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Full Length Research Paper

Characterisation of *Saccharomyces cerevisiae* hybrids selected for low volatile acidity formation and the production of aromatic Sauvignon blanc wine

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Wine yeasts (*Saccharomyces cerevisiae*) vary in their ability to develop the full aroma potential of Sauvignon blanc wine due to an inability to release volatile thiols. Subsequently, the use of 'thiol-releasing' wine yeasts (TRWY) has increased in popularity. However, anecdotal evidence suggests that some commercially available TRWY intermittently exhibit undesirable characteristics for example, volatile acidity (VA) formation. Therefore, a trial was undertaken to select and evaluate *S. cerevisiae* hybrids for the production of Sauvignon blanc wine with enhanced fruity and tropical aromas, but low VA. Hybrids were characterised by clamped homogeneous electrical field (CHEF) DNA karyotyping and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) biotyping, and subsequently trialled against top commercial TRWY that is, Zymaflore VL3 and Zymaflore X5 (Laffort Oenologie), and Fermicru 4F9 (DSM Oenology) in laboratory-scale Sauvignon blanc vinifications during 2013. Most hybrids produced wines with VA levels significantly lower than those produced with Zymaflore VL3, Zymaflore X5 and Fermicru 4F9. Low VA forming hybrids also produced wines with tropical wine aroma notes. Wines produced by Fermicru 4F9 had the lowest acetic acid (the main volatile acid) of the commercial TRWY in this study. However, some hybrid yeasts produced wines with less acetic acid on average than wines produced by Fermicru 4F9. Overall, hybrids NH 6, NH 48, NH 56, NH 88 and NH 145 produced wines with enhanced tropical fruity aroma, but lower VA compared to wines produced by commercial TRWY.

Key words: Hybrid yeasts, CHEF, MALDI-TOF/TOF MS biotyping, Sauvignon blanc, tropical fruit aroma, volatile acidity.

INTRODUCTION

Wine aroma is comprised of compounds emanating directly from the grapes, compounds produced by the yeast such as esters and higher alcohols, and yeast mediated compounds for example, volatile thiols (King, 2010; Bovo et al., 2015). Wine yeasts (*Saccharomyces cerevisiae*) vary in their ability to develop the full aroma potential of Sauvignon blanc wine due to an inability to release volatile thiols (King et al., 2011). Retention of

these bound thiols implies that the full aroma potential of the wine is not realised, as the bound thiols can only be released by wine yeasts during fermentation (Swiegers et al., 2006; Holt et al., 2011). Subsequently, the use of 'thiol-releasing' *S. cerevisiae* commercial wine yeasts (TRWY) for the production of aromatic Sauvignon blanc wine has increased in popularity (Swiegers et al., 2009). These yeast strains can release 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) from the respective cysteine-bound precursors. Other yeast strains can convert the aromatic 3MH (passion fruit aroma) to 3MHA (tropical and citrus aromas). However, anecdotal evidence suggests that some commercial TRWY intermittently produce undesirable high levels of volatile acidity (VA), which imparts vinegar-like nuances to the wines (Du Toit and Pretorius, 2000; Ugliano et al., 2007; Vilela-Moura et al., 2011). Acetic acid is the main contributor to VA in wine with odour detection levels ranging between 0.7 and 1.1 g/L (Byarugaba-Bazirake, 2008; Vilela-Moura et al., 2010). Even though, excessive levels of VA are mainly caused by lactic acid bacteria, acetic acid bacteria and wild yeasts, wine yeasts also contribute to VA, by producing acetic acid during alcoholic fermentation (Cordente et al., 2013; Luo et al., 2013). Other steam distillable acids, that is lactic, formic, butyric, and propionic acids can also contribute to VA (Erasmus et al., 2004; Moss, 2015). Currently, in South Africa the legal limit of VA permissible in wine is 1.2 g/L (OIV, 2010; Sirén et al., 2015). However, the sensory threshold of VA is generally accepted to be 0.8 g/l (Du Toit, 2000).

Reduction of yeast derived VA formation can be done by using genetically modified (GM) yeasts (Swiegers et al., 2007) or improved *S. cerevisiae* hybrid yeasts bred through classical mating (Pérez-Torrado et al., 2015). Although genetic modification can address VA formation by wine yeasts, the use of genetically modified organisms (GMO) is illegal (Berrie, 2011). The Cape Winemakers Guild (CWG) and South African Wine Industry Council (SAWIC) is also largely against the use of GMO in wine production (CWG, 2015). Both CGW and SAWIC emphasises that the SA wine industry is too dependent on the highly sensitive European market for exports, which are largely against GM food products. Sauvignon blanc was chosen for this study because this cultivar was previously shown to produce grapes containing aroma-inactive, non-volatile, bound thiols (metabolites) that can only be released by the wine yeast *Saccharomyces cerevisiae* during fermentation (Von Mollendorf, 2013). Therefore, the aim of this study was to select and evaluate *S. cerevisiae* hybrids for the production of wine with enhanced fruity and tropical fruit aromas, but low VA.

MATERIALS AND METHODS

Wine yeast strains

One hundred and thirty-six hybrid strains (NH 1 to 10, 12, 13, 15 to 18, 20, 22 to 25, 27 to 78, 80 to 95, 97 to 104, 106 to 119, 121 to 145), four *S. cerevisiae* parental yeast strains (PS 1 to 4), three commercial TRWY references (Zymaflore VL3, Zymaflore X5 [Laffort Oenologie, France], and Fermicru 4F9 [DSM Oenology, Netherlands]) used in this study are conserved in the ARC Infruitec-Nietvoorbij micro-organism culture collection (ARC Inf-Nvbij CC). Hybrids were bred at the ARC Infruitec-Nietvoorbij microbiology laboratory through classical mating, as part of an ongoing hybrid breeding programme as described by Steensels et al. (2014) and Snoek et al. (2015).

Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping

DNA karyotyping of yeast strains was conducted according to the embedded agarose procedure described by Carle & Olson (1985), and Van der Westhuizen et al. (1992). The procedure was adapted by conducting chromosome separation in TBE (50 mM Tris, 41.3 mM boric acid, and 0.5 mM EDTA [Sigma-Aldrich, USA]) buffer at 14°C with pulse-times of 30 and 215 sec for 34 hours using clamped homogenous electric field (CHEF) gel electrophoresis (CHEF-DR II, Bio-Rad Laboratories, Richmond, USA). Yeast strain PS1 was run parallel to CHEF DNA size marker #1703605 (Bio-Rad, Madrid, Spain) as an internal standard to determine respective chromosomal band sizes. Chromosomal banding patterns were visualised on a Bio-Rad image analyser following staining with 0.01% (v/v) ethidium bromide. Subsequently, the genetic relatedness of the various yeast strains was determined by subjecting CHEF DNA karyotypes to cluster analysis using FP Quest software FP 4.5 software (Bio-Rad, Madrid, Spain). Cluster analysis was based on the Dice coefficient and an un-weighted pair group method with arithmetic mean (UPGMA), with 1% tolerance and 0.5% optimisation.

Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/TOF MS) biotyping

Yeast strains were also identified by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/TOF MS) biotyping as an alternative to CHEF DNA karyotyping. Formic acid protein extraction for subsequent MALDI-TOF biotyping was conducted as described by Pavlovic et al. (2013). One microliter of wine yeast protein extract was spotted onto a MTP 384 polished steel target plate as described by Moothoo-Padayachie et al. (2013) and Deak et al. (2015). Thereafter, the spotted target plate was inserted into a Bruker UltrafleXtreme MALDI-TOF/TOF MS (Bruker Daltonics, Bremen, Germany) apparatus. Generation of yeast protein mass spectra using MALDI-TOF/TOF MS was conducted according to the standard National Agricultural Proteomics Research and Services Unit method (obtainable from the National Agricultural Proteomics Research and Services Unit (NAPRSU), University of the Western Cape, South Africa). Mass spectra for all strains were acquired in triplicate.

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Laboratory-scale fermentation trials

Wet culture wine yeasts were evaluated in laboratory-scale fermentation trials as described by Rossouw et al. (2010) and Maarman et al. (2014). Frozen Sauvignon blanc grape must (total sugar = 21.9°B; total acidity = 9.3 g/L; pH = 3.28) was thawed and 250 ml aliquots were transferred into fermentation vessels (340 ml glass bottles). The yeast cultures were grown at 28°C for 48 h in 10 ml YPD (1% [w/v] yeast extract, 2% [w/v] peptone, and 2% [w/v] dextrose [Biolab, Merck]), and subsequently used to inoculate the Sauvignon blanc grape must at a concentration of 2% (v/v). Commercial TRWY Zymaflore VL3, Zymaflore X5 (Laffort Oenologie, France), and Fermicru 4F9 (DSM Oenology, Netherlands) were included in fermentation trials as references. Fermentation vessels were stoppered with a fermentation lock filled with water. Fermentations were conducted on an orbital shaker in an insulated temperature-controlled room, which were electronically regulated at 14.5°C, and monitored by CO_2 weight loss for 30 days. All fermentations were conducted in triplicate in a completely randomised block design (Addelman, 1970).

Fourier transform infra-red (FTIR) spectroscopy

Wines were subjected to residual glucose/fructose, ethanol, VA, titratable acidity (TA) and pH analyses using an Oenofoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) after fermentations stabilised.

Gas chromatography-mass spectrometry (GC-MS)

Wines with the most prominent fruity aromas as determined by the sensory panel were subjected to GC-MS analysis. Flavour compounds *viz*. esters, total fatty acids and higher alcohols were quantified by means of calibration mixtures of the applicable aroma compounds in conjunction with gas chromatography (GC) as described by van Jaarsveld et al. (2009), Zhang et al. (2012) and Vilanova et al. (2013).

Sensory evaluation

Wines were subjected to descriptive sensory evaluation by a panel of 14 experienced wine judges. Judges were requested to indicate aroma intensities on a unipolar six point numerical scale (absent [0], very low [1], low [2], medium [3], high [4] and very high [5]), and also to specify the most prominent aroma/s perceived that is, 'tropical fruit' for example banana, guava, peach, passion fruit and citrus; 'vegetative' for example, asparagus, herbaceous, green pepper, green beans, cut grass, green olive and gooseberry; or 'floral' for example rose, orange blossom etc. The wines were served as coded samples in international wine tasting glasses (approximately 50 ml) in a completely randomised order for each judge.

Statistical analyses

Chemical and sensory analyses data were subjected to principal component analysis (PCA) to determine the relationship between variables and treatments (yeasts) (Pearson, 1896; 1901; Zou et al., 2006). The data matrix consisted of four chemical variables that is VA, ethanol, total acidity and pH; and three sensory aroma descriptors that is, 'tropical fruit', 'vegetative' and 'floral'. Pearson's correlation was performed to study the linear relationship between the chemical and sensory variables. The Pearson's correlation matrix was used to standardise the data before performing the PCA. The PCA was performed using XLSTAT software (Addinsoft, 2013) with the principal components (PC's) as factors (that is, F1 and F2).

RESULTS AND DISCUSSION

Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping

Wine chemical and sensory quality was affected by the yeast strain used to carry out the alcoholic fermentation (Sharma et al., 2012; Usbeck et al., 2014). As a result, differentiation of yeast strains is essential to ensure that the correct yeast strain is used to inoculate grape must. Previous studies showed that PFGE/CHEF DNA karyotyping allowed for the delineation of closely related yeast strains (Sheehan et al., 1991; van Breda et al., 2013). Similarly, CHEF DNA karyotyping was useful in this investigation to differentiate closely related *S. cerevisiae* hybrid strains descending from mutual parental yeast strains (Figure 1). Distinctive variations in the DNA karyotypes between hybrids can be seen especially for the smaller chromosomes (bottom bands). Four pairs of hybrids that is, NH 33 and NH 34; NH 63 and NH 64; NH 75 and NH 76; and NH 86 and NH 89 had similar DNA karyotypes, whilst the remainder of yeast strains had distinguishable DNA karyotypes. Therefore, 139 CHEF DNA karyotyping profiles of the 143 strains were generated with genetic similarity ranging from 58 to 100%. The larger chromosomes (top bands) were common to most hybrids and parental yeast strains. It is evident that chromosomal DNA of the hybrids originated from more than one parental strain. It can be envisaged that some characteristics, including flavour compound (metabolite) release during fermentation, should be similar, different or enhanced compared to parental strains.

