components of plants (Ralph et al. 1994); being a simple phenolic molecule, it is also a precursor molecule for the production of many other aromatic compounds. There have been several reports of ferulic acid being a potent antioxidant based on *in vitro* and *in vivo* studies (Itagaki et al. 2009; Kanski et al. 2002). Ferulic acid is commercially used as food preservative (Graf 1992) and UV protectant (Murray et al. 2008).

This is to our knowledge the first reported isolation of ferulic acid from fermentation products of *Phyllosticta capitalensis* or *Guignardia mangiferae*. The relatively small amounts of ferulic acid produced (12.5 mg) did however raise the question of its actual role and importance as a fungal metabolite. Ferulic acid production was not purposefully induced in any way during cultivation and fermentation; the light it was exposed to was purely artificial light from fluorescent lamps kept to a minimum, oxygen was as atmospheric compositions, and temperature was kept to 24°C. In light of this, the following hypotheses were proposed to explain the presence of ferulic acid in the extract.

4.4.1 Role of ferulic acid in biosynthesis pathways. There is a distinct possibility that ferulic acid was present as a precursor molecule in biosynthetic pathways of the fungal strain. Ferulic acid is a simple cinnamic acid derivative which can be modified to give variant molecules to suit the purposes of an organism as explained below.

4.4.2 Protocathechuic acid – a ferulic acid-derived antioxidant. Another pathway ferulic acid can take is demethylation, followed by side-chain shortening; this two-step reaction would yield 3, 4-dihydroxybenzoic acid (Tillet and Walker 1990), a phenolic anti-oxidant. More commonly known as protocathechuic acid, this metabolite has been isolated from fungi such as the medicinal *Phellinus linteus* (Lee et al. 2008), and the edible *Agaricus bisporus* (Signore et al. 1997). Recent research has shown that protocathechuic acid has a very wide range of bioactivity, including anticancer, anti-inflammatory, antidiabetic, and antibacterial (Kakkar and Bais 2014).

4.4.3 Phenol formation via degradation pathways. One notable fungal biotransformation reaction of ferulic acid involves a decarboxylation step to yield 4-ethenyl-2-methoxyphenol by *Paecilomyces variotii* and *Pestalotia palmarum* (Rahouti et al. 1989), and *Saccahromyces cerevisiae* (Huang et al. 1993). The metabolite 4-ethenyl-2-methoxyphenol is credited for the distinctive "clove" flavour found in some wheat beers such as Witbier and Weißbier. It was also found that subsequent degradation may also occur to yield vanillyl alcohol, vanillinic acid and several other derivatives (Rahouti et al. 1989).

4.4.4 Horizontal gene transfer. While there can be many potential forms in which ferulic acid can be transformed into, the cause of its presence is less certain. One hypothesis is that a horizontal gene transfer from host plant may have occurred during the long evolution of this fungal species (Richards et al. 2009). The higher plants which play host to this endophytic fungus are often capable of generating ferulic acid from the basic amino acid phenylalanine (Pinçon et al. 2001). There has been substantial amount of research done which indicates long term co-evolution of endophytic fungi and their hosts has led to horizontal gene transfer (Strobel et al. 1996; Rosewich and Kistler 2000; Aly et al. 2010) of genes responsible for metabolite synthesis. Genes coding for biosynthetic pathways commonly associated with higher plants can make its way into the DNA of endophytic fungi and vice versa.

4.4.5 Ferulic acid an indicator of lignocellulose degradation. One other cause of isolating ferulic acid from *P. capitalensis* extract is that it may be a product of lignocellulosic degradation by cell wall degrading enzymes, or CWDEs (de Souza 2013). Lignocellulose make up the bulk of plant biomass generated from photosynthesis; it is the general terms used for a combination of ligneous and cellulosic material which makes up plant cell walls (Sánchez 2009). Ferulic acid is commonly bound to cell wall lignin in the form of feruloyl esters (Burr and Fry 2009). The release of ferulic acid from cell walls of rice grains can be achieved via enzymatic action by feruloyl esterases (Mathew and Abraham 2004) possibly synthesised by *P. capitalensis*. This is in agreement with the absence of ferulic acid from extracts of the negative control. The production of CWDEs such as lignases, cellulases, and hemicellulases by *P. capitalensis* would be unsurprising as such enzymes can be very useful for any organism with an endophytic lifestyle to thrive within the tissue of plants. CWDEs are a class of enzymes primarily responsible for

degradation of plant cell walls, and such, allow the endophytic fungus to proliferate and spread to other parts of the plant. Certain endophytic fungi, including *Phyllosticta* sp., have been found to thrive under senescent conditions, and are thought play a significant role in leaf litter degradation (Kumaresan and Suryanarayanan 2002). If such a mechanism is proven to be the reason for isolation of ferulic acid from the endophytic fungus, a more in-depth study into the enzymes responsible for potential lignocellulosic activity may result in isolation of novel enzymes for industrial applications (Quiroz-Castañeda and Folch-Mallol 2013).

4.5 Conclusion

We believe that the isolation of ferulic acid from *Phyllosticta capitalensis* was more likely due to lignocellulosic degradation of lignin-containing substrate as the existence of a ferulic acid synthesis pathway has yet to be confirmed for fungi. Follow-up studies on the genome of the isolated fungal strain will be needed to validate this hypothesis. Our results further highlight that there are still many scientific discoveries to be made in the field of endophytic fungi research.

5. Optimisation of growth media for cultivation of mangrove endophytic fungi from Kuching, Sarawak

5.0 Abstract

The coastal regions between land and sea experience perpetual fluxes in physicochemical conditions, undergoing bi-daily cycles of inundation and drying, changes in temperature between night and day, seasonal variations in erosion and sedimentation rates, as well as unpredictable changes in pH and salinity. Organisms which thrive in such an extreme environment typically possess traits which have helped them adapt; mangrove trees especially have been found with distinct properties which puts them in a position to be the dominant flora of many tropical coastlines. Mangrove endophytic fungi are a unique class of organisms found to inhabit the internal tissue of mangrove plants. These fungi spend virtually their entire life cycle within the mangrove plants and contribute to the survival of the trees which host them. One frequent problem with research on mangrove endophytic fungi is slow growth on standard mycological medium, resulting in biases during isolation steps of fungal diversity studies, as well as low yields of metabolites during natural products screening. In this study, we attempted manipulation of several parameters of fungal growth medium in the cultivation of three strains of mangrove endophytic fungi. Although there was not one defined medium which gave an absolute increase in the growth of fungal strains, we recommend that future isolation and cultivation of mangrove endophytic fungi be carried out on growth media with pH at 5.0 and 9.0. This should enable greater number of fungal strains isolated per plant sample by reducing bias in cases of fungi with narrow range of optimum growth pH. Manipulation in nitrogen supplement and salinity of medium can be performed to induce higher growth rates and secondary metabolism.

5.1 Introduction

The word "mangrove" is attributed to the collection of plants which colonised and dominate the intertidal areas of tropical regions (Gilman et al. 2008). There has been much work done regarding the diversity of mangrove tree species (Field et al. 1998; Burke et al. 2001), but a lot of research remains to be done in assessing the mycobiota of mangroves (Cheng et al. 2009; Alias et al. 2010). The fungi which colonise mangrove trees, and all higher plants for that matter, can be generally classified into two main groups – the exophytes which grow on the outside of the plant, and endophytes which grow from within the host (Amadi 2005).

Endophytic fungi are a special group of fungi gaining ever more prominence in the field of natural products discovery (Debbab et al. 2011). There have been many papers published in recent years describing the isolation of bioactive molecules from mangrove endophytic fungi (Kjer et al. 2009; Bhimba et al. 2012; Hammerschmidt et al. 2014). It is hoped that some such compounds can be further researched on for medicinal uses, not unlike the anti-cancer drug paclitaxel, currently marketed as Taxol©. Paclitaxel was originally isolated from the bark of Pacific yew trees during a massive plant screening programme in the USA (Wani et al. 1971); in the years to follow, demand for the tree bark skyrocketed and new methods to procure the valuable chemotherapeutic drugs were sought after. It was much later found to be also synthesised by several strains of endophytic fungi (Stierle et al. 1993; Strobel et al. 1996) albeit with small yields. Further work is still being carried out in order to improve the quantities of extracts (Soca-Chafre et al. 2010; Wang and Tang 2011).

As endophytes of several species of higher plants, mangrove endophytic fungi have adapted to life within the confines of host plant tissue (Petrini 1991); as endophytes of extreme-tolerant plants, they too have co-evolved to survive in the same extreme ecological niche (Debbab et al. 2013). It is believed that this natural selection have resulted in a class of organisms capable of synthesising a wide variety of secondary metabolites which aid in the survival of host plant, as well as itself (Scherlach and Hertweck 2009).

One of the problems faced by mycologists working on mangrove endophytes today is that some endophytic fungi are unable to thrive in laboratory defined cultures (Chintapenta et al. 2014); and some of them which do, simply grow at too slow a rate *in vitro* for industrial

applications. This trend has also been observed in works pertaining to isolation of other non-mangrove endophytic fungi (Zhu et al. 2008), and therein lies the need for optimisation of fungal growth in order to fully study them.

Fungi and yeasts have been used in the ancient fermentation arts of making wine, cheese, and vinegar. But modern mycology did not actually take root until much later in the 1940‟s whereby strain optimisation of the medically important fungus *Penicillium sp.* was carried out (Chain et al. 1940). The fungal fermentation product, Penicillin, was one of the first anti-bacterial agents to be manufactured at industrial scale (Florey 1944). In reaching the required levels of production, several steps have been taken to enhance the growth of chosen *Penicillium chrysogenum* strain thus enhancing the synthesis of commercially-valuable secondary metabolites (Peñalva et al. 1998).

A preliminary study to the chemical compounds produced by these strains of *Phyllostica* and *Neosartorya* indicated that they may be new sources of several uncharacterised secondary metabolites (Onn et al. 2012b), but their slow growth on standard laboratory medium has hampered efforts to obtain a large enough sample for proper analysis. Given that the strains were isolated as endophytes of halo-tolerant plants from marine environment, it stands to reason that their metabolism may be uniquely tailored to their natural surroundings (Nayak et al. 2012). It is also more than likely that they are highly resilient strains of fungi capable of growing at a variety of non-standard conditions including a wide range of salt concentrations, temperature, nutrient compositions, and pH (Petrini et al. 1992; Hyde and Sarma 2006).

In this study, we aim to develop suitable media for cultivation of mangrove endophytic fungi isolated from Sarawak mangrove plants with the view of inducing faster growth and secondary metabolism. During the course of fungal cultivation, it was also a secondary goal to develop proper measuring technique of fungal strains growing on test medium, especially when colony morphologies were irregularly shaped.

5.2 Methodology

5.2.1 Fungal Material. The fungi strains used in this study were isolated from leaves and roots of a young mangrove plant *Avicennia* sp. The plant material was collected in 2010 as described by Onn (2013). The collected plant materials were cut into 1 cm fragments before being surface sterilized by immersing them sequentially in 70% ethanol for 3 minutes and 0.5% sodium hypochlorite for 1 min. Thereafter, the fragments were rinsed thoroughly with sterile distilled water and surface-dried before being placed onto Potato Dextrose Agar (PDA). The plates were incubated at 28°C for 1 week. After incubation period, hyphal tips of fungi growing out from the plant fragments were transferred to new PDA plates. Subsequent transfers were performed to ensure axenic culture was obtained.

5.2.2 Strain identification. The fungal strains were identified using molecular method of amplification and sequencing of the internal transcribed spacer (ITS) region of fungal genomic DNA. Polymerase chain reaction (PCR) of the ITS region was performed with the primer pair: forward primer ITS1 (5‟-TCCGTAGGTGAACCTGCGG-3‟) and reverse primer ITS4 (5‟-TCCTCCGCTTATTGATATGC-3‟) according to the method described by White et al. (1990) using the following amplification program: 94°C for 1 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, then a final extension at 72°C for 10 min. PCR products were stored at -20°C until purification workup and sequencing. Sequencing was carried out in Beijing Genomics Institute (BGI) using the Sanger chain termination method.

5.2.3 Media development. The standard medium chosen for this study was potato dextrose agar (PDA) with the following composition: Potato infusion (4.0 g/L), dextrose (20.0 g/L), and bacto agar (15.0 g/L) in demineralised water. The final pH of medium is 5.6 ± 0.2 in accordance with manufacturer specifications.

Modifications were made to the standard medium based on two considerations – firstly, simulation of natural growth environment and secondly, optimum pH for most productive growth of fungal strains. The extent of simulating endophytic fungi natural environment was categorised as an ordinal variable; this parameter cannot be defined as an absolute mirror of the living conditions of the host plant but can be objectively studied *in vitro* by changing the medium composition one “step” at a time. For all preparations, modifications

to nutrient composition were carried out first, followed by adjustment of pH, then autoclaved at 121°C for 15 minutes.

5.2.3.1 Media modifications. The following three modified media were prepared for enhancing fungal growth. The first medium was made with the addition of organic nitrogen source (yeast extract, 1.5 g/L) to give potato dextrose yeast agar (PDYA; **I**). The second was made by further supplementing PDYA with 3% artificial sea salt (Red Sea Coral Pro Salt) to give potato dextrose yeast artificial salt agar (PDYASA; **II**); the third growth medium was created by making PDYA using filtered sea water instead of demineralised water to give potato dextrose yeast natural salt agar (PDYNSA; **III**). A summary of changes made to standard PDA is given in **Table 22**.

Table 22: Alterations carried out on standard medium in this study, including organic nitrogen source and salinity (artificial and natural).

Additional nutrients to PDA	Rationale / remarks	Abbreviation
Yeast extract (1.5 g/L)	Addition of a rich nitrogen source	**PDYA; I**
Yeast extract (1.5 g/L) Red Sea Coral Pro Salt (30.0 g/L)	Salt water aquarium salts	**PDYASA; II**
Yeast extract (1.5 g/L) Filtered natural sea water	Natural sea water contains naturally occurring salts and nutrients	**PDYNSA; III**

By supplying an effective source of organic nitrogen for fungal utilisation, it is hoped that an increase in growth rate can be observed. The addition of salts in growth medium allows for simulation of marine conditions *in vitro*; particularly useful for the cultivation of halophilic organisms. Two types of saline media were made; first using commercially available salt mix of standardised composition to give PDYASA and secondly, sea water collected from sampling site during high tide to give PDYNSA. Usage of artificial salt mix allows for greater consistency of growth medium composition in future works; whereas the use of filtered sea water from sampling site ensures a more accurate replication of mangrove forest salinity at the expense of uniformity between experiments. This also allowed for comparison between efficacies of different sources of salinity in terms of enhancing fungal growth.

5.2.3.2 Adjustment of pH. The initial pH of each modified media (**I** to **III**) was adjusted to three different points ranging from acidic to alkaline (**Table 23**) before autoclaving. The necessary adjustments were carried out using analytical grade HCl and NaOH solutions, measured using pH meter.