Cluster analysis of yeast CHEF DNA karyotypes allowed for the differentiation of yeast strains with common ancestry as described by Hoff (2012), Choi and Woo (2013) and Gallego et al. (2014). A dendogram comprising of sixteen clusters (I to XVI) was observed at a genetic similarity limit of 80% for all 143 strains (Figure 1). Four hybrids that is, NH 6, NH 67, NH 73 and NH 112 exhibiting the ability to produce wines with tropical fruit aroma (hereafter abbreviated as TFPH) clustered with the commercial TRWY reference Zymaflore X5, whilst another two TFPH and low VA producing hybrids (LVPH) that is, NH 56 and NH 57 clustered with the commercial TRWY references Fermicru 4F9 and Zymaflore VL3, respectively. Both hybrids also clustered with tropical fruit wine producing PS 1 at a 74% genetic similarity cut-off. Moreover, both hybrids clustered with the lower VA producing PS 2, PS 3 and PS 4 (Figure 1). Therefore, these hybrids exhibiting the sought-after tropical fruit aroma enhancing and low VA forming qualities,

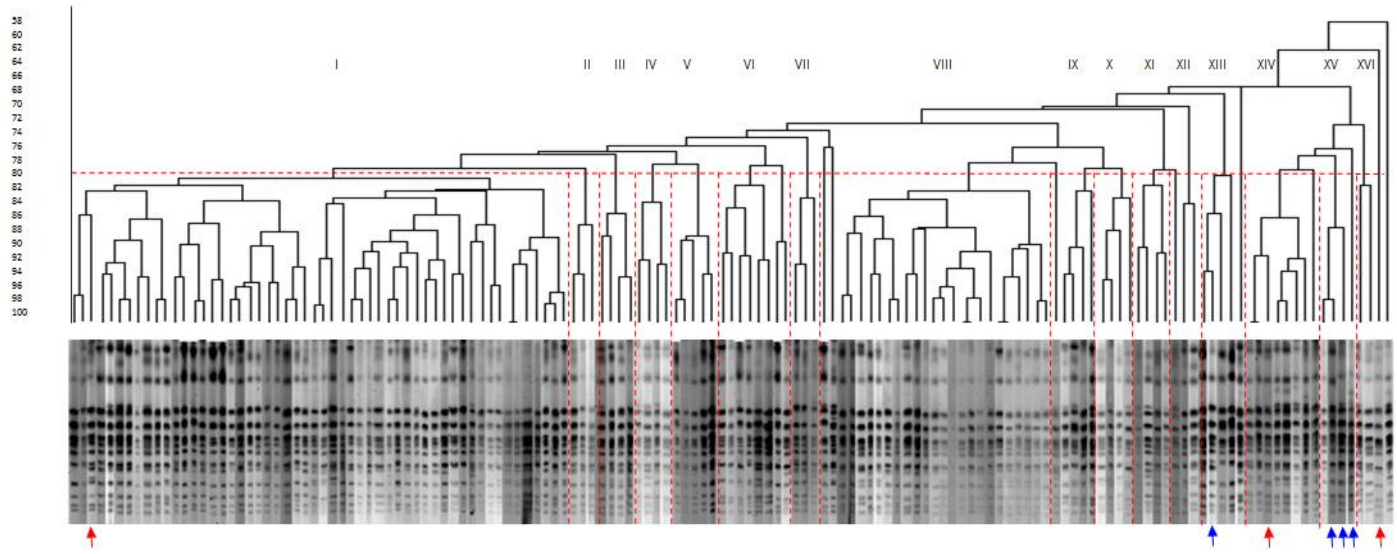

Figure 1. Dendogram showing the genetic similarity among three commercial 'thiol-releasing' wine yeasts (TRWY)(red arrows), four parental yeast (PS)(blue arrows) and 136 hybrid yeast (NH) strains. Cluster analyses was performed using a UPGMA algorithm. Yeast strains with 80% similarity (dotted line) were assigned to the same cluster indicated by Roman numerals. Dice (Opt:0.50%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%] **Cluster I:** NH 143, NH 132, ZYMAFLORE X5, NH12, NH 68, NH 66, NH 67, NH 125, NH 69, NH 24, NH 73, NH 10, NH 112, NH 107, NH 42, NH 113, NH 114, NH 133, NH 135, NH 134, NH 138, NH 35, NH 36, NH 117, NH 6, NH 9, NH 3, NH 47, NH 41, NH 15, NH 17, NH 54, NH 53, NH 94, NH 5, NH 7, NH 16, NH 52, NH 22, NH 25, NH 1, NH 106, NH 37, NH 13, NH 20, NH 91, NH 99, NH 33, NH 34, NH 32, NH 31, NH 4, NH 70, NH 30; **Cluster II:** NH 43, NH 55, NH 78; **Cluster III:** NH 130, NH 98, NH 48, NH 62; **Cluster IV:** NH 27, NH 81, NH 49, NH 97; **Cluster V:** NH 108, NH 127, NH 129, NH 136, NH 137; **Cluster VI:** NH 28, NH 50, NH 110, NH 29, NH 139, NH 142, NH 11, NH 18; **Cluster VII:** NH 39, NH 40, NH 38; **Cluster VIII:** NH 109, NH 2, NH 115, NH 116, NH 100, NH 45, NH 88, NH 23, NH 95, NH 44, NH 72, NH 77, NH 92, NH 93, NH 61, NH 75, NH 76, NH 74, NH 128, NH 86, NH 89, NH 83, NH 85, NH 87, NH 90; **Cluster IX:** NH 80, NH 101, NH 144, NH 71, NH 118; **Cluster X:** NH 123, NH 141, NH 103, NH 119; **Cluster XI:** NH 122, NH 8, NH 126, NH 82; **Cluster XII:** NH 121, NH 131, NH 84; **Cluster XIII:** NH 124, PS 2, NH 145, NH 140, NH 46; **Cluster XIV:** NH 63, NH 64, FERMICRU 4F9, NH 56, NH 59, NH 58, NH 60, NH 65; **Cluster XV:** NH 51, PS 1, PS 3, PS 4; **Cluster XVI:** NH 102, NH 104, ZYMAFLORE VL3, NH 57.

inherited it from the respective parental strains.

Yeast profiling with MALDI-TOF/TOF MS Biotyper

Biotyping using MALDI-TOF/TOF MS was successfully deployed to match ribosomal protein originating from commercial TRWY references, PS, and NH strains to that of a database described by Bizzini et al. (2010), Xiao et al. (2014) and Ghosh et al. (2015). All strains were identified as *Candida robusta,* the anamorph to *S. cerevisiae* (Diddens and Lodder, 1942; Kurtzman et al., 2011) following biotyping (mass spectra can be requested from the National Agricultural Proteomics Research and Services Unit (NAPRSU), University of the Western Cape, South Africa). Overall 79.72% of the strains were reliably identified as *Candida robusta* with scores of > 2 as described by Moothoo-Padayachie et al. (2013). Nonetheless no cut-off score for reliable MALDI-TOF/TOF MS biotyping was established, as all strains were shown by DNA karyotyping to be *S. cerevisiae*. Also noteworthy is that Cheng et al. (2013) showed that a lower cut-off score (1.7) sufficiently differentiate *Candida* yeast strains. Therefore, the lowest cut-off score (>1.8) for some strains used during this trial is acceptable.