Table 23: Selected media pH values which were used in this study.

pH of medium	Rationale / remarks
5	Typical pH for terrestrial fungi
7	Neutral pH; mid-point between acidic and basic conditions
9	Slightly more alkaline than average sea water

5.2.4 Preparation of agar plates. A fixed volume of each medium (10.0 mL) was pipetted into sterile plastic petri dishes (8.0 cm diameter). The plates were allowed to cool at room temperature while irradiated with sterilizing UV light. This method ensures constant depth of medium, therefore preventing inconsistency of fungal growth rate due to varying nutrient depletion rates.

5.2.5 Preparation of inocula and inoculation. Inocula were prepared by cultivating the purified fungal strains on half strength PDA, incubated at 24°C for a period of 7 days. Actively growing hyphal tips were removed carefully with sterilised needle for inoculation onto fresh plates of prepared medium modified from single spore isolation method of Choi et al. (1999); care was taken to ensure minimal amounts of agar was taken with each transfer to ensure the new growth is solely dictated by tested medium and not due to other nutrient sources. Only one inoculation was performed for each plate, inoculants were placed on the exact centre of plates. Spore suspensions were not used in the experiment as strains YL1 and YL2 did not exhibit spores which were easily picked up; YL3 produces fruiting bodies, and can easily be broken to prepare spore suspension. For consistency among the fungal strains used, only actively growing conidiophores were taken for inoculation. Quadruplicates inoculations were performed for each strain which allowed for more reliable statistical analysis in the event of contamination. All three fungal strains were grown on PDA and twelve (12) other modified media at 27°C.

5.2.6 Measurement of fungal growth. Growth was recorded by measuring the area covered by each colony using a transparency sheet with accurately printed boxes of 1 mm × 1 mm. Measurements were taken every 2-3 days beginning with T = 0 at time of inoculation to T = 21 on the 21st day of fermentation. When taking measurements of area under 100 mm^2, the values obtained were rounded to the nearest 0.5 mm^2; when the total area of colony exceeds 100 mm^2, the values obtained were rounded to the nearest 5.0 mm^2. Occasionally, when the growth of colonies were a perfect circle, growth area was calculated using $A = \pi r^2$, where A is area of colony and r is radius of colony in mm^2.As quadruplicate data was obtained for each medium inoculated with fungi, the means of three closest values of the quadruplicates prepared

5.2.7 Statistical treatment. For each quadruplicate measurement of colony surface area, the least significant value was rejected to give three values by which the mean surface area (MSA) was calculated. The growth of fungi strains tested can be examined via growth curves generated by plotting MSA (mm^2) versus time (Day). The mean surface area on Day $\boldsymbol{X}$ (MSA$_X$) for each fungus grown on non-standard media was compared to the corresponding fungal strain grown on standard growth medium – PDA, with substantially large values then subjected to a simple T-test to determine level of significance. This allows for an objective method of determining the extent of influence a particular medium has on enhancing the growth of mangrove endophytic fungi.

5.3 Results and Discussion

5.3.1 Isolation and purification of fungi. Isolation of fungal strains were carried out using standard endophytic fungi isolation steps, inclusive of adequate surface sterilisation and preparation of negative control plates to ensure none of the chosen strains were exophytes or saprophytes. During the initial purification steps, a pure culture of green-pigmented fungus was obtained on PDA. However, a sectoring event was observed after subsequent subcultures on the same medium, giving two distinct morphological types as follows:

Type 1. White to sandy brown upon secondary metabolism; radially sulcate; velutinous texture; umbonate; slight undulate margins. Reverse: cream coloured mycelia which

turned sandy brown upon secondary metabolism. This morphological type was assigned strain **YL1**.

Type 2. White to pale green upon secondary metabolism; radially sulcate; velutinous texture; umbonate; wavy margins. Reverse: cream coloured mycelia which turned dull green upon secondary metabolism. This morphological type was assigned strain **YL2**.

Upon single spore isolation, the purified strains of the morphological types remained fairly stable with rare recurrences of sectoring in subsequent cultivars. Subsequent sectoring events produced some fast growing colonies, but attempts to sub-culture and purify faster growing morphological types were unsuccessful due to high rate of reversion to slow growing strains. It seems likely that two slower growing types were more stable and was more likely to be observed (**Figure 24A** and **24B**). It is not uncommon to find different forms for one fungus (Lacap et al. 2003) with same genetic material.

One other strain which was isolated and purified in this study often exhibited non-circular growth pattern. Morphologically, it has flat-growing, black-green coloured filamentous hyphae, with substantial amount of shiny black fruiting bodies (**Figure 24C**); actively growing vegetative hyphae was thick and translucent white, turning dark green upon secondary metabolism and always extending just slightly further (approximately 2.0 mm) than aerial mycelia. This fungus was assigned strain **YL3**.

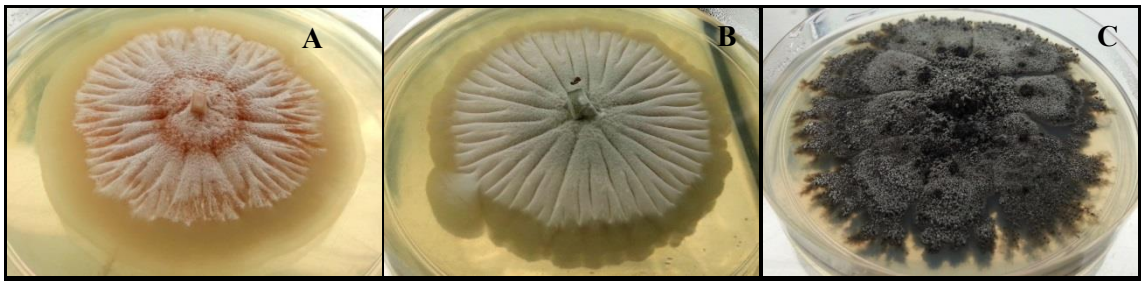


Figure 24: Surface morphology of the three fungal strains used in this study. (A: YL1 - White morphotype of *Neosartorya hiratsukae*; B – Green morphotype of *Neosartorya hiratsukae*; C - *Phyllosticta capitalensis*)

5.3.2 Strain identification. Identification of the fungal strains (**Figure 25**) was carried out using molecular method; sequences of the ITS region are distinctly different between each fungi species, allowing for accurate differentiation and identification. The returned sequences were cleaned up using Chromas 2.22, and subjected to NCBI nucleotide BLAST

in order to search for closest match among the submitted samples. The sequences obtained for our fungi were then aligned with several submitted sequences for construction of a phylogenetic tree by Neighbour-Joining Method based on the Tamura-Nei Model using the MEGA 6.0 programme. The sequences obtained for YL1 and YL2 were exactly the same based on alignment of 546 base pairs with no gaps or nucleotide substitutions, this confirms that the two morphologically different strains derived from a common isolation plate was the result of a sectoring event instead of contamination or strain mutation. This was further confirmed using phylogenetic analysis whereby the sequences were placed together in the same subtree 98% of the time when subjected to bootstrap value of 1000.

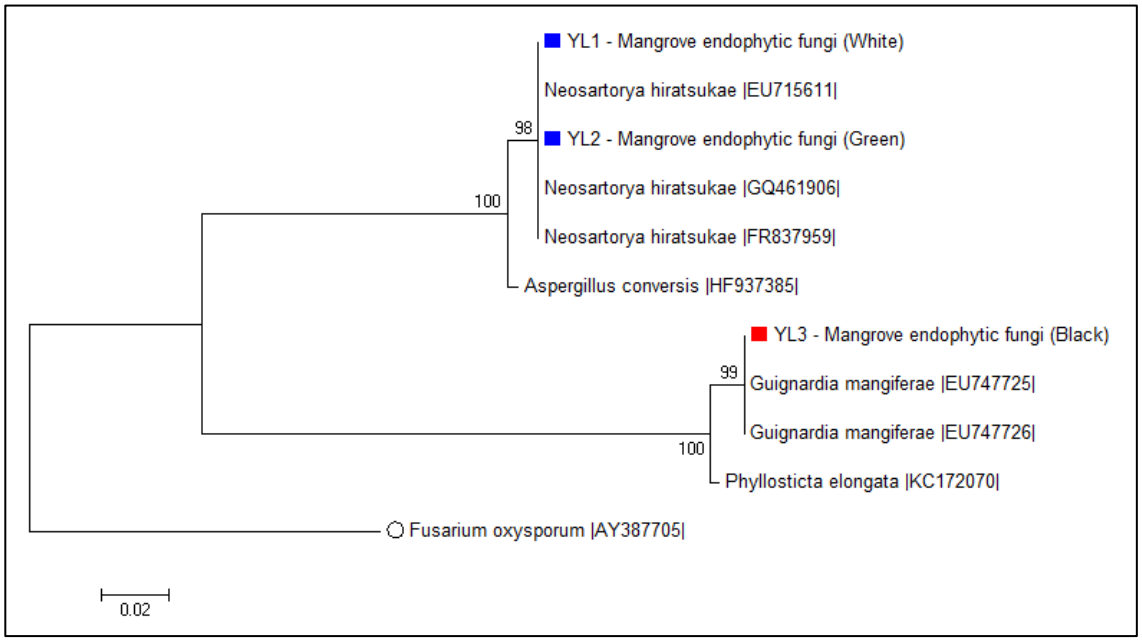


Figure 25: Phylogenetic tree constructed based on ITS sequences of the 18S rDNA region.

5.3.3 Measurement of fungal growth. The growth curves generated were grouped based on each strain‟s performance at a given pH (YL1 and YL2) or nutrient conditions (YL3). The extent of fungi growth was measured based on mean surface area (MSA) covered by media mycelia of fungus colony on each type of medium. This method was the best choice for reasonable estimation of fungal biomass increase in a solid agar medium. The most established methods of measuring fungal colony diameter and radial growth rate were not applicable to this study due to non-uniformed growth patterns of fungi. All fungal strains

tend to produce colonies frequently taking irregular forms and would have resulted in gross inaccuracy. Alternatively, direct measurements of fungal dry weights at intervals or indirect measurements of nutrient depletion rates would require destruction of samples, and are more suited to measure growth rate in submerged cultures. Growth of some filamentous fungi in submerged cultures has been reported to be logarithmic (Borrow et al. 1964), yet others have been found to not grow exponentially (Prosser and Tough 1991). This may be due to different growth patterns observed by unbranched hyphae and mycelial hyphae in submerged cultures (Trinci and Banbury 1967).

Based on the obtained growth profiles of the three fungal strains tested, it was found that mean surface area on the 21st day (MSA_{21}) was the most reliable indicator for extent of fungal growth. This arbitrary decision was also supported by Morris et al. (1996) who reported that detection of clinical mould isolates was only 81% on the 7th day of incubation, rising up to 96% on the 14th day. In our study, it is believed that an extra seven days beyond the initial 14 days should be sufficient for adequate comparison between growths on different media. In three instances, increased growth on modified medium was significantly observed only after the 14th day of growth (**Figures 26, 27A,** and **28A**).

The growth profiles obtained was indicated the stage of growth each fungal strain was experiencing. In most cases, it was found that the fungal strains tested experienced linear increases in MSA measurements up to day 21; very few growth profiles yielded exponential increase in mycelia coverage throughout the duration of incubation. There was only one clear observation of secondary metabolism achieved by fungi in modified medium tested (**Figure 28B**) – strain YL3 experienced exponential growth for the first 7 days of incubation, deceleration of growth began approximately on the 8th day, and began secondary metabolism around the 15th day of incubation.

5.3.4 Effect of pH on fungal growth. The *Neosartorya* strains YL1 and YL2 showed elevated MSA_{21} when subjected to pH 5.0, (**Figure 26; Figure 27A**). A slightly higher MSA_{21} was also observed for YL2 grown at pH 7.0. Thus, a pH of 5.0 was determined as optimum for growth of *N. hiratsukae* strains tested. The growth of *Phyllosticta* strain YL3 was not significantly influenced by pH, a visible increase in MSA_{21} was observed across all three pH values tested when strain YL3 was grown on PDYA compared to standard PDA

(**Figure 28A**). It was noted that alkaline conditions were preferred when strain YL3 was grown on saline media PDYASA and PDYNSA. It could be said that this *P. capitalensis* strain was uniquely suited to marine habitats in terms of pH conditions.

Testing of growth under acidic and alkaline conditions takes into consideration that terrestrial fungi are known to favour acidic pH ranges (Rousk et al. 2009), yet the typical marine environment has pH levels of 8.08-8.33 (Marion et al. 2011). The fungi used in this study were mangrove endophytic fungi, a unique class of adaptable marine fungi (Bugni and Ireland 2004); it was not surprising that pH preference of tested different strains was not as straightforward as compared to their land-based counterparts.

5.3.5 Effect of nitrogen source. The *Phyllosticta* strain YL3 responded well to PDYA growth medium which was made by addition of yeast extract and pH adjustments. A significant increase in MSA_{21} values for all three pH tested was clearly observed ($p<0.04$) as compared to growth on PDA (**Figure 28A**).

Supplemented yeast extract (1.5 g/L) was a major source of organic nitrogen, and to a lesser extent – micronutrients such as vitamins and minerals (Grant and Pramer 1962). Yeast extract was a constant feature in all modified medium as it was assumed its nutrient content mimics host plant conditions better than defined laboratory media. The enhanced growth of marine fungi grown on nitrogen-rich medium has been reported (Miao et al. 2006), thus it was within reason to find that the *Phyllosticta* strain YL3 grew better when yeast extract was added into growth medium.

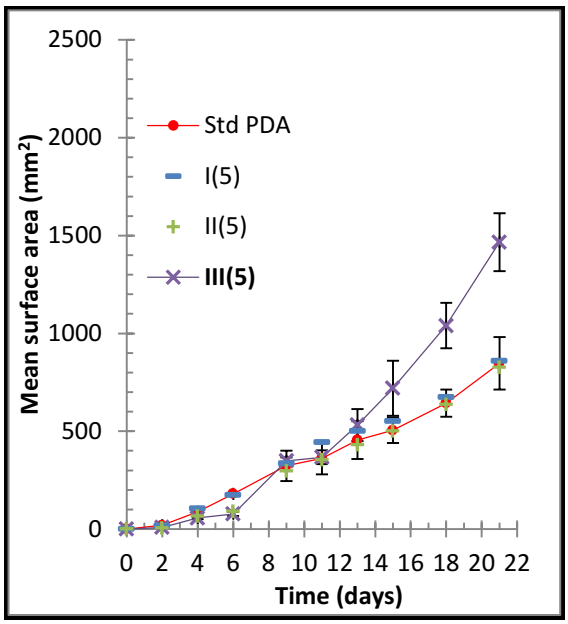


Figure 26: Growth curves for strain YL1 (growth at pH 5.0). Baseline data (growth on PDA) showed here as red line. (I: PDYA; II: PDYASA; III: PDYNSA).

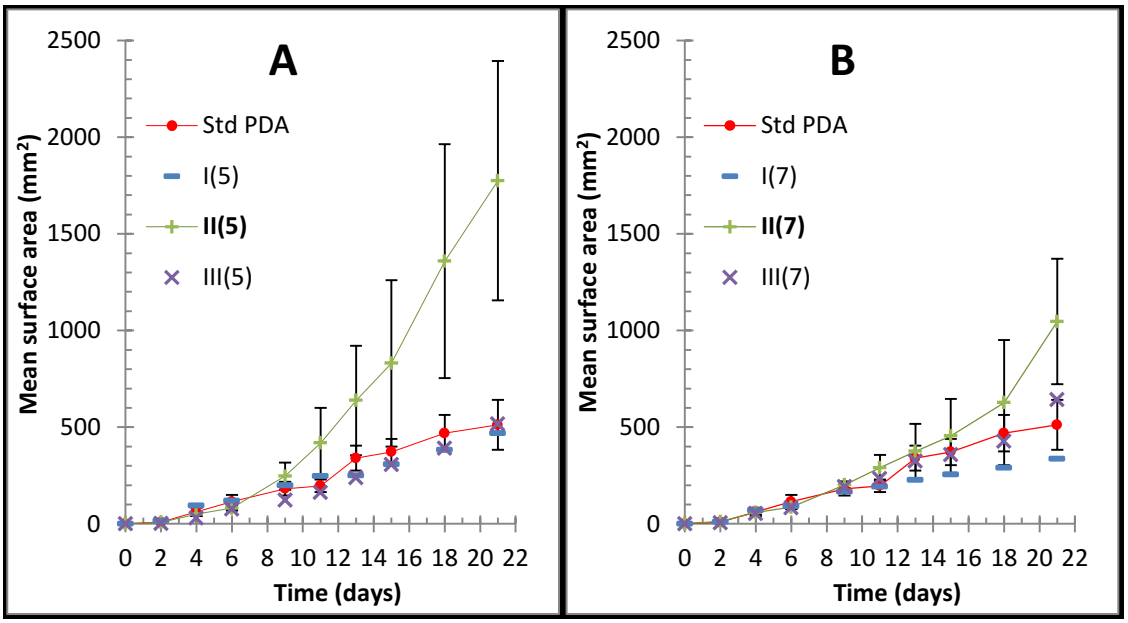


Figure 27: Growth curves for strain YL2 (A: growth at pH 5.0; B: growth at pH 7.0). Baseline data (growth on PDA) showed here as red line. (I: PDYA; II: PDYASA; III: PDYNSA).

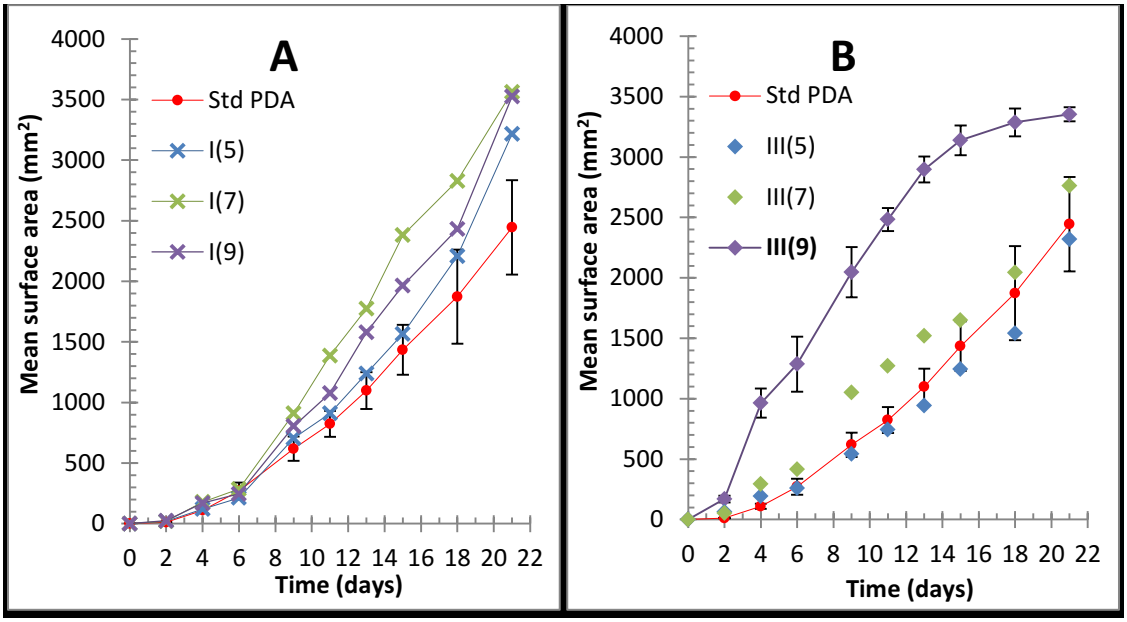


Figure 28: Growth curves for strain YL3 (A: growth in medium I; B: growth in medium III). Baseline data (growth on PDA) showed here as red line. (I: PDYA; III: PDYNSA).

5.3.6 Effect of salinity. The *N. hiratsukae* strain YL2 responded positively towards growth medium which incorporated reef-salts giving MSA_{21} of 1775 mm^2 when grown on PDYASA at pH 5.0 (**Figure 27A**) as compared to MSA_{21} of 511.7 mm^2 when subjected to standard PDA growth medium; growth on PDYASA at pH 7.0 yielded a final MSA_{21} of 1046.7 mm^2 (**Figure 27B**).

The other *N. hiratsukae* strain YL1 and *P. capitalensis* strain YL3 performed better when grown in medium made using filtered sea water. The MSA_{21} achieved by YL1 when grown on PDYNSA at pH 5.0 was 1466.7 mm^2, as opposed to 846.7 mm^2 when grown on PDA (**Figure 24**). The strain YL3 gave an MSA_{21} of 3353.3 mm^2 when grown on PDYNSA at pH 9.0, as compared to 2455.0 mm^2 when grown on standard PDA (**Figure 6B**).

Table 24: Two key parameters which differentiate media made using artificial saline from media made using filtered sea water.

Parameters	PDYASA	PDYNSA
Salinity	30.0 ppt	14.1 ppt
Nitrate	-	1.0 ppm

Artificial salinity was achieved in PDYASA medium with addition of commercial salt mix of known composition; this is in contrast with natural salinity achieved in PDYNSA medium which was made using filtered sea water collected from mangrove plant sampling site. One local study (Holt, personal communication) reported that sea water near the sample collection site contained nitrate up to 1.0 mg/L, this inorganic nutrient can be a contributing factor to the enhanced growth of the strains YL1 ($p<0.005$) and YL3 ($p<0.030$) on that PDYNSA. This was in direct contrast with the observed enhanced growth of YL2 in a nitrate-free medium. Such an observation is not unique as Finlay et al. (1991) did report significant growth variations between different isolates belonging to the same species.

5.3.7 Growth of *Neosartorya* sp. strains. The fungi strains YL1 and YL2 were found to exhibit distinctly different growth profiles despite being very similar on a genetic level. The ITS region is a critical region which allows for accurate differentiation between species from the same genus and the ITS-PCR results indicated that the two strains were an exact base-to-base match for a region of 546 bp in length.

The growth profile and pigmentation between the two strains were very different throughout the duration of the experiment, responding in different ways towards the manipulated variables. It was observed that the two strains have a well-defined preference for low pH levels for growth. The highest MSA_{21} values recorded for both strains were at pH 5.0, with only one other MSA_{21} on modified medium slightly higher than on PDA at pH 7.0. Aside from preference towards low pH range, the two strains were similar in terms of a non-response towards supplemented yeast extract. This hinted at the two fungi strains being most likely non-fastidious, the presence of additional nutrients does not significantly enhance their growth on agar.

Strain YL1 grew well on PDYNSA, indicative of a preference for moderate salinity levels and the presence of nitrate in the medium – conditions which are frequently associated with brackish water of mangrove swamps and river mouths. Interestingly, a study by Rau and Molitoris (1991) did find that many marine fungi were capable of producing nitrate reductase enzymes thereby allowing for utilisation of nitrate as nitrogen source. Strain YL2 favoured the growth on PDYASA, which hints at halophilism as significant increase of

MSA_{21} was found at salinity level equivalent to open ocean waters. Although both strains grew well in saline medium, their optimal pH range were found to be around 5.0-7.0, implying that distribution of these endophytes will be limited by physicochemical conditions of host tissue.

The two strains related to *N. hiratsukae* in this study were isolated from same original colony which more closely resembled strain YL2 in terms of pigment colour, colony morphologies were highly similar. When subjected to varied nutrient conditions, two strains of same species reacted differently. These variations between individuals of same genetic make-up can be attributed to phenotypical plasticity, or simply the ability of organisms to respond to environmental signals by altering its morphology and/or physiology (West-Eberhard 1989). Phenotypic plasticity in fungi is also termed "fungal pleomorphism" and serves an important role to enhance the survivability of organism by means of picking the right implements from its genetic toolbox (Price et al. 2003).

Phenotypic plasticity is a widespread characteristic within fungi, none more so than the cosmopolitan fungus *Aureobasidium pullulans* (Slepecky and Starmer 2009) which has been isolated from a great variety of surfaces. The fungal strain used in that study was able to reversibly take various forms in response to the conditions it was subjected to; which include variations in light, temperature, epigenetic modifiers, and carbon sources. Resultant morphologies were diverse, which led to the implication that many other fungi previously isolated and characterised could just be variants of the same species but were unknowingly mistaken for speciation (Butin 1993).

It was a sectoring event which led to isolation of strains YL1 and YL2, two strains which remained relatively stable throughout the duration of study. It might be that an unidentified switch was activated at some point, leading to distinct changes in fungal morphotype (Kilpatrick and Chilvers 1981). The resultant changes were retained as the conditions which fungi were subjected to during experimentation did not lead to a switching back. It seemed more likely that a few other variants were generated in response to manipulation of pH, nitrogen source, and salinity but may not be obvious as morphological differences were minimal within strains.

Neosartorya hiratsukae falls within the category of *Aspergillus* section *Fumigati*, a section of fungi well known for polyphasic natures and frequent mistaken identification (Samson et al. 2007). Further work would be required to properly study the various morphotypes of this fungal strain. Careful, stringent control and manipulation of environmental variables will be critical in ensuring quality results. Improving our understanding of phenotypic plasticity and genetic polymorphism might be the key to proper taxonomy and accurate speciation of *Neosartorya* spp. and related *Aspergillus* strains (Forsman 2014).

5.3.8 Growth of *Phyllosticta* sp. strain. The strain YL3 grew very well on modified medium PDYA regardless of initial pH of medium. Significant increases of MSA_{21} from growths on that set of media were observed. When salts were added to give PDYASA and PDYNSA, the growth of this *Phyllosticta* strain was inhibited, giving MSA_{21} values on saline modified medium comparable or lesser than growth on PDA. High pH of medium was found to reasonably counteract the inhibitory effects of salinity; growth on PDYNSA at pH 9.0 gave significantly high MSA_{21}, as well as a growth curve which indicated secondary metabolism being achieved within the 21 days of incubation.

P. capitalensis is a species of endophytic fungi which has been found to inhabit a wide variety of higher plant species (Okane et al. 2003). In this study, the strain tested was found to possess good tolerance towards a wide pH range. One inference which can be made was that this fungal strain has been capable of colonising the internal tissue of many different plants due to its remarkable adaptability towards environmental pH. The fungal growth rate and final biomass were found to increase when media pH was raised; it may also be possible that this growth increment allows the fungi to achieve secondary metabolism sooner under mildly alkaline conditions, allowing it to out-compete bacteria which tend to thrive at such environments.

The growth enhancing components of yeast extract include organic nitrogen in the form of amino acids, potassium, magnesium, zinc and several many other micronutrients (Grant and Pramer 1962). When yeast extract was the sole additive for PDYA growth medium, it was found that the resultant MSA_{21} on nitrogen-rich medium were significantly larger than that

on PDA. This indicates that growth of *P. capitalensis* strain YL3 was enhanced by increase in available organic nitrogen and perhaps exogenous factors.

Nitrogen metabolism has been reported to be an important factor in the biological functions of fungi including sporulation in yeast (McClary et al. 1959) and host infection by phytopathogens (Krappmann and Braus 2005; Bolton and Thomma 2008). Thus, it was unsurprising to find growth rate of a cosmopolitan endophyte to be enhanced under the influence of a rich source of nitrogenous compounds as a certain degree of opportunism would be regarded as beneficial to attune itself to the intercellular spaces of a variety of host plants (Wikee et al. 2013).

One factor which was not well-tolerated by strain YL3 was salinity. Although all saline-containing medium was supplemented with yeast extract, the inhibitory effect of high sodium concentrations (Jones and Jennings 1964) outweighed the growth-promoting effects of nitrogen-rich additive (Aleklett and Wallander 2012). Although the data was limited, there seems to be a correlation between the extent of inhibition and the overall salinity of growth medium, with further testing on a range of salinity values required for verification.

It seems likely that strain YL3, closely related to *Phyllosticta capitalensis*, was an alkali-tolerant fungi and well-adapted to the pH fluxes of mangrove environment. The studied strain did not favour saline conditions except when medium pH was buffered to 9.0, suggesting a combinatory effect of mild salinity and marine pH conditions to be a trigger for fast growth and secondary metabolism.

Table 25: List of strains and the growth medium which resulted in enhanced growth and the measurements taken for each instance.

Strain	Medium	MSA_{21} (mm^2)	Standard deviation	Ratio of MSA_{21} Experiment : PDA	P-value	Modifications
YL1	**III(5)**	1466.7	147.7	1.732	0.00295	Yeast extract, filtered seawater, pH 5.0
	PDA	846.7	133.8	-	-	Baseline medium
YL2	**II(5)**	1775.0	619.0	3.469	0.03298	Yeast extract, artificial sea salt, pH 5.0
	II(7)	1046.7	325.1	2.046	0.04452	Yeast extract, artificial sea salt, pH 7.0
	PDA	511.7	128.9	-	-	Baseline medium
YL3	**I(5)**	3215.0	5.0	1.315	0.03801	Yeast extract, pH 5.0
	I(7)	3563.3	248.3	1.457	0.00973	Yeast extract, pH 7.0
	I(9)	3525.0	105.0	1.442	0.01680	Yeast extract, pH 9.0
	III(9)	3353.3	57.7	1.371	0.02680	Yeast extract, filtered seawater, pH 9.0
	PDA	2445.0	390.4	-		Baseline medium

5.3.9 Considerations for mangrove endophytic fungi growth medium. With regards to the two species of fungi (three strains) and media modifications tested, there was no clear trend of a best medium for growth of endophytic fungi. As shown in **Table 25**, different strains react very differently to environmental changes. It was revealed however that certain strains may have very specific niches when it comes to pH of growth medium. Supplement of nitrogenous compounds might enhance the growth of some fungi while doing very little for others. For mangrove endophytes, it appears that salinity of medium does have a profound impact on stimulating fungal growth; different salinity levels have varying effects on the tested strains.