A dendrogram consisting of nine clusters (I to IX) was generated following cluster analysis of the mass spectra at phylogenetic distance level of 0.80 indicated by dotted line (Figure 2). Hybrid strains were spread throughout the various mass spectral clusters. Some of the mass spectral clustering complemented DNA karyotype clustering, since TFPH, that is NH 56 and NH 57, clustered with the commercial TRWY reference Zymaflore VL3. Moreover, LVPH, that is NH 124; and NH 3, NH 88, NH 140, NH 13 and NH 81, were shown by MALDI-TOF/TOF MS biotyping (Bruker Daltonics, Bremen, Germany) to have a close phylogenetic relationship with the low VA producing PS 3 and PS 4, respectively (Figure 2). Also noteworthy is that TFPH and LVPH, that is NH 6, NH 132 and NH 134 was shown by biotyping to have a close phylogenetic relationship with parental strains that is PS 1 and PS 2, and PS 3, which was shown to produce wines with tropical fruit aroma (hereafter abbreviated to as TFPP). This provides more evidence supporting the notion that promising hybrids inherited desirable traits from the respective PS.

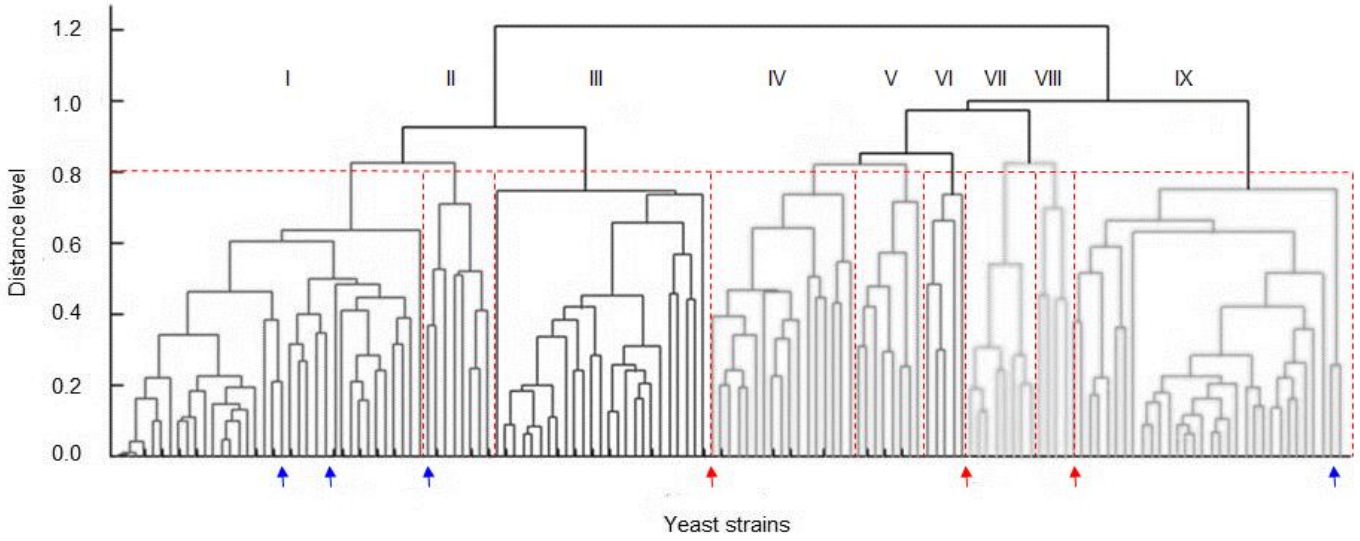

Figure 2. Principal component analysis (PCA) dendogram generated from matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF MS) biotyping spectra of three commercial 'thiol-releasing' wine yeasts (TRWY)(red arrows), four parental yeast (PS)(blue arrows) and 136 hybrid yeast (NH) strains, generated by cluster analysis using BIOTYPER software (Bruker Daltonics). Dendrogram based on identification score values and distance level is indicative of phylogenetic distance amongst yeast strains. Blue and red arrows indicate parental and commercial reference strains, respectively. Yeast strains were assigned to the same cluster at a 0.80 distance level (dotted line) indicated by Roman numerals. **Cluster I:** NH 104, NH 123, NH 24, NH 16, NH 47, NH 108, NH 90, NH 2, NH 9, NH 20, NH 23, NH 22, NH 44, NH 111, NH 75, NH 89, NH 145, NH 52, NH 114, NH 126, PS 1, NH 92, NH 144, NH 66, NH 107, PS 2, NH 6, NH 43, NH 82, NH 127, NH 72, NH 106, NH 29, NH 122, NH 115, NH 18 ; **Cluster II:** PS 3, NH 141, NH 103, NH 15, NH 124, NH 41, NH 93, NH 143; **Cluster III:** NH 37, NH 73, NH 86, NH 74, NH 31, NH 68, NH 132, NH 78, NH 125, NH 55, NH 87, NH 35, NH 38, NH 39, NH 134, NH 139, NH 137, NH 83, NH 36, NH 77, NH 131, NH 133, NH 46; **Cluster IV:** ZYMAFLORE VL3, NH 84, NH 91, NH 110, NH 33, NH 17, NH 56, NH 109, NH 57, NH 76, NH 61, NH 94, NH 135, NH 69, NH 112, NH 85; **Cluster V:** NH 7, NH 25, NH 48, NH 49, NH 102, NH 60, NH 65, NH 59; **Cluster VI:** NH 8, NH 50, NH 71, NH 42, NH 53; **Cluster VII:** FERMICRU 4F9, NH 40, NH 142, NH 54, NH 129, NH 51, NH 99, NH 100; **Cluster VIII:** NH 1, NH 10, NH 97, NH 101; **Cluster IX:** ZYMAFLORE X5, NH 30, NH 36, NH 140, NH 3, NH 13, NH 4, NH 5, NH 138, NH 80, NH 63, NH 32, NH 58, NH 67, NH 81, NH 98, NH 130, NH 116, NH 64, NH 45, NH 62, NH 88, NH 27, NH 28, NH 70, NH 95, NH 34, NH 118, NH 119, PS 4, NH 113.

Identification of microorganisms according to ribosomal protein spectra was reported by Gekenidis et al. (2014) and Oumeraci et al. (2015). In this study, distinctive ribosomal protein mass spectra of hybrid yeasts compared to parental strains were observed (Figure 3) (all data can be obtained from the National Agricultural Proteomics Research and Services Unit (NAPRSU), University of the Western Cape, South Africa). This study complemented research done by Bărbulescu et al. (2015), and shows that MALDI-TOF/TOF MS biotyping is a reliable yeast strain identification method that complemented CHEF DNA karyotyping. Biotyping proved to be a rapid identification method resulting in 143 mass spectra, whilst the laborious CHEF DNA karyotyping generated 139 karyotypes. However, CHEF DNA karyotyping still remains the cheaper option. Both techniques allowed for the delineation of genetically related hybrids.

Laboratory-scale fermentation trials

Most hybrids were able to ferment the grape must at a rate similar to commercial TRWY references and PS 1, PS 2, PS 3 and PS 4 (Figure 4). Most fermentations were shown to stabilise after 25 days following inoculation with the respective yeast strains. However, hybrids NH 36 and NH 34 fermented at rates noticeably different than the remaining strains included in this trial. Both hybrids produced wines with more vegetative aroma descriptors. Therefore, it can be tentatively surmised that fermentation rates nearby those of commercial TRWY references and TFPP are linked to production of wines with the sought-after fruity and tropical fruit aroma notes, since TFPH (for example, NH 56, NH 48, NH 88, NH 57, NH 3, NH 77, NH 124, NH 24, NH 29, NH 6) had similar rates to that of the commercial references and parental strains. This study complemented previous research which showed that faster fermentation rates improved the sensory quality of wines (Bell and Henschke, 2005). Also noteworthy is that, Shinohara et al. (1994) showed that hybrid yeast strains with similar fermentation rates as aromatic wine producing parental strains, was able to produced wines with aroma enhancing metabolites. Nonetheless, both NH 36 and NH 34 were shown to be LVPH and will be used in further breeding programs to

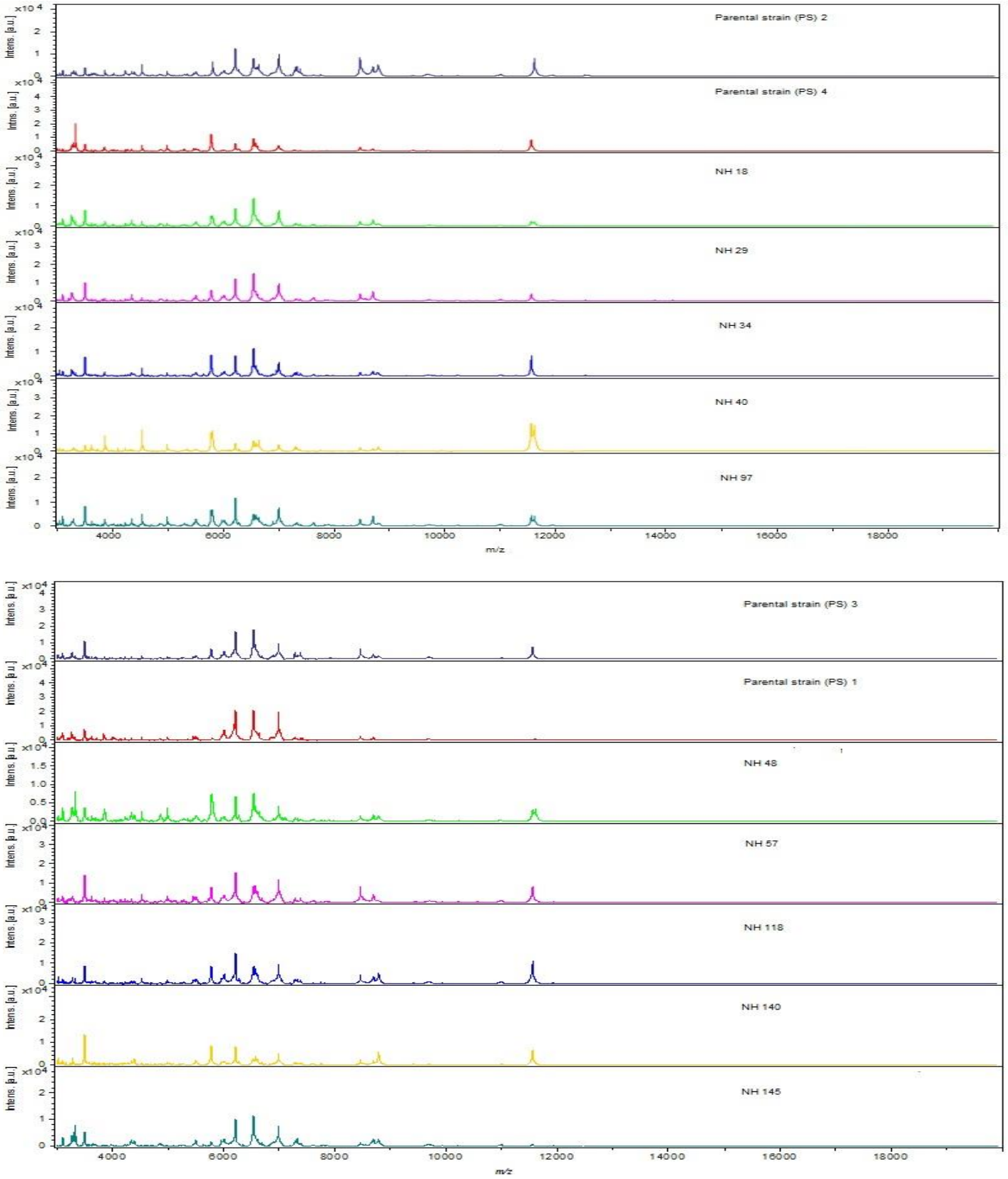

Figure 3. Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) spectral fingerprints of four parental strains (PS) and ten hybrid strains (NH). The absolute intensities of the ions and mass-to-charge (m/z) ratios are represented on the y- and x-axis, respectively.