A combination of the appropriate pH, salinity, and nutrient was required to enhance growth of each fungal strain. Therefore, medium used for isolation and growth of mangrove endophytic fungi be made with considerations given to the factors tested. Currently, the standard medium for general isolation of endophytic fungi is Yeast Extract Glucose Chloramphenicol Agar, or YGC Agar, buffered to a final pH of 6.6 ± 0.2. We would like to make a recommendation specific for mangrove endophytic fungi, which is the preparation of two sets of isolation plates buffered to different pH levels of approximately 5.0 and 9.0. This should allow for isolation of fungi with typical requirement of low pH for growth as well as alkaliphilic strains, this inference was also put forward by Nagai et al. (1995) in explaining discrepancies in fungal species isolated using media of different pHs. Obligate halophilic fungi strains are virtually incompatible to endophytic lifestyle; hence salinity modifications should only be reserved for growth rate enhancement after initial isolation.

For growth of isolated fungi strains, minor modifications to typical medium such as PDA and MEA should also be carried out; pH adjustment being most important, with addition of sea salts and yeast extract supplement highly dependent on the nature of fungi studied.

5.4 Conclusion

It was found that pH and salinity are two major factors which influenced the growth of mangrove endophytic fungi tested. At optimum pH of 5.0 and suitable salinity levels, *N. hiratsukae* strains grew significantly faster than standard growth medium conditions. The growth of the *P. capitalensis* was more dependent on salinity, and was generally inhibited by the presence of salts in the medium. The presence of yeast extract supplement was crucial for the growth enhancement of *P. capitalensis* only in the absence of salinity, but yielded no positive effect for the two *N. hiratsukae* strains tested.

It was found that there was not one attribute which directly enhances growth of mangrove endophytic fungi. The one instance of secondary metabolism observed most likely occurred due to a combination of high pH, moderate medium salinity levels, and presence of nitrogen supplement. Improvements in mangrove endophytic fungi research should be made in the fungi isolation stage using media at two or more different pH levels to enable cultivation of fungal strains which may grow very slowly on acidic medium but prolific on alkaline medium, and vice versa.

Further work will be required to study the individual and combinatory effects of each variable on growth of mangrove endophytes. An improved experimental set-up can be made to include a larger sample size in terms of fungi tested; this would allow for conclusive development an optimum general growth medium for mangrove endophytic fungi.

6. Conclusions

Without a doubt, in an environment of adversity, only the strong prevail. Strong individuals area able to reproduce, giving offspring which carry within them valuable genetic information to be passed down the generations. It is through evolutionary adaptations which has allowed mangrove plant species to dominate the ever-shifting coastal landscape of the South China Sea, and indeed mangroves can be found all over the world‟s coastal regions where their seeds may take root and surface water temperatures are favourable.

The fungi which reside within mangrove plant tissues are no less remarkable to their hosts. Not only must they tolerate extreme natural phenomena of osmotic pressure changes, temperature fluxes, and tidal inundations, they have to do all that within the confines of another organism which elicits its own responses to stress experienced. Mangrove endophytic fungi are a curious group of organisms which deserve greater attention in our research, including but not limited to biodiversity, inter-species relationships, natural product chemistry, industrial enzymes, and even climate change.

In the first part of this work, it was found that a mangrove endophytic fungus related to *Phyllosticta capitalensis* (strain YL3) gave the antioxidant trans-ferulic acid when grown in solid rice medium. To our knowledge, this was the first time this compound was isolated from a fermentation reaction by *P. capitalensis* and related species. As trans-ferulic acid is a ubiquitous component of plant cell walls, it remains unknown whether the isolated ferulic acid was a product of metabolite synthesis or degradation of growth medium by feruloyl esterase.

In an effort to resolve this question, a more detailed study on the enzymes produced by this strain of fungi can be carried out. In a recent work by Arfi et al. (2013) salt-adapted lignocellulolytic enzymes were isolated from a mangrove fungus of *Pestalotiopsis* sp. An experiment modelled upon the study of secretomes and salinity-based assays will be very useful in verifying the presence of novel fungal enzymes which may release ferulic acid from biomass of solid culture media.

In the second part, we explored the issue of mangrove endophytic fungi exhibiting slow growth on laboratory medium. It was found that different strains of fungi have varying preferences. The two mangrove endophytic strains related to *Neosartorya hiratsukae* (YL1 and YL2) were found to strongly favour low pH levels and brackish to marine salinity conditions; whereas *Phyllosticta*-related strain (YL3) grew significantly well when supplemented with yeast extract, but was inhibited by salinity. Strain YL3 showed good tolerance to pH levels, but was found to grow extremely fast only when a combinatory influence was exerted by high pH, moderate salinity, and organic nitrogen source.

An extension of this research would be to carry out even more detailed work on cultivation methodology. Given that distinctly different preferences were observed amongst the two strains tested, new isolation methods should be developed in order to fully isolate and characterise the endophytic fungi which inhabit mangrove plants (Arnold 2007). Manipulation of pH seems to be a critical factor to achieve enhanced growth rates (Kladwang et al. 2003), and should be the starting point for development of new protocols for isolation of extremotolerant fungi. An ecological survey based on novel isolation and cultivation methods may further lead to advances in applications of fungi technology (Suryanarayanan 2013).

7. References

Abraham WR, Arfmann HA (1992) Rearranged tetrahydrofurans from *Chaetomium cochlioides*. Phytochemistry **31**(7): 2405-2408

Adl SM, Simpson AGB, Lane CE, Lukeš J, Bass D, Bowser SS, Brown MW, Burki F, Dunthorn M, Hampl V, Heiss A, Hoppenrath M, Lara E, Gall LL, Lynn DH, McManus H, Mitchell EAD, Mozley-Stanridge SE, Parfrey LW, Pawlowski J, Rueckert S, Shadwick L, Scoch CL, Smirnov A, Spiegel FW (2012) The revised classification of eukaryotes. Journal of Eukaryotic Microbiology **59**(5): 429-493

Aleklett K, Wallander H (2012) Effects of organic amendments with various nitrogen levels on arbuscular mycorrhizal fungal growth. Applied Soil Ecology **60**(1): 71-76

Alias SA, Zainuddin N, Jones EBG (2010) Biodiversity of marine fungi in Malaysian mangroves. Botanica Marina **53**(6): 545-554

Allam AI, Hollis JP (1972) Sulfide inhibition of oxidases in rice roots. Phytopathology **6**(2): 634-639

Alongi DM (2002) Present state and future of the world‟s mangrove forests. Environmental Conservation **29**(3): 331-349

Alongi DM, Pfitzner J, Trott LA, Tirendi F, Dixon P, Klumpp DW (2005) Rapid sediment accumulation and microbial mineralization in forests of the mangrove *Kandelia candel* in the Jiulongjiang Estuary, China. Estuarine, Coastal and Shelf Science **63**(4): 605-618

Aly AH, Debbab A, Kjer J, Proksch P (2010) Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Diversity **41**(1): 1-16

Amadi JE (2005) Endophytic and exophytic fungi isolated from the seeds of *Tetracarpidium conophorum* in Ilorin, Nigeria. Nigerian Journal of Pure & Applied Sciences **20**(1): 1757-1761

An Z, Wang C, Liu X, Bennett JW (2010) China‟s fungal genomics initiative: a whitepaper. Mycology **1**(1): 1-8

Arabatzis M, Kambouris M, Kyprianou M, Chrysaki A, Foustoukou M, Kanellopoulou M, Kondyli L, Kouppari G, Koutsia-Karouzou C, Lebessi E, Pangalis A, Petinaki E, Stathi A, Trika-Graphakos E, Vartzioti E, Vogiatzi A, Vyzantiadis TA, Zerva L, Velegraki A (2011) Polyphasic identification and susceptibility to seven antifungals of 102 *Aspergillus* isolates recovered from immunocompromised hosts in Greece. Antimicrobial agents and Chemotherapy **55**(6): 3025-3030

Arfi Y, Chevret D, Henrissat B, Berrin JG, Levasseur A, Record E (2013) Characterization of salt-adapted secreted lignocellulolytic enzymes from the mangrove fungus *Pestalotiopsis* sp. Nature communications **4**(5): 1810

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biology Reviews **21**(2-3): 51-66

Arnold AE, Mejia LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences **100**(26): 15649-15654

Ball MC (1998) Mangrove species richness in relation to salinity and waterlogging: A case study along the Adelaide River floodplain, northern Australia. Global Ecology and Biogeography Letters **7**(1): 73–82

Bhimba BV, Franco DAAD, Mathew JM, Jose GM, Joel EL, Thangaraj M (2012) Anticancer and antimicrobial activity of mangrove derived fungi *Hypocrea lixii* VB1. Chinese Journal of Natural Medicines **10**(1): 77-80

Bigger JW (1944) Treatment of staphylococcal infections with penicillin by intermittent sterilisation. The Lancet **244**(6320): 497-500

Bohlmann F, Ziesche J (1979) Ein ungewöhnliches Tetrahydrofuran-Derivat aus Helichrysum aureo-nitens. Phytochemistry **18**(4): 664-665

Bolton MD, Thomma BPHJ (2008) The complexity of nitrogen metabolism and nitrogen-regulated gene expression in plant pathogenic fungi. Physiological and Molecular Plant Pathology **72**(4): 104-110

Borrow A, Brown S, Jefferys EG, Kessell RH, Lloyd EC, Lloyd PB, Rothwell A, Rothwell B, Swait JC (1964) The kinetics of metabolism of *Gibberella fujikuroi* in stirred culture. Canadian Journal of Microbiology **10**(3): 407-444

Brookes D, Tidd BK, Turner WB (1963) 1028. Avenaciolide, an antifungal lactone from *Aspergillus avenaceus*. Journal of the Chemical Society **1**(1): 5385-5391

Bugni TS, Ireland CM (2004) Marine-derived fungi: a chemically and biologically diverse group of microorganisms. Natural Products Reports **21**(1): 143-163

Burke L, Kura Y, Kassem K, Revenga C, Spalding M, McAllister D, Caddy J (2001), Coastal Ecosystems. Washington, DC: World Resources Institute, 77p

Burr SJ, Fry SC (2009) Extracellular cross-linking of maize arabinoxylans by oxidation of feruloyl esters to form oligoferuloyl esters and ether-like bonds. The Plant Journal **58**(4): 554-567

Burrows BF (1967) A new fungal tetrahydrofuran. Chemical Communications (London) **12**(1): 597-598

Butin H (1993) Morphological adaptation and spore pleomorphism in the form-complex *Dichomera-Camarosporium* and *Fusicoccum-Dothiorella*. Sydowia **45**(1): 161-166

Butt TM, Jackson C, Magan N (2001) Fungi as biocontrol agents: progress problems and potential. CABI Publishing 385p

Byrne PJ, Jones EBG (1975) Effect of salinity on the reproduction of terrestrial and marine fungi. Transactions British Mycological Society **65**(2): 185-200

Chaeprasert S, Piapukiew J, Whalley AJS, Sihanonth P (2010) Endophytic fungi from mangrove plant species of Thailand: their antimicrobial and anticancer potential. Botanica Marina **53**(6): 555-564

Chain E, Florey HW, Gardner AD, Heatley NG, Jennings MA, Orr-Ewing J, Sanders AG (1940) Penicillin as a chemotherapeutic agent. The Lancet **236**(6104): 226-228

Chen G, Zhu Y, Wang HZ, Wang SJ, Zhang RQ (2007) The metabolites of a mangrove endophytic fungus, *Penicillium thomi*. Journal of Asian Natural Products Research **9**(2): 159-164

Chen GY, Lin YC, Wen L, Vrijmoed LLP, Jones EBG (2003) Two new metabolites of a marine endophytic fungus (No. 1893) from an estuarine mangrove on the South China Sea coast. Tetrahedron **59**(26): 4907-4909

Cheng ZS, Pan JH, Tang WC, Chen QJ, Lin YC (2009) Biodiversity and biotechnological potential of mangrove-associated fungi. Journal of Forestry Research **20**(1): 63-72

Cheng ZS, Tang WC, Su ZJ, Cai Y, Sun SF, Chen QJ, Wang FH, Lin YC, She ZG, Vrijmoed LLP (2008) Identification of mangrove endophytic fungus 1403 (*Fusarium proliferatum*) based on morphological and molecular evidence. Journal of Forestry Research **19**(3): 219-224

Chintapenta LK, Rath CC, Maringinti B, Ozbay G (2014) Culture conditions for growth and pigment production of a mangrove penicillium species. Journal of Multidisciplinary Scientific Research **2**(3): 1-5

Choi YW, Hyde KD, Ho WWH (1999) Single spore isolation of fungi. Fungal Diversity **3**(1): 29-38

Chong VC, Sasekumar A, Leh MUC, D' Cruz R (1990) The fish and prawn communities of a Malaysian coastal mangrove system, with comparisons to adjacent mud flats and inshore waters. Estuarine, Coastal and Shelf Science **31**(5): 703-722

Cram WJ, Torr PG, Rose DA (2002) Salt allocation during leaf development and leaf fall in mangroves. Trees **16**(2-3): 112-119

Dahdouh-Guebas F, Bondt RD, Abeysinghe PD, Kairo JG, Cannicci S, Triest L, Koedam N (2004) Comparative study of the disjunct zonation pattern of the grey mangrove *Avicennia marina* (Forsk.) Vierh. in Gazi Bay (Kenya). Bulletin of Marine Science **74**(2): 237-252

Dahdouh-Guebas F, Koedam N (2008) Long-term retrospection on mangrove development using transdisciplinary approaches: A review. Aquatic Botany **89**(2): 80-92

Day JW, Conner WH, Ley-Lou F, Day RH, Navarro AM (1987) The productivity and composition of mangrove forests, Laguna de Términos, Mexico. Aquatic Botany **27**(3): 267-284

de Souza WR (2013) Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization. Intech 247p

Debbab A, Aly AH, Proksch P (2011) Bioactive secondary metabolites from endophytes and associated marine derived fungi. Fungal Diversity **49**(1): 1-12

Debbab A, Aly AH, Proksch P (2013) Mangrove derived fungal endophytes – a chemical and biological perception. Fungal Diversity **61**(1): 1-27

Deng CM, Liu SX, Huang CH, Pang JY, Lin YC (2013) Secondary metabolites of a mangrove endophytic fungus *Aspergillus terreus* (No. GX7-3B) from the South China Sea. Marine Drugs **11**(7): 2616-2624

Duke NC, Ball MC, Ellison JC (1998) Factors influencing biodiversity and distributional gradients in mangroves. Global Ecology and Biogeography Letters **7**(1): 27-47

Ebrahim W, Aly AH, Mándi A, Totzke F, Kubbutat MHG, Wray V, Lin WH, Dai HF, Proksch P, Kurtán T, Debbab A (2012a) Decalactone derivatives from *Corynespora cassiicola*, an endophytic fungus of the mangrove plant *Laguncularia racemosa*. European Journal of Organic Chemistry **2012**(18): 3476-3484