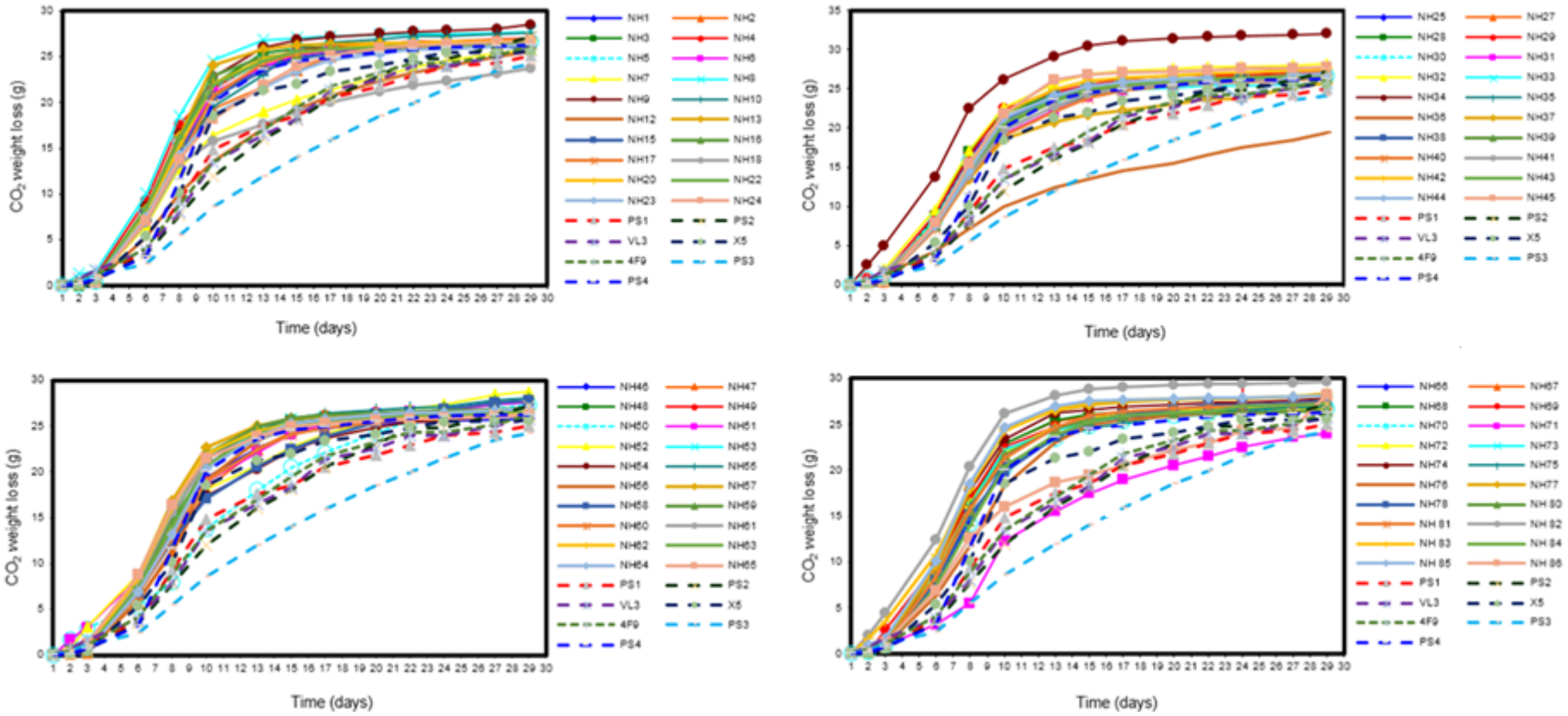

Figure 4. CO_2 weight loss of Sauvignon blanc grape must fermented at an ambient temperature of 14.5°C at the ARC Infruitec-Nietvoorbij microbiology laboratory using three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains.

improve progeny in this regard.

Fourier transform infra-red (FTIR) spectroscopy

Principle component analysis (PCA) biplot of FTIR spectroscopy generated data showed that promising hybrids, including NH 56, NH 48, NH 88, NH 57, NH 3, NH 77, NH 124, NH 24, NH 29, NH 6 situated in the left quadrants produced wines had a negative correlation with VA (Figure 5). The same observation was made with regard to PS 3, PS 2, PS 4 that was shown to be low VA producers (hereafter referred to as LVPP) and the commercial TRWY reference Fermicru 4F9. Overall, most hybrid strains produced wine with VA below 0.20 g/L (data not shown), whereas commercial TRWY references Zymaflore VL3 (0.31 ± 0.20 g/L) and Zymaflore X5 (0.50 ± 0.21 g/L) produced wines with significantly higher VA. These results support anecdotal evidence that some commercially available yeast strains can be implicated in VA formation. However, all commercial references produced wines with VA levels that comply with legislation. Strain PS 3 (0.02 ± 0.02 g/L) produced wines with the lowest