Ebrahim W, Kjer J, Amrani ME, Wray V, Lin WH, Ebel R, Lai DW, Proksch P (2012b) Pullularins E and F, two new peptides from the endophytic fungus *Bionectria ochroleuca* isolated from the mangrove plant *Sonneratia caseolaris*. Marine Drugs **10**(5): 1081-1091

Ellison JC (1998) Impacts of sediment burial on mangroves. Marine Pollution Bulletin **37**(8): 420-426

Ellison AM, Farnsworth EJ, Merkt RE (1999) Origins of mangrove ecosystems and the mangrove biodiversity anomaly. Global Ecology and Biogeography **8**(2): 95-115

Evidente A, Cimmino A, Andolfi A, Vurro M, Zonno MC, Motta A (2008) Phyllostoxin and phyllostin, bioactive metabolites produced by *Phyllosticta cirsii*, a potential mycoherbicide for *Cirsium arvense* biocontrol. Journal of agricultural and food chemistry **56**(3): 884-888

Field CB, Osborn JG, Hoffman LL, Polsenberg JF, Ackerly DD, Berry JA, Björkman O, Held A, Matson PA, Mooney HA (1998) Mangrove biodiversity and ecosystem function. Global Ecology and Biogeography Letters **7**(1): 3-14

Finlay RD, Frostegård Å. Sonnerfeldt AM (1992) Utilization of organic and inorganic nitrogen sources by ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Dougl. ex Loud. New Phytologist **120**(1): 105-115

Fleming A (1929) On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. British journal of experimental pathology **10**(3): 226

Florey HW (1944) Penicillin: A survey. British Medical Journey **2**(4361): 169

Forsman A (2014) Rethinking phenotypic plasticity and its consequences for individuals, populations and species. Heredity **1**(1): 1-9

Fromard F, Puig H, Mougin E, Marty G, Betoulle JL, Cadamuro L (1998) Structure, above-ground biomass and dynamis of mangrove ecosystems: a new data from French Guiana. Oecologia **115**(1-2): 39-53

Furukawa K, Wolanski E (1996) Sedimentation in mangrove forests. Mangroves and Salt Marshes **1**(1): 3-10

Ennos AR (2000) The mechanics of root anchorage. Advances in Botanical Research **33**(1): 133-157

Ercoli N, Lewis MN, Moench LJ (1945) The antibacterial activity of penicillin in experimental infections of mice with *C. diphtheria*. Journal of Pharmacology and Experimental Therapeutics **84**(2): 120-127

Gessner MO (1997) Fungal biomass, production and sporulation associated with particulate organic matter in streams. Limnetica **13**(2): 33-44

Gilman EL, Ellison J, Duke NC, Field C (2008) Threats to mangroves from climate change and adaptation options: A review. Aquatic Botany **89**(2): 237-250

Giri C, Ochieng E, Tieszen LL, Zhu Z, Singh A, Loveland T, Masek J, Duke N (2011) Status and distribution of mangrove forests of the world using earth observation satellite data. Global Ecology and Biogeography **20**(1): 154-159

Glienke C, Pereira OL, Stringari D, Fabris J, Kava-Cordeiro V, Galli-Terasawa L, Cunnington J, Shivas RG, Groenewald JZ, Crous PW (2011) Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. Persoonia **26**(1): 47-56

Godhantaraman N (1994) Species composition and abundance of tintinnids and copepods in the Pichavaram mangroves (South India). Ciencias Marinas **20**(3): 371-391

Graf E (1992) Antioxidant potential of ferulic acid. Journal of Free Radical Biology & Medicine **13**(4): 435-448

Grant CL, Pramer D (1962) Minor element composition of yeast extract. Journal of Bacteriology **84**(4): 869

Guarro J, Kallas EG, Godoy P, Karenina A, Gené J, Stchigel A, Colombo AL (2002) Cerebral aspergillosis caused by *Neosartorya hiratsukae*, Brazil. Emerging Infectious Diseases **8**(9): 989-991

Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. Journal of Natural Products **69**(3): 509-526

Guo ZY, Shao CL, She ZG, Cai XL, Liu F, Vrijmoed LLP, Lin YC (2007a) ^{1}H and ^{13}C NMR assignments for two oxaphenalenones bacillosporin C and D from the mangrove endophytic fungus SBE-14. Magnetic Resonance in Chemistry **45**(9): 439-441

Guo ZY, She ZG, Shao CL, Wen L, Liu F, Zheng ZH, Lin YC (2007b) ^{1}H and ^{13}C NMR signal assignments of Paecilin A and B, two new chromone derivatives from mangrove endophytic fungus *Paecilomyces* sp. (tree 1–7). Magnetic Resonance in Chemistry **45**(9): 777-780

Hammerschmidt L, Debbab A, Ngoc TD, Wray V, Hemphill CP, Lin WH, Broetz-Oesterhelt H, Kassack MU, Proksch P, Aly AH (2014) Polyketides from the mangrove-derived endophytic fungus *Acremonium strictum*. Tetrahedron Letters **55**(24): 3463-3468

Han Z, Mei WL, Zhao YX, Deng YY, Dai HF (2009) A new cytotoxic isocoumarin from endophytic fungus *Penicillium* sp. 091402 of the mangrove plant *Bruguiera sexangula*. Chemistry of Natural Compounds **45**(6): 805-807

Hanada RE, Pomella AW, Coasta HS, Bezerra JL, Loquercio LL, Pereira JO (2010) Endophytic fungal diversity in *Theobroma cacao* (cacao) and *T. grandiflorum* (cupuaçu) trees and their potential for growth promotion and biocontrol of black-pod disease. Fungal Biology **114**(11): 901-910

Hennings P (1908) Fungi S. Paulenses a cl. Puttemans collecti. Hedwigia **48**(4): 1-20

Holguin G, Vazquez P, Bashan Y (2001) The role of sediment microorganisms in the productivity, conservation, and rehabilitation of mangrove ecosystems: an overview. Biology and Fertility of Soils **33**(4): 265-278

Hovenden MJ, Allaway WG (1994) Horizontal structures on pneumatophores of *Avicennia marina* (Forsk.) Vierh. – a new site of oxygen conductance. Annals of Botany **73**(4): 377-383

Hsu YH, Nakagawa M, Hirota A, Shima S, Nakayama M (1988) Structure of Myrocin B, a new diterpene antibiotic produced by *Myrothecium verrucaria*. Agricultural and Biological Chemistry **52**(5): 1305-1307

Huang CH, Pan JH, Chen B, Yu M, Huang HB, Zhu X, Lu YJ, She ZG, Lin YC (2011a) Three bianthraquinone derivatives from the mangrove endophytic fungus *Alternaria* sp. ZJ9-6B from the South China Sea. Marine Drugs **9**(5): 832-843

Huang HB, Feng XJ, Xiao Z, Liu L, Li HX, Ma L, Lu YJ, Ju JH, She ZG, Lin YC (2011b) Azaphilones and *p*-terphenyls from the mangrove endophytic fungus *Penicillium chermesinum* (ZH4-E2) isolated from the South China Sea. Journal of Natural Products **74**(5): 997-1002

Huang ZJ, Cai XL, Shao CL, She ZG, Xia XK, Chen YG, Yang JX, Zhou SN, Lin YC (2008) Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. Phytochemistry **69**(7): 1604-1608

Huang ZJ, Guo ZY, Yang RY, Yin XH, Li XY, Luo WQ, She ZG, Lin YC (2009a) Chemistry and cytotoxic activities of polyketides produced by the mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. Chemistry of Natural Compounds **45**(5): 625-628

Huang ZJ, Shao CL, Chen YG, She ZG, Lin YC, Zhou SN (2007) A new isocoumarin from mangrove endophytic fungus (No. dz17) on the South China Sea coast. Chemistry of Natural Compounds **43**(6): 655-658

Huang ZJ, Yang RY, Guo ZY, She ZG, Lin YC (2010) A new naphtho-γ-pyrone from mangrove endophytic fungus ZSU-H26. Chemistry of Natural Compounds **46**(1): 15-18

Huang ZJ, Yang RY, Yin XH, She ZG, Lin YC (2009b) Structure elucidation and NMR assignments for two xanthone derivatives from a mangrove endophytic fungus (No. ZH19). Magnetic Resonance in Chemistry **48**(1): 80-82

Huang ZX, Dostal L, Rosazza JPN (1993) Microbial Transformations of ferulic acid by *Saccharomyces cerevisiae* and *Pseudomonas fluorescens*. Applied and Environmental Microbiology **59**(7): 2244-2250

Hukka A, Vitanen HA (1999) A mathematical model of mould growth on wooden material. Wood Science and Technology **33**(6): 475-485

Hwang YH, Chen SC (1995) Salt tolerance in seedlings of the mangrove *Kandelia candel* (L.) Druce, Rhizophoraceae. Botanical Bulletin of Academia Sinica **36**(1): 25-31

Hyde KD, Alias SA (1999) *Linocarpon angustatum* sp. nov., and *Neolinocarpon nypicola* sp. nov. from petioles of *Nypa fruticans*, and a list of fungi from aerial parts of this host. Mycoscience **40**(2): 145-149

Hyde KD, Alias SA (2000) Biodiversity and distribution of fungi associated with decomposing *Nypa fruticans*. Biodiversity and Conservation **9**(3): 393-402

Hyde KD, Lee SY (1995) Ecology of mangrove fungi and their role in nutrient cycling: what gaps occur in our knowledge?. Hydrobiologia **295**(1-3): 107-118

Hyde KD, Sarma VV (2006) Biodiversity and ecological observations on filamentous fungi of mangrove palm *Nypa fruticans* Wurumb (Liliopsida – Arecales) along the Tutong River, Brunei. Indian Journal of Marine Sciences **35**(4): 297-307

Hyde KD, Soytong K (2008) The fungal endophyte dilemma. Fungal Diversity **33**(1): 163-173

Isaka M, Suyarnsestakorn C, Tanticharoen M, Kongsaeree P, Thebtaranonth Y (2002) Aigialomycins A-E, new resorcylic macrolides from the marine mangrove fungus *Aigialus parvus*. Journal of Organic Chemistry **67**(5): 1561-1566

Itagaki S, Kurokawa T, Nakata C, Saito Y, Oikawa S, Kobayashi M, Hirano T, Iseki K (2009) *In vitro* and *in vivo* antioxidant properties of ferulic acid: A comparative study with other natural oxidation inhibitors. Food Chemistry **114**(2): 466-471

Jin PF, Zuo WJ, Guo ZK, Mei WL, Dai HF (2013) Metabolites from the endophytic fungus *Penicillium* sp. FJ-1 of *Ceriops tagal*. Acta Pharmaceutica Sinica **48**(11): 1688-1691

Joel EL, Bhimba BV (2012) Fungi from mangrove plants: their antimicrobial and anticancer potentials. International Journal of Pharmacy and Pharmaceutical Sciences **4**(3): 139-142

Jones EBG, Jennings DH (1964) The effect of salinity on the growth of marine fungi in comparison with non-marine species. Transactions of the British Mycological Society **47**(4): 619-625

Jones EBG, Jennings DH (1965) The effect of cations on the growth of fungi. New Phytologist **64**(1): 86-100

Jones HE, Etherington JR (1970) Comparative studies of plant growth and distribution in relation to waterlogging: I. The survival of Erica cinerea L. and E. tetralix L. and its apparent relationship to iron and manganese uptake in waterlogged soil. The Journal of Ecology **58**(1): 487-496

Kakkar S, Bais S (2014) A review on protocatechuic acid and its pharmacological potential. ISRN Pharmacology **2014**(1): 943-952

Kanski J, Aksenova M, Stoyanova A, Butterfield DA (2002) Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems *in vitro*: structure-activity studies. Journal of Nutritional Biochemistry **13**(5): 273-281

Kataria HR (1988) Sectoring in cultures of *Ceratobasidium cereale* (*Rhizoctonia cerealis*). Transactions of the British Mycological Society **91**(3): 529-532

Kathiresan K, Bingham BL (2001) Biology of mangroves and mangrove ecosystems. Advances in Marine Biology **40**(1): 81-251

Kavaler L (1965) Mushrooms, molds, and miracles. New York: John Day, 318p

Kendrick B (2011) Fungi and the history of mycology. eLS

Khan R, Shahzad S, Choudhary MI, Khan SA, Ahmad A (2010) Communities of endophytic fungi in medicinal plant *Withania Somnifer*. Pakistan Journal of Botany **42**(2): 1281-1287

Kilpatrick JA, Chilvers GA (1981) Variation in natural population of *Epicoccum purpurascens*. Transactions of the British Mycological Society **77**(3): 497-508

Kitaya Y, Yabuki K, Kiyota M, Tani A, Hirano T, Aiga I (2002) Gas exchange and oxygen concentration in pneumotophores and prop roots of four mangrove species. Trees **16**(2-3): 155-158

Kjer J, Debbab A, Aly AH, Proksch P (2010) Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. Nature protocols **5**(3): 479-490

Kjer J, Wray V, Edrada-Ebel R, Ebel R, Pretsch A, Lin WH, Proksch P (2009) Xanalteric acids I and II and related phenolic compounds from an endophytic *Alternaria* sp. isolated from the mangrove plant *Sonneratia alba*. Journal of Natural Products **72**(11): 2053-2057

Kladwang W, Bhumirattana A, Hywel-Jones N (2003) Alkaline-tolerant fungi from Thailand. Fungal Diversity **13**(1): 69-83

Klaiklay S, Rukachaisirikul V, Phongpaichit S, Pakawatchai C, Saithong S, Buatong J, Preedanon S, Sakayaroj J (2012a) Anthraquinone derivatives from the mangrove-derived fungus *Phomopsis* sp. PSU-MA214. Phytochemistry Letters **5**(4): 738-742

Klaiklay S, Rukachaisirikul V, Tadpetch K, Sukpondma Y, Phongpaichit S, Buatong J, Sakayaroj J (2012b) Chlorinated chromone and diphenyl ether derivatives from the mangrove-derived fungus *Pestalotiopsis* sp. PSU-MA69. Tetrahedron **68**(10): 2299-2305

Klemke C, Kehraus S, Wright AD, König GM (2004) New secondary metabolites from the marine endophytic fungus *Apiospora montagnei*. Journal of Natural Products **67**(6): 1058-1063

Krappmann S, Braus GH (2005) Nitrogen metabolism of *Aspergillus* and its role in pathogenicity. Medical Mycology **43**(1): 31-40

Kristensen E, Bouillon S, Dittmar T, Marchand C (2008) Organic carbon dynamics in mangrove ecosystems: A review. Aquatic Botany **89**(2): 201-219

Kumaresan V, Suryanarayanan TS (2002) Endophyte assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. Fungal Diversity **9**(1): 81-91

Lacap DC, Hyde KD, Liew ECY (2003) An evaluation of the fungal „morphotype" concept based on ribosomal DNA sequences. Fungal Diversity **12**(1): 53-66

Lee YS, Kang YH, Jung JY, Lee S, Ohuchi K, Shin KH, Kang IJ, Park JHY, Shin HK, Lim SS (2008) Protein glycation inhibitors from the fruiting body of *Phellinus linteus*. Biological and Pharmaceutical Bulletin **31**(10): 1968-1972

Li DL, Li XM, Li TG, Dang HY, Proksch P, Wang BG (2008) Benzaldehyde derivatives from *Eurotium rubrum*, an endophytic fungus derived from the mangrove plant *Hibiscus tiliaceus*. Chemical & Pharmaceutical Bulletin **56**(9): 1282-1285

Li HJ, Lin YC, Yao JH, Vrijmoed LLP, Jones EBG (2004) Two new metabolites from the mangrove endophytic fungus no. 2524. Journal of Asian Natural Products Research **6**(3): 185–191

Li J, Yang X, Lin YY, Yuan J, Lu YJ, Zhu X, Li J, Li MF, Lin YC, He JG, Liu L (2014) Meroterpenes and azaphilones from marine mangrove endophytic fungus *Penicillium 303#*. Fitoterapia **97**(2014): 241-246

Li KK, Lu YJ, Song XH, She ZG, Wu XW, An LK, Ye CX, Lin YC (2010) The metabolites of mangrove endophytic fungus Zh6-B1 from the South China Sea. Bioorganic & Medicinal Chemistry Letters **20**(11): 3326-3328

Li MY, Xiao Q, Pan JY, Wu J (2009) Natural products from semi-mangrove flora: source, chemistry and bioactivities. Natural Product Reports **26**(2): 281-298

Liang S, Zhou RC, Dong SS, Shi SH (2008) Adaptation to salinity in mangroves: Implication on the evolution of salt-tolerance. Chinese Science Bulletin **53**(11): 1708-1715

Lin YC, Wu XY, Feng S, Jiang GC, Luo JH, Zhou SN, Vrijmoed LLP, Jones EBG, Krohn K, Steingröver K, Zsila F (2001a) Five unique compounds: xyloketals from mangrove fungus *Xylaria* sp. from the South China Sea coast. Journal of Organic Chemistry **66**(19): 6252-6256

Lin YC, Wu XY, Feng S, Jiang GC, Zhou S, Vrijmoed LLP, Jones EBG (2001b) A novel *N*-cinnamoylcyclopeptide containing an allenic ether from the fungus *Xylaria* sp. (strain c2508) from the South China Sea. Tetrahedron Letters **42**(3): 449-451

Lin ZJ, Zhu TJ, Fang YC, Gu QQ, Zhu WM (2008) Polyketides from *Penicillium* sp. JP-1, an endophytic fungus associated with the mangrove plant *Aegiceras corniculatum*. Phytochemistry **69**(5): 1273-1278

Liu F, Li Q, Yang H, Cai XL, Xia XK, Chen SP, Li MF, She ZG, Lin YC (2009) Structure elucidation of three diphenyl ether derivatives from the mangrove endophytic fungus SBE-14 from the South China Sea. Magnetic Resonance in Chemistry **47**(5): 453-455

Maddau L, Cabras A, Franceschini A, Linaldeddu BT, Crobu S, Roggio T, Pagnozzi D (2009) Occurrence and characterization of peptaibols from *Trichoderma citrinoviride*, an endophytic fungus of cork oak, using electrospray ionization quadrupole time-of-flight mass spectrometry. Microbiology **155**(10): 3371-3381

Magan N (2007) Fungi in extreme environments. Environmental and microbial relationships **4**(6): 5-24

Maitland DP (1990) Carapace and branchial water circulation and water related behaviours in the semaphore crab *Heloecius cordiformis* (Decapoda: Brachyura: Ocypodidae). Marine Biology **105**(2): 275-286

Marion GM, Millero FJ, Camões MF, Spitzer P, Feistel R, Chen CTA (2011) pH of seawater. Marine Chemistry **126**(1): 89-96

Martins A, Vieira H, Gaspar H, Santos S (2014) Marketed marine natural products in the pharmaceutical and cosmeceutical industries: tips for success. Marine Drugs **12**(2): 1066-1101

Marwah RG, Fatope MO, Deadman ML, Al-Maqbali YM, Husband J (2007) Musanahol: a new aureonitol-related metabolite from a *Chaetomium* sp. Tetrahedron **63**(34): 8174-8180

Mathew S, Abraham TE (2004) Ferulic acid: an antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. Critical reviews in Biotechnology **24**(2-3): 59-83

Mazda Y, Magi M, Kogo M, Hong PN (1997) Mangroves as a coastal protection from waves in the Tong King delta, Vietnam. Mangroves and Salt Marshes **1**(2): 127-135

McClary DO, Nulty WL, Miller GR (1959) Effect of potassium versus sodium in the sporulation of *Saccharomyces*. Journal of bacteriology **78**(3): 362

McGivan JD, Chappell JB (1970) Avenaciolide: a specific inhibitor of glutamate transport in rat liver mitochondria. Biochemical Journal **116**(4): 37-38

McGuinness KA (1997) Dispersal, establishment and survival of *Ceriops tagal* propagules in a north Australian mangrove forest. Oecologia **109**(1): 80-87

McKee KL (1993) Soil physiochemical patterns and mangrove species distribution – reciprocal effects?. Journal of Ecology **81**(3): 477-487

Mei WL, Zheng B, Zhao YX, Zhong HM, Chen XL, Zeng YB, Dong WH, Huang JL, Proksch P, Dai HF (2012) Meroterpenes from endophytic fungus A1 of mangrove plant *Scyphiphora hydrophyllacea*. Marine Drugs **10**(9): 1993-2001

Meijaard E, Nijman V (2000) Distribution and conservation of the proboscis monkey (*Nasalis larvatus*) in Kalimantan, Indonesia. Biological Conservation **92**(1): 15–24

Miao L, Kwong TFN, Qian PY (2006) Effect of culture conditions on mycelial growth, antibacterial activity, and metabolite profiles of the marine-derived fungus *Arthrinium* c.f. *saccharicola*. Applied Microbiology and Biotechnology **72**(5): 1063-1073

Montaser R, Luesch H (2011) Marine natural products: a new wave of drugs?. Future Medicinal Chemistry **3**(12): 1475-1489

Morris AJ, Byrne TC, Madden JF, Reller LB (1996) Duration of incubation of fungal cultures. Journal of Clinical Microbiology **34**(6): 1583-1585

Murray JC, Burch JA, Streilein RD, Iannacchione MA, Hall RP, Pinnell SR (2008) A topical antioxidant solution containing vitamins C and E stabilized by ferulic acid provides protection for human skin against damage caused by ultraviolet irradiation. Journal of the American Academy of Dermatology **59**(3): 418-425

Nagai K, Sakai T, Rantiatmodjo RM, Suzuki K, Gams W, Okada G (1995) Studies on the distribution of alkalophilic and alkali-tolerant soil fungi I. Mycoscience **36**(3): 247-256

Nagelkerken I, Blaber SJM, Bouillon S, Green P, Haywood M, Kirton LG, Meynecke JO, Pawlik J, Penrose HM, Sasekumar A, Somerfield PJ (2008) The habitat function of mangroves for terrestrial and marine fauna: A review. Aquatic Botany **89**(2): 155-185

Nayak SS, Gonsalves V, Nazareth SW (2012) Isolation and salt tolerance of halophilic fungi from mangroves and solar salterns in Goa – India. Journal of Geo-Marine Sciences **41**(2): 164-172

Okane I, Lunyong S, Nakagiri A (2003) Extensive host range of an endophytic fungus, *Guignardia endophyllicola* (anamorph: *Phyllosticta capitalensis*). Mycoscience **44**(5): 353-363

Ong JE (1993) Mangroves - a carbon source and sink. Chemosphere **27**(6): 1097-1107

Onn ML (2013) Screening of mangrove endophytic fungi for bioactive compounds. Masters thesis.

Onn ML, Lim PT, Mujahid A, Müller M (2012a) Biosorption of copper (Cu) and zinc (Zn) by mangrove endophytic fungi. Marine & Freshwater Research (Manuscript ID: MF12341)

Onn ML, Lim PT, Mujahid A, Proksch P, Müller M (2012b) Preliminary results of screening of mangrove endophytic fungi for bioactive compounds. Journal of Basic Microbiology (Manuscript ID: jobm.201200752)

Pang KL, Vrijmoed LLP, Goh TK, Plaingam N, Jones EBG (2008) Fungal endophytes associated with *Kandelia candel* (Rhizophoraceae) in Mai Po Nature Reserve, Hong Kong. Botanic Marina **51**(3): 171-178

Parkes KC (1990) A revision of the mangrove vireo, *Vireo pallens* (Aves: Vireonidae). Annals of the Carnegie Museum **59**(1): 49-60

Pedersen DS, Rosenbohm C (2001) Dry Column Vacuum Chromatography. Synthesis **2001**(16): 2431-2434

Peñalva MA, Rowlands RT, Turner G (1998) The optimization of penicillin biosynthesis in fungi. Trends in biotechnology **16**(11): 483-489

Petrini O (eds) (1991) Microbial Ecology of Leaves. Springer – Verlag New York Inc., 180p

Petrini O, Sieber TN, Toti L, Viret O (1992) Ecology, metabolite production, and substrate utilization in endophytic fungi. Natural Toxins **1**(3): 185-196

Pinçon G, Chabannes M, Lapierre C, Pollet B, Ruel K, Joseleau JP, Boudet AM, Legrand M (2001) Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-O-methyltransferase I and cinnamoyl-coenzyme A reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. Plant Physiology **126**(1): 145-155

Polidoro BA, Carpenter KE, Dahdouh-Guebas F, Ellison JC, Koedam NE, Yong JWH (2014) Global patterns of mangrove extinction risk: Implications for ecosystem services and biodiversity loss. Coastal Conservation **19**(1): 15-36

Price TD, Qvarnström A, Irwin DE (2003) The role of phenotypic plasticity in driving genetic evolution. Proceedings of the Royal Society of London. Series B: Biological Sciences **270**(1523): 1433-1440

Prihanto AA, Firdaus M, Nurdiani R (2011) Endophytic fungi isolated from mangrove (*Rhyzopora mucronata*) and its antibacterial activity on *Staphylococcus aureus* and *Escherichia coli*. Journal of Food Science and Engineering **1**(5): 386-389

Promputtha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD, Jeewon R (2007) A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. Microbial Ecology **53**(4): 579-590

Prosser JI, Tough AJ (1991) Growth mechanisms and growth kinetics of filamentous microorganisms. Critical reviews in biotechnology **10**(4): 253-274

Pudhom K, Teerawatananond T, Chookpaiboon S (2014) Spirobisnaphthalenes from the mangrove-derived fungus *Rhytidhysteron* sp. AS21B. Marine Drugs **12**(3): 1271-1280

Quiroz-Castañeda RE, Folch-Mallol JL (eds.) (2013) Sustainable degradation of lignocellulosic biomass – Techniques, applications and commercialization. Intech, 275p

Radić N, Štrukelj B (2012) Endophytic fungi – The treasure chest of antibacterial substances. Phytomedicine **19**(14): 1270-1284

Rahouti M, Seigle-Murandi F, Steiman R, Eriksson KE (1989) Metabolism of ferulic acid by *Paecilomyces variotii* and *Pestalotia palmarum*. Applied and environmental microbiology **55**(9): 2391-2398

Rajendran N (1997) Studies on mangrove - associated prawn seed resources of the Pichavaram, Southeast coast of India. Ph.D. thesis. Annamalai University, India, 131p

Ralph J, Quideau S, Grabber JH, Hatfield RD (1994) Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. Journal of the Chemical Society Perkin Transactions 1 **23**: 3485-3498

Rau R, Molitoris HP (1991) Determination and significance of nitrate reductase in marine fungi. Kieler Meeresforschungen: Sonderheft **8**(1): 369-375

Redman RS, Dunigan D, Rodriguez RJ (2001) Fungal symbiosis from mutualism to parasitism: Who controls the outcome, host or invader?. New Phytologist **151**(3): 705-716

Richards TA, Soanes DM, Foster PG, Leonard G, Thornton CR, Talbot NJ (2009) Phylogenomic analysis demonstrates a pattern of rare and ancient horizontal gene transfer between plants and fungi. The Plant Cell **21**(7): 1897-1911

Rodriguez R, Redman R (2008) More than 400 million years of evolution and some plants still can‟t make it on their own: Plant stress tolerance via fungal symbiosis. Journal of Experimental Botany **59**(5): 1109-1114

Rönsberg D, Debbab A, Mándi A, Wray V, Dai HF, Kurtán T, Proksch P, Aly AH (2013) Secondary metabolites from the endophytic fungus *Pestalotiopsis virgatula* isolated from the mangrove plant *Sonneratia caseolaris*. Tetrahedron Letters **54**(25): 3256-3259

Rosewich UL, Kistler HC (2000) Role of horizontal gene transfer in the evolution of fungi 1. Annual review of phytopathology **38**(1): 325-363

Rousk J, Brookes PC, Bååth E (2009) Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Applied and Environmental Microbiology **75**(6): 1589-1596

Rukachaisirikul V, Rodglin A, Phongpaichit S, Buatong J, Sakayaroj J (2012) α-Pyrone and seiricuprolide derivatives from the mangrove-derived fungi *Pestalotiopsis* spp. PSU-MA92 and PSU-MA119. Phytochemistry Letters **5**(1): 13-17

Rukachaisirikul V, Sommart U, Phongpaichit S, Sakayaroj J, Kirtikara K (2008) Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. Phytochemistry **69**(3): 783-787

Samson RA, Hong S, Peterson SW, Frisvad JC, Varga J (2007) Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. Studies in Mycology **59**(14): 147-203

Sánchez C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnology advances **27**(2): 185-194

Sanderson PL, Armstrong W (1980) The responses of conifers to some of the adverse factors associated with waterlogged soils. New Phytologist **8**(5): 351-362

Schardl CL, Leuchtmann A (2005) The Epichloë endophytes of grasses and the symbiotic continuum 23. Mycology Series, 475p

Scherlach K, Hertweck C (2009) Triggering cryptic natural product biosynthesis in microorganism. Organic Biochemical Chemistry **7**(9): 1753-1760

Scholander PF, Hammel HT, Hemmingsen E, Garey W (1962) Salt balance in mangroves. Plant Physiology **37**(6): 722

Sebastianes FLS, Ramão-Dumaresq AS, Lacava PT, Harakava R, Azevedo JL, de Melo IS, Pizzirani-Kleiner AA (2013) Species diversity of culturable endophytic fungi from Brazilian mangrove forests. Current Genetics **59**(3): 153-166

Shao CL, Guo ZY, Xia XK, Liu Y, Huang ZJ, She ZG, Lin YC, Zhou SN (2007) Five nitro-phenyl compounds from the South China Sea mangrove fungus. Journal of Asian Natural Products Research **9**(7): 643-648

Shao CL, Wang CY, Gu YC, Wei MY, Pan JH, Deng DS, She ZG, Lin YC (2010) Penicinoline, a new pyrrolyl 4-quinolinone alkaloid with an unprecedented ring system from an endophytic fungus *Penicillium* sp. Bioorganic & Medicinal Chemistry Letters **66**(19): 6252-6256