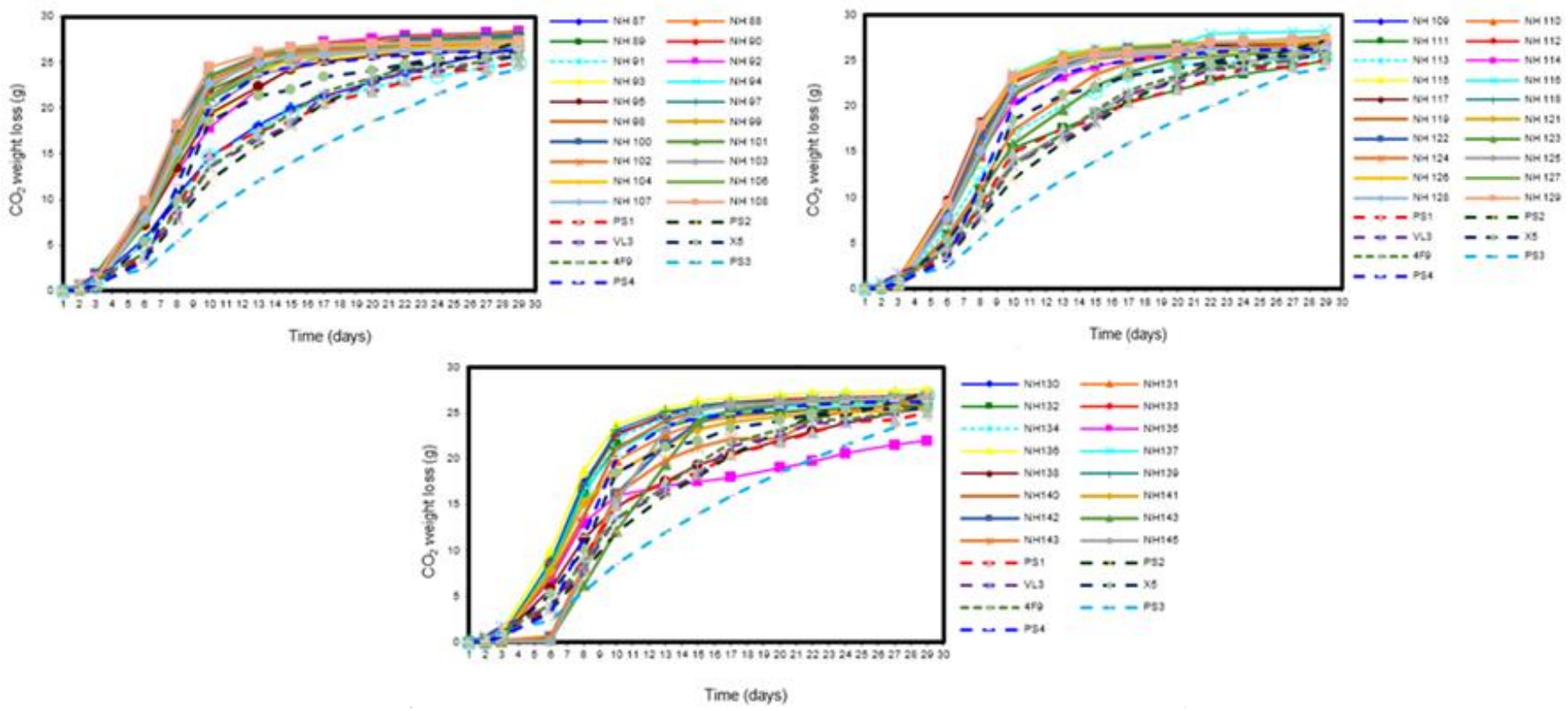

Figure 4. Contd.

VA of all the PS included in this study. Low VA forming hybrids must have inherited this trait from the respective PS that displayed this quality.

Most TFPH produced wines with a more positive association with pH compared to wines produced with commercial TRWY references that is, Fermicru 4F9, Zymaflore VL3 and Zymaflore X5 (Figure 5). However, all yeast strains included in this study on average produced wines with desired pH values (pH 3.3 ± 0.01) as described (Gauntner, 1997; Pambianchi, 2001). It was also observed that plenty of hybrids, including the TFPH already mentioned had a positive association with the titratable acidity (TA) that are closely related to pH, hence these wines were perceived to be fruitier, a wine aroma normally perceived within this pH range.

Also noteworthy is that, climate change together with a desire by wine producers to harvest grapes at optimal ripeness has led to grapes harvested with high sugar levels (Palliotti et al., 2014). Subsequently, these wines have undesirable high alcohol levels. Wine yeast strains suitable for the production of lower alcohol from grapes with higher sugar were identified as a global industry priority (Gardner et al., 2007; Contreras et al., 2014). Therefore, this study adds value to this priority, since promising LVPH (for example, NH 24, NH 73, NH 77, NH 124 and NH 145) also produced wines with lower alcohol levels

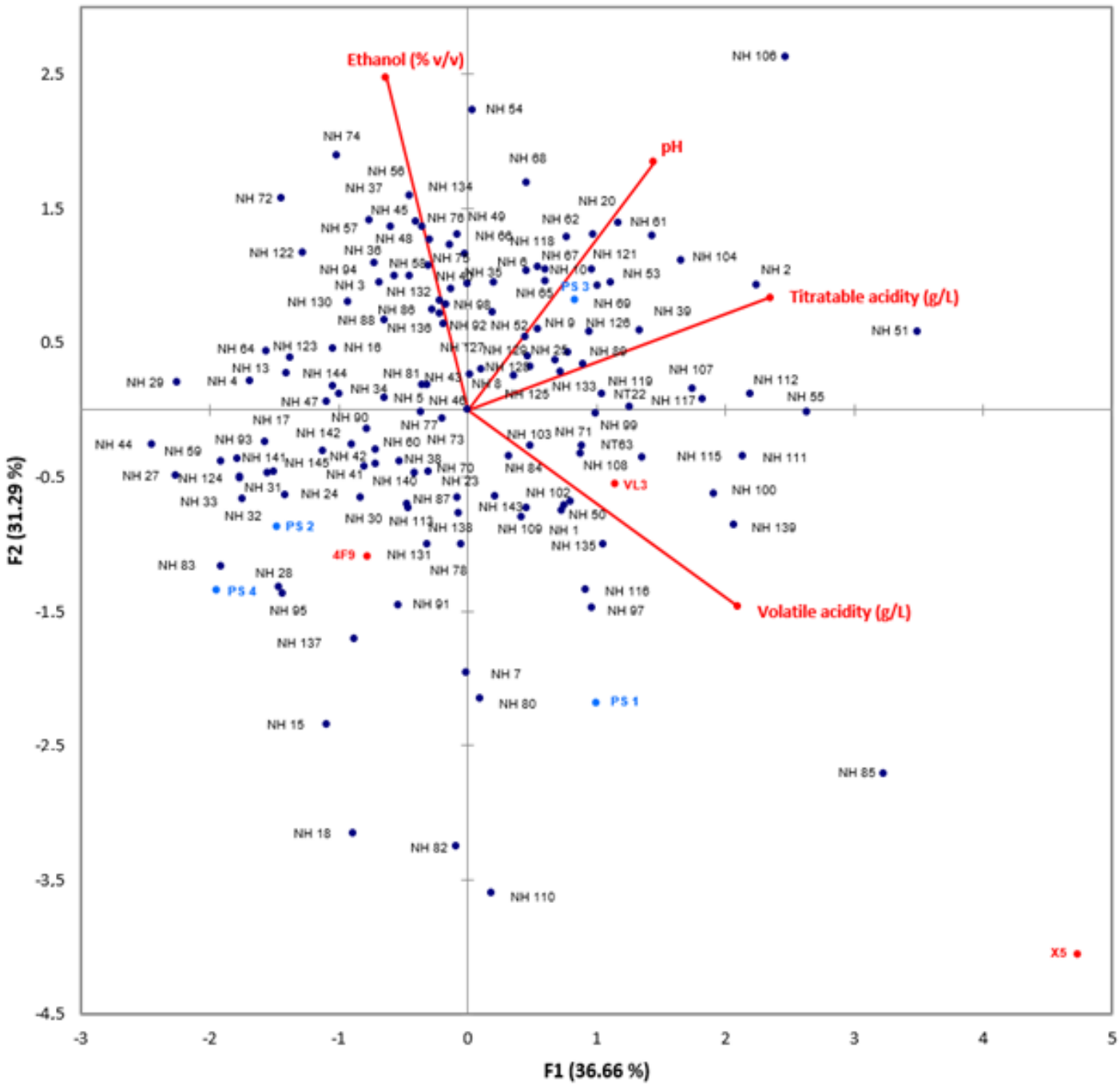

Figure 5. Biplot of basic chemical parameters of laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains. Average values of triplicate fermentations.