Shearer CA, Descals E, Kohlmeyer B, Kohlmeyer J, Marvanova L, Padgett D, Porter D, Raja HA, Schmit JP, Thorton HA, Voglymayr H (2007) Fungal biodiversity in aquatic habitats. Biodiversity Conservation **16**(1): 49-67

Signore AD, Romeo F, Giaccio M (1997) Content of phenolic substances in basidiomycetes. Mycological Research **101**(5): 552-556

Sigurdsson JB, Sundari G (1990) Colour changes in the shell of the tree-climbing bivalve *Enigmonia aenigmatica* (Holten, 1802)(Anomidae). The Raffles Bulletin of Zoology **38**(2): 213-218

Sivakumar T (2013) A review on biodiversity of marine and mangrove fungi. International Journal of Current Research and Academic Review **1**(1): 26-44

Sivasothi N, Burhanuddin HMN (1994) A review of otters (Carnivora: Mustelidae: Lutrinae) in Malaysia and Singapore. Hydrobiologia **285**(1-3): 151-170

Skamnioti P, Gurr SJ (2009) Against the grain: safeguarding rice from rice blast disease. Trends in biotechnology **27**(3): 141-150

Slepecky RA, Starmer WT (2009) Phenotypic plasticity in fungi: a review with observations on *Aureobasidium pullulans*. Mycologia **101**(6): 823-832

Smith III TJ (1993) Tropical Mangrove Ecosystems. American Geophysical Union, 101p

Soca-Chafre G, Rivera-Orduña FN, Hidalgo-Lara ME, Hernandez-Rodriguez C, Marsch R, Flores-Cotera LB (2011) Molecular phylogeny and paclitaxel screening of fungal endophytes from *Taxus globose*. Fungal biology **115**(2): 143-156

Sommart U, Rukachaisirikul V, Trisuwan K, Tadpetch K, Phongpaichit S, Preedanon S, Sakayaroj J (2012) Tricycloalternarene derivatives from the endophytic fungus *Guignardia bidwellii* PSU-G11. Phytochemistry Letters **5**(1): 139-143

Song YX, Wang JJ, Huang HB, Ma L, Wang J, Gu YC, Liu L, Lin YC (2012) Four eremophilane sesquiterpenes from the mangrove endophytic fungus *Xylaria* sp. BL321. Marine Drugs **10**(2): 340-348

Sridhar KR (2004) Mangrove fungi in India. Current science **86**(12): 1586-1587

Stamets P (2000) Growing gourmet and medicinal mushrooms 3. Berkeley: Ten Speed Press, 22p

Stierle A, Strobel G, Stierle D (1993) Taxol and taxol production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science **260**(5105): 214-216

Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews **67**(4): 491-502

Strobel G, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. Journal of Natural Products **67**(2): 257-268

Strobel G, Yang X, Sears J, Kramer R, Sidhu RS, Hess WM (1996) Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. Microbiology **142**(2): 435-440

Strobel GA (2003) Endophytes as sources of bioactive products. Microbes and Infection **5**(6): 535-544

Stuebing RB, Ismail G, Ching LH (1994) The distribution and abundance of the Indo-Pacific crocodile *Crocodylus porosus* Schneider in the Klias River, Sabah, east Malaysia. Biological Conservation **69**(1): 1-7

Suryanarayanan TS (2013) Endophyte research: Going beyond isolation and metabolite documentation. Fungal Ecology **6**(6): 561-568

Suryanarayanan TS, Ravishankar JP, Venkatesan G, Murali TS (2004) Characterization of the melanin pigment of a cosmopolitan fungal endophyte. Mycological Research **108**(8): 974-978

Tao LY, Zhang JY, Liang YJ, Chen LM, Zhen LS, Wang F, Mi YJ, She ZG, To KKW, Lin YC, Fu LW (2010) Anticancer effect and structure-activity analysis of marine

products isolated from metabolites of mangrove fungi in the South China Sea. Marine Drugs **8**(4): 1094-1105

Tao YW, Zeng XJ, Mou CB, Li J, Cai XL, She ZG, Zhou SN, Lin YC. (2008) ^{1}H and ^{13}C NMR assignments of three nitrogen containing compounds from the mangrove endophytic fungus (ZZF08). Magnetic Resonance in Chemistry **46**(5): 501-505

Thatoi H, Behera BC, Mishra RR, Dutta SK (2013) Biodiversity and biotechnological potential of microorganisms from mangrove ecosystems: A review. Annals of Microbiology **63**(1): 1-19

Tillett R, Walker JRL (1990) Metabolism of ferulic acid by a *Penicillium* sp. Archives of microbiology **154**(2): 206-208

TRBA (2002) Ausschuss für Biologische Arbeitsstoffe. Technische Regel für Biologische Arbeitsstoffe 460: Einstufung von Pilzen in Risikogruppen. Bekanntmachung des BMA vom 1.8.2002. BArbBl Heft 10/2002, 78–84

Trinci APJ (1969) A kinetic study of the growth of *Aspergillus nidulans* and other fungi. Journal of General Microbiology **57**(1): 11-24

Trinci APJ, Banbury GH (1967) A study of the growth of the tall conidiophores of *Aspergillus giganteus*. Transactions of the British Mycological Society **50**(4): 525-538

Udagawa S, Tsubouchi H, Horie Y (1991) *Neosartorya hiratsukae*, a new species of food-borne Ascomycetes. Transactions of the Mycological Society of Japan **32**(1): 23–29

Udayanga D, Liu X, McKenzie EHC, Chukeatirote E, Bahkali AHA, Hyde KD (2011) The genus *Phomopsis*: biology, application, species concepts and names of common phytopathogens. Fungal Diversity **50**(1): 189-225

UNEP (2004) Mangroves in the South China Sea. UNEP/GEF/SCS Technical Publication No. 1

UNEP-WCMC (2001) Distribution of coral, mangrove and seagrass diversity. UNEP/GRID-Arendal

Valiela I, Bowen JL, York JK (2001) Mangrove forests: one of the world"s threatened major tropical environments. BioScience **51**(10): 807-815

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-tolerance, disease resistance, and higher yield. Proceedings of the National Academy of Sciences of the United States of America **102**(38): 13386-13391

Wang X, Mao ZG, Song BB, Chen CH, Xiao WW, Hu B, Wang JW, Jiang XB, Zhu YH, Wang HJ (2013) Advances in the study of the structures and bioactivities of metabolites isolated from mangrove-derived fungi in the South China Sea. Marine Drugs **11**(10): 3601-3616

Wang Y, Tang K (2011) A new endophytic taxol-and baccatin III-producing fungus isolated from *Taxus chinensis* var. *mairei*. African Journal of Biotechnology **10**(72): 16379-16386

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. Journal of the American Chemical Society **93**(9): 2325-2327

Wee AKS, Takayama K, Asakawa T, Thompson B, Onrizal, Sungkaew S, Tung NX, Nazre M, Soe KK, Tan HTW, Watano Y, Baba S, Kajita T, Webb EL (2014) Oceanic currents, not land masses, maintain the genetic structure of the mangrove *Rhizophora mucronata* Lam. (Rhizophoraceae) in Southeast Asia. Journal of Biogeography **41**(5): 954-964

Wen L, Cai XL, Xu F, She ZG, Chan WL, Vrijmoed LLP, Jones EBG, Lin YC (2009) Three metabolites from the mangrove endophytic fungus *Sporothrix* sp. (#4335) from the South China Sea. Journal of Organic Chemistry **74**(3): 1093-1098

Wen L, Chen G, She ZG, Yan CY, Cai JN, Mu L (2010) Two new paeciloxocins from a mangrove endophytic fungus *Paecilomyces* sp. Russian Chemical Bulletin **59**(8): 1656-1659

Wen L, Lin YC, She ZG, Du DS, Chan WL, Zheng ZH (2008) Paeciloxanthone, a new cytotoxic xanthone from the marine mangrove fungus *Paecilomyces* sp. (Tree1-7). Journal of Asian Natural Products Research **10**(1-2): 133-137

West-Eberhard MJ (1989) Phenotypic plasticity and the origins of diversity. Annual Review of Ecology and Systematics **20**(1): 249-278

White TJ, Bruns T, Lee S, Taylor J (eds) (1990) PCR protocols: A guide to methods and applications 18. Academic Press, San Diego, 322p

Wikee S, Lombard L, Crous PW, Nakashima C, Motohashi K, Chukeatirote E, Alias SA, McKenzie EHC, Hyde KD (2013) *Phyllosticta capitalensis*, a widespread endophyte of plants. Fungal diversity **60**(1): 91-105

Wikee S, Udayanga D, Crous PW, Chukeatirote E, McKenzie EHC, Bahkali AH, Dai DQ, Hyde KD (2011) *Phyllosticta* – An overview of current status of species recognition. Fungal Diversity **51**(1): 43-61

Woodroffe C (1993) Tropical mangrove ecosystems. American Geophysical Union, 41p

World Health Organization (2010) Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. WHO, Geneva, Stop TB Department, 17p

Wulandari NF, To-Anun C, Cai L, Abd-Elsalam KA, Hyde KD (2010) *Guignardia*/*Phyllosticta* species on banana. Cryptogamie-Mycologie **31**(4): 403-419

Xing XK, Chen J, Xu MJ, Lin WH, Guo SX (2011) Fungal endophytes associated with *Sonneratia* (Sonneratiaceae) mangrove plants on the south coast of China. Forest Pathology **41**(4): 334-340

Xu J, Aly AH, Wray V, Proksch P (2011) Polyketide derivatives of endophytic fungus *Pestalotiopsis* sp. Isolated from the Chinese mangrove plant *Rhizophora mucronata*. Tetrahedron Letters **52**(5): 21-25

Xu J, Kjer J, Sendker J, Wray V, Guan HS, Edrada R, Müller WEG, Bayer M, Lin WH, Wu J, Proksch P. (2009) Cytosporones, coumarins, and an alkaloid from the endophytic fungus *Pestalotiopsis* sp. isolated from the Chinese mangrove plant *Rhizophora mucronata*. Bioorganic & Medicinal Chemistry **17**(20): 7362-7367

Yancey PH (2001) Water stress, osmolytes and proteins. American Zoologist **41**(4): 699-709

Yang JX, Xu F, Huang CH, Li J, She ZG, Pei Z, Lin YC (2010) Metabolites from the mangrove endophytic fungus *Phomopsis* sp. (#zsuH76). European Journal of Organic Chemistry **2010**(19): 3692–3695

Yasumoto E, Adachi K, Kato M, Sano H, Sasamoto H, Baba S, Ashihara H (1999) Uptake of inorganic ions and compatible solutes in cultured mangrove cells during salt stress. In Vitro Cellular & Developmental Biology-Plant **35**(1): 82-85

Yong JWH, Sheue CR (2014) A comparative guide to the Asian mangroves. Open access dataset, 2p

Yoshioka T, Inokuchi T, Fujioka S, Kimura Y (2004) Phenolic compounds and flavonoids as plant growth regulators from fruit and leaf of *Vitex rotundifolia*. Zeitschrift fur Naturforschung **59**(7/8): 509-514

Zeng YB, Wang H, Zuo WJ, Zheng B, Yang T, Dai HF, Mei WL. A fatty acid glycoside from a marine-derived fungus isolated from mangrove plant *Scyphiphora hydrophyllacea*. Marine Drugs **10**(3): 598-603

Zetola N, Francis JS, Nuermberger EL, Bisha WR (2005) Community-acquired meticillin-resistant *Staphylococcus aureus*: an emerging threat. The Lancet Infectious Diseases **5**(5): 275-286

Zhang JY, Tao LY, Liang YJ, Chen LM, Mi YJ, Zheng LS, Wang F, She ZG, Lin YC, To KKW, Fu LW (2010) Anthracenedione derivatives as anticancer agents isolated from secondary metabolites of the mangrove endophytic fungi. Marine Drugs **8**(4): 1469-1481

Zhou X, Gong Z, Su Y, Lin J, Tang K (2009) Cordyceps fungi: natural products, pharmacological functions and developmental products. Journal of Pharmacy and Pharmacology **61**(3): 279-29

Zhu GS, Yu ZN, Gui Y, Liu ZY (2008) A novel technique for isolating orchid mycorrhizal fungi. Fungal Diversity **33**(1): 123-137

Zhu JK (2003) Regulation of ion homeostasis under salt stress. Current Opinion in Plant Biology **6**(5): 441-445

8. Appendix

This section contains raw data for MSA measurements mentioned in Parts 3.4, 5.2, and 5.3.

The strains and media codes are given as below:

Strains used:

Assigned code	Taxonomical identity
YL1	*Neosartorya hiratsukae*
YL2	*Neosartorya hiratsukae*
YL3	*Phyllosticta capitalensis*

Media type:

Media code	Media type (abbreviations in Table 22)
Std PDA	Standard PDA
I(5)	PDYA; pH 5.0
I(7)	PDYA; pH 7.0
I(9)	PDYA; pH 9.0
II(5)	PDYASA; pH 5.0
II(7)	PDYASA; pH 7.0
II(9)	PDYASA; pH 9.0
III(5)	PDYNSA; pH 5.0
III(7)	PDYNSA; pH 7.0
III(9)	PDYNSA; pH 9.0
IV(5)	PDYMNSA; pH 5.0
IV(7)	PDYMNSA; pH 7.0

YL1	Std PDA				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	25.0	13.5	19.0	19.2	5.8
4	95.5	76.5	92.0	88.0	10.1
6	180.0	175.0	185.0	180.0	5.0
9	300.0	260.0	410.0	323.3	77.7
11	345.0	290.0	450.0	361.7	81.3
13	415.0	385.0	565.0	455.0	96.4
15	445.0	495.0	575.0	505.0	65.6
18	640.0	575.0	715.0	643.3	70.1
21	725.0	825.0	990.0	846.7	133.8

YL1	I(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	19.0	18.5	11.5	16.3	4.2
4	105.0	105.0	105.0	105.0	0.0
6	175.0	180.0	165.0	173.3	7.6
9	340.0	355.0	315.0	336.7	20.2
11	415.0	475.0	440.0	443.3	30.1
13	475.0	550.0	480.0	501.7	41.9
15	550.0	580.0	525.0	551.7	27.5
18	725.0	675.0	625.0	675.0	50.0
21	865.0	945.0	770.0	860.0	87.6

YL1	I(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	14.5	12.5	15.0	14.0	1.3
4	115.0	110.0	110.0	111.7	2.9
6	160.0	155.0	155.0	156.7	2.9
9	315.0	310.0	290.0	305.0	13.2
11	375.0	420.0	360.0	385.0	31.2
13	420.0	475.0	405.0	433.3	36.9
15	525.0	525.0	565.0	538.3	23.1
18	550.0	590.0	550.0	563.3	23.1
21	775.0	775.0	750.0	766.7	14.4

YL1	I(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	12.5	10.5	22.0	15.0	6.1
4	105.0	110.0	115.0	110.0	5.0
6	130.0	145.0	140.0	138.3	7.6
9	315.0	320.0	310.0	315.0	5.0
11	375.0	345.0	350.0	356.7	16.1
13	460.0	445.0	465.0	456.7	10.4
15	540.0	475.0	570.0	528.3	48.6
18	700.0	575.0	715.0	663.3	76.9
21	990.0	675.0	950.0	871.7	171.5

YL1	II(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	1.0	10.5	4.5	5.3	4.8
4	73.0	66.0	57.0	65.3	8.0
6	98.5	110.0	65.0	91.2	23.4
9	395.0	250.0	245.0	296.7	85.2
11	415.0	355.0	290.0	353.3	62.5
13	475.0	445.0	375.0	431.7	51.3
15	550.0	515.0	440.0	501.7	56.2
18	705.0	640.0	565.0	636.7	70.1
21	915.0	825.0	740.0	826.7	87.5

YL1	II(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	9.5	8.0	15.0	10.8	3.7
4	66.0	72.0	76.5	71.5	5.3
6	100.0	115.0	115.0	110.0	8.7
9	275.0	280.0	270.0	275.0	5.0
11	355.0	365.0	350.0	356.7	7.6
13	465.0	445.0	460.0	456.7	10.4
15	525.0	495.0	525.0	515.0	17.3
18	565.0	635.0	625.0	608.3	37.9
21	805.0	865.0	850.0	840.0	31.2

YL1	II(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	4.0	2.5	5.0	3.8	1.3
4	49.5	22.0	34.0	35.2	13.8
6	82.5	29.0	50.0	53.8	27.0
9	220.0	125.0	165.0	170.0	47.7
11	280.0	195.0	220.0	231.7	43.7
13	325.0	215.0	265.0	268.3	55.1
15	390.0	230.0	285.0	301.7	81.3
18	570.0	515.0	370.0	485.0	103.3
21	645.0	365.0	425.0	478.3	147.4

YL1	III(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	8.5	9.0	8.5	8.7	0.3
4	52.0	63.0	54.0	56.3	5.9
6	78.5	87.5	66.3	77.4	10.7
9	350.0	365.0	330.0	348.3	17.6
11	405.0	360.0	335.0	366.7	35.5
13	625.0	500.0	470.0	531.7	82.2
15	880.0	665.0	615.0	720.0	140.8
18	1175.0	1000.0	955.0	1043.3	116.2
21	1610.0	1475.0	1315.0	1466.7	147.7

YL1	III(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	6.5	1.0	5.0	4.2	2.8
4	66.5	19.5	63.5	49.8	26.3
6	110.0	155.0	105.0	123.3	27.5
9	270.0	275.0	175.0	240.0	56.3
11	380.0	350.0	310.0	346.7	35.1
13	525.0	480.0	395.0	466.7	66.0
15	680.0	580.0	515.0	591.7	83.1
18	845.0	705.0	675.0	741.7	90.7
21	1080.0	965.0	880.0	975.0	100.4

YL1	III(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	6.0	3.0	2.0	3.7	2.1
4	95.0	78.5	76.5	83.3	10.2
6	120.0	105.0	95.0	106.7	12.6
9	240.0	225.0	230.0	231.7	7.6
11	370.0	315.0	310.0	331.7	33.3
13	380.0	390.0	325.0	365.0	35.0
15	525.0	490.0	505.0	506.7	17.6
18	665.0	615.0	590.0	623.3	38.2
21	890.0	805.0	815.0	836.7	46.5

YL2	Std PDA				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	4.0	14.5	6.0	8.2	5.6
4	57.0	85.5	44.0	62.2	21.2
6	115.0	150.0	78.5	114.5	35.8
9	175.0	220.0	150.0	181.7	35.5
11	215.0	215.0	160.0	196.7	31.8
13	285.0	325.0	410.0	340.0	63.8
15	325.0	340.0	450.0	371.7	68.3
18	365.0	490.0	550.0	468.3	94.4
21	395.0	490.0	650.0	511.7	128.9

YL2	I(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	13.5	17.0	14.0	14.8	1.9
4	93.5	95.5	95.0	94.7	1.0
6	120.0	115.0	120.0	118.3	2.9
9	210.0	195.0	190.0	198.3	10.4
11	235.0	255.0	250.0	246.7	10.4
13	255.0	235.0	260.0	250.0	13.2
15	325.0	305.0	295.0	308.3	15.3
18	365.0	415.0	365.0	381.7	28.9
21	425.0	540.0	440.0	468.3	62.5

YL2	I(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	15.0	7.0	5.0	9.0	5.3
4	72.5	69.5	66.5	69.5	3.0
6	95.0	78.5	97.5	90.3	10.3
9	155.0	215.0	125.0	165.0	45.8
11	165.0	250.0	160.0	191.7	50.6
13	195.0	315.0	170.0	226.7	77.5
15	215.0	375.0	175.0	255.0	105.8
18	270.0	375.0	225.0	290.0	77.0
21	320.0	415.0	275.0	336.7	71.5

YL2	I(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	5.0	4.5	7.5	5.7	1.6
4	58.0	66.5	79.5	68.0	10.8
6	78.5	95.0	82.0	85.2	8.7
9	155.0	165.0	155.0	158.3	5.8
11	205.0	215.0	200.0	206.7	7.6
13	175.0	220.0	200.0	198.3	22.5
15	240.0	260.0	205.0	235.0	27.8
18	280.0	280.0	255.0	271.7	14.4
21	245.0	330.0	300.0	291.7	43.1

YL2	II(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	5.5	7.0	11.0	7.8	2.8
4	41.5	59.5	48.5	49.8	9.1
6	67.5	95.0	82.5	81.7	13.8
9	190.0	225.0	325.0	246.7	70.1
11	350.0	280.0	625.0	418.3	182.4
13	650.0	350.0	915.0	638.3	282.7
15	800.0	415.0	1275.0	830.0	430.8
18	1690.0	660.0	1725.0	1358.3	605.0
21	2000.0	1075.0	2250.0	1775.0	619.0

YL2	II(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	11.0	14.5	11.0	12.2	2.0
4	69.5	55.0	52.0	58.8	9.4
6	82.5	97.5	78.5	86.2	10.0
9	185.0	200.0	220.0	201.7	17.6
11	235.0	270.0	365.0	290.0	67.3
13	265.0	325.0	535.0	375.0	141.8
15	340.0	350.0	675.0	455.0	190.6
18	430.0	450.0	1000.0	626.7	323.5
21	800.0	925.0	1415.0	1046.7	325.1

YL2	II(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	3.0	3.0	7.0	4.3	2.3
4	35.0	30.0	34.0	33.0	2.6
6	44.0	42.5	46.5	44.3	2.0
9	110.0	95.0	110.0	105.0	8.7
11	100.0	110.0	120.0	110.0	10.0
13	130.0	125.0	150.0	135.0	13.2
15	130.0	165.0	165.0	153.3	20.2
18	175.0	195.0	190.0	186.7	10.4
21	245.0	275.0	215.0	245.0	30.0

YL2	III(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	3.0	2.0	1.5	2.2	0.8
4	30.0	27.5	30.5	29.3	1.6
6	75.5	75.5	77.0	76.0	0.9
9	135.0	115.0	115.0	121.7	11.5
11	175.0	155.0	155.0	161.7	11.5
13	255.0	260.0	200.0	238.3	33.3
15	315.0	320.0	285.0	306.7	18.9
18	415.0	450.0	305.0	390.0	75.7
21	540.0	560.0	450.0	516.7	58.6

YL2	III(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	3.5	6.5	6.0	5.3	1.6
4	55.5	52.5	48.5	52.2	3.5
6	78.5	91.5	80.0	83.3	7.1
9	175.0	195.0	205.0	191.7	15.3
11	245.0	200.0	255.0	233.3	29.3
13	315.0	310.0	350.0	325.0	21.8
15	315.0	315.0	440.0	356.7	72.2
18	380.0	350.0	550.0	426.7	107.9
21	525.0	475.0	925.0	641.7	246.6

YL2	III(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	2.5	5.0	5.5	4.3	1.6
4	34.5	39.0	65.5	46.3	16.8
6	75.5	62.5	72.5	70.2	6.8
9	170.0	200.0	190.0	186.7	15.3
11	190.0	225.0	260.0	225.0	35.0
13	205.0	225.0	255.0	228.3	25.2
15	240.0	255.0	315.0	270.0	39.7
18	285.0	315.0	400.0	333.3	59.7
21	415.0	450.0	670.0	511.7	138.2

YL4	Std PDA				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	9.0	12.0	13.5	11.5	2.3
4	98.5	135.0	95.0	109.5	22.2
6	305.0	195.0	315.0	271.7	66.6
9	625.0	515.0	715.0	618.3	100.2
11	855.0	705.0	910.0	823.3	106.1
13	1075.0	960.0	1260.0	1098.3	151.4
15	1390.0	1255.0	1660.0	1435.0	206.2
18	1810.0	1520.0	2290.0	1873.3	388.9
21	2465.0	2045.0	2825.0	2445.0	390.4

YL4	I(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	19.0	20.0	21.5	20.2	1.3
4	125.0	110.0	125.0	120.0	8.7
6	215.0	225.0	200.0	213.3	12.6
9	705.0	705.0	705.0	705.0	0.0
11	910.0	910.0	910.0	910.0	0.0
13	1260.0	1260.0	1195.0	1238.3	37.5
15	1590.0	1590.0	1520.0	1566.7	40.4
18	2290.0	2125.0	2205.0	2206.7	82.5
21	3210.0	3215.0	3220.0	3215.0	5.0

YL4	I(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	18.0	23.0	21.5	20.8	2.6
4	170.0	185.0	180.0	178.3	7.6
6	275.0	295.0	285.0	285.0	10.0
9	910.0	910.0	910.0	910.0	0.0
11	1520.0	1255.0	1385.0	1386.7	132.5
13	1995.0	1590.0	1735.0	1773.3	205.2
15	2645.0	2210.0	2290.0	2381.7	231.5
18	2640.0	2825.0	3020.0	2828.3	190.0
21	3850.0	3420.0	3420.0	3563.3	248.3

YL4	I(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	25.0	24.0	24.0	24.3	0.6
4	175.0	185.0	150.0	170.0	18.0
6	255.0	290.0	195.0	246.7	48.0
9	805.0	805.0	855.0	821.7	28.9
11	1020.0	1075.0	1135.0	1076.7	57.5
13	1590.0	1570.0	1575.0	1578.3	10.4
15	2125.0	1810.0	1960.0	1965.0	157.6
18	2825.0	2660.0	1810.0	2431.7	544.7
21	3630.0	3420.0	3525.0	3525.0	105.0

YL4	II(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	85.0	88.0	65.0	79.3	12.5
4	125.0	155.0	120.0	133.3	18.9
6	195.0	210.0	220.0	208.3	12.6
9	375.0	300.0	255.0	310.0	60.6
11	460.0	560.0	475.0	498.3	53.9
13	550.0	640.0	580.0	590.0	45.8
15	590.0	750.0	690.0	676.7	80.8
18	710.0	940.0	790.0	813.3	116.8
21	1175.0	1385.0	1050.0	1203.3	169.3

YL4	II(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	52.0	72.0	70.0	64.7	11.0
4	170.0	175.0	180.0	175.0	5.0
6	210.0	240.0	220.0	223.3	15.3
9	590.0	550.0	575.0	571.7	20.2
11	805.0	790.0	830.0	808.3	20.2
13	855.0	925.0	960.0	913.3	53.5
15	1165.0	1380.0	1135.0	1226.7	133.6
18	1520.0	1660.0	1150.0	1443.3	263.5
21	1965.0	2045.0	1450.0	1820.0	322.9

YL4	II(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	210.0	215.0	185.0	203.3	16.1
4	580.0	565.0	520.0	555.0	31.2
6	630.0	660.0	595.0	628.3	32.5
9	1450.0	1255.0	1085.0	1263.3	182.6
11	1590.0	1440.0	1195.0	1408.3	199.4
13	1850.0	1600.0	1265.0	1571.7	293.5
15	2125.0	1665.0	1320.0	1703.3	403.9
18	2290.0	1965.0	1385.0	1880.0	458.4
21	2290.0	2375.0	1520.0	2061.7	471.0

YL4	III(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	51.0	63.0	78.0	64.0	13.5
4	150.0	165.0	265.0	193.3	62.5
6	225.0	255.0	300.0	260.0	37.7
9	500.0	505.0	625.0	543.3	70.8
11	705.0	690.0	840.0	745.0	82.6
13	880.0	870.0	1075.0	941.7	115.6
15	1255.0	1105.0	1375.0	1245.0	135.3
18	1660.0	1590.0	1375.0	1541.7	148.5
21	2290.0	2205.0	2465.0	2320.0	132.6

YL4	III(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	43.0	60.0	55.0	52.7	8.7
4	290.0	280.0	310.0	293.3	15.3
6	410.0	360.0	475.0	415.0	57.7
9	1075.0	910.0	1165.0	1050.0	129.3
11	1385.0	1025.0	1400.0	1270.0	212.3
13	1265.0	1650.0	1645.0	1520.0	220.9
15	1590.0	1700.0	1660.0	1650.0	55.7
18	2125.0	1885.0	2125.0	2045.0	138.6
21	2640.0	2825.0	2825.0	2763.3	106.8

YL4	III(9)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	195.0	175.0	140.0	170.0	27.8
4	1075.0	980.0	835.0	963.3	120.9
6	1275.0	1520.0	1065.0	1286.7	227.7
9	1810.0	2125.0	2205.0	2046.7	208.8
11	2525.0	2375.0	2550.0	2483.3	94.6
13	2840.0	2830.0	3020.0	2896.7	106.9
15	3025.0	3120.0	3270.0	3138.3	123.5
18	3220.0	3220.0	3420.0	3286.7	115.5
21	3320.0	3320.0	3420.0	3353.3	57.7

YL4	IV(5)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	32.0	12.0	11.0	18.3	11.8
4	340.0	315.0	285.0	313.3	27.5
6	485.0	525.0	375.0	461.7	77.7
9	910.0	1075.0	855.0	946.7	114.5
11	1210.0	855.0	1020.0	1028.3	177.6
13	1560.0	1805.0	1265.0	1543.3	270.4
15	2045.0	2290.0	1735.0	2023.3	278.1
18	2375.0	1810.0	2290.0	2158.3	304.6
21	3020.0	2550.0	2825.0	2798.3	236.1

YL4	IV(7)				
Day	a	b	c	mean	std dev
0	0.0	0.0	0.0	0.0	0.0
2	28.0	38.0	42.0	36.0	7.2
4	165.0	160.0	170.0	165.0	5.0
6	260.0	255.0	240.0	251.7	10.4
9	530.0	505.0	530.0	521.7	14.4
11	660.0	705.0	575.0	646.7	66.0
13	965.0	1020.0	855.0	946.7	84.0
15	1350.0	1485.0	1135.0	1323.3	176.5
18	1810.0	1660.0	1255.0	1575.0	287.1
21	2375.0	2375.0	1590.0	2113.3	453.2