(negative association ethanol). It is envisioned that this observation will be investigated further as part of another study.

Sensory evaluation

The biplot of wine sensory data showed no distinct clusters, but rather a spread over the entire sensory space (Figure 6). Both commercial TRWY references Zymaflore VL3 and Zymaflore X5 produced wines with a positive association with tropical fruit aromas (Figure 6). Moreover, both TRWY were previously recommended for the production of aromatic white wines due to the yeast's 'thiol-releasing' abilities (Personal communication, 2010). The TRWY Fermicru 4F9 produced wines with relative

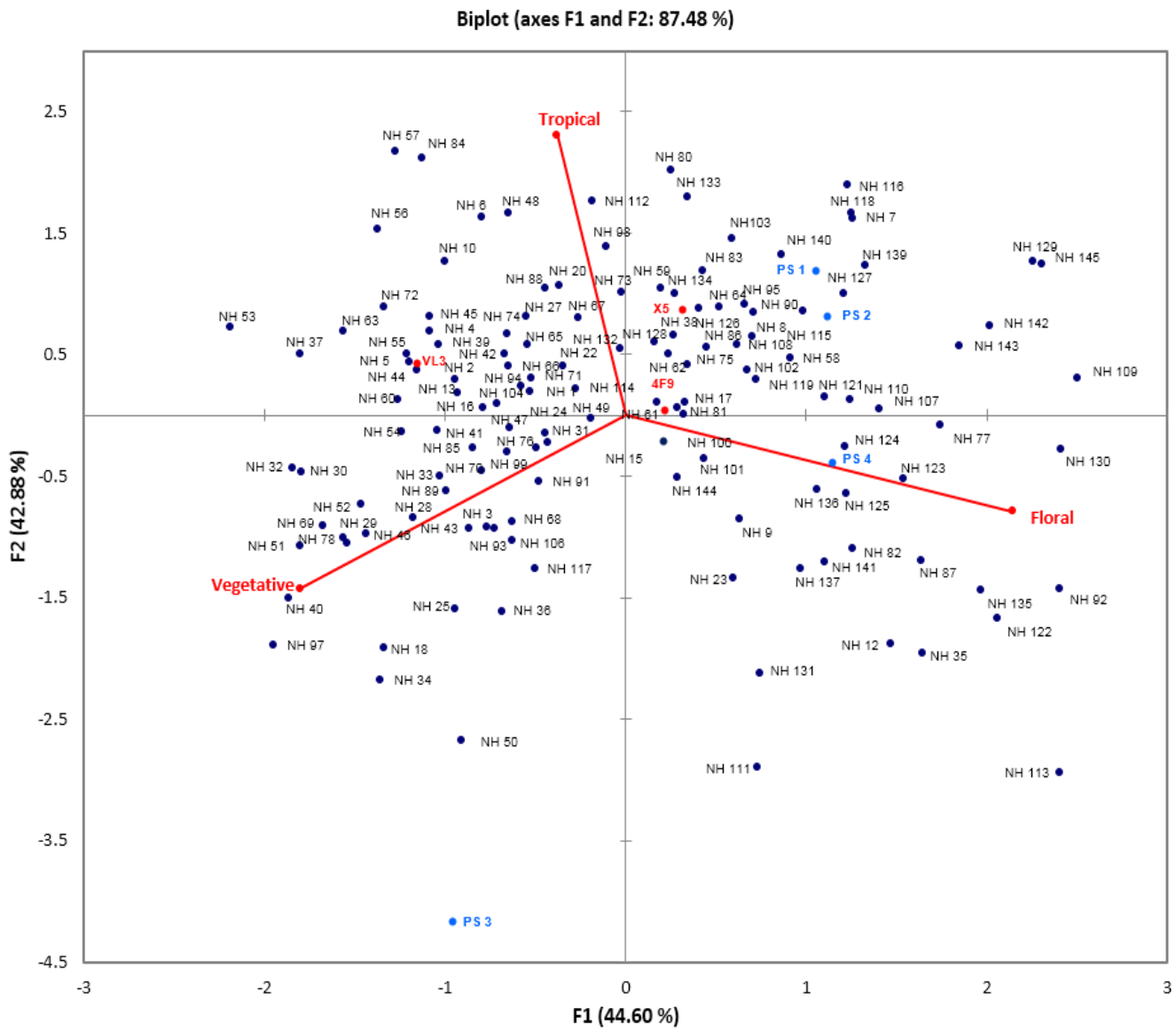

Figure 6. Biplot of descriptive sensory evaluation of laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains. Average values of triplicate fermentations.

less tropical fruit aroma than afore-mentioned TRWY, however the wines had a greater association with tropical aroma compared to wine produced with for example, PS 3. It is noteworthy that the Zymaflore VL3 produced wines had hints of vegetative aromas, whilst Fermicru 4F9 produced wine with a slight hint of floral aroma. It can tentatively be said that marginal vegetative aromas perceived in the Zymaflore VL3 produced wine is the result of the positive association with VA (Figure 5), whilst the hints of floral aroma perceived in the Fermicru 4F9 produced wines were due to overpowering tropical aroma. Therefore, higher VA levels observed in wines produced by Zymaflore X5 and Zymaflore VL3 were somehow masked by the overall positive aromas perceived. Nevertheless, commercial references produced wines with desired aroma notes and VA levels that complies with legislation.

Numerous hybrids, amongst others, NH 112, NH 98, NH 88, NH 84, NH 73, NH 67, NH 57, NH 56, NH 48 and NH 6 are considered TFPH, since they produced wines with enhanced tropical fruit aromas compared to commercial TRWY and TFPP. Some of these TFPH were similarly identified as LVPH (Figure 5). These hybrids, therefore, comply with both criteria put forward in the overall objective of this study. Wines with tropical fruit aroma and low VA levels are an industry priority, and the production thereof was previously achieved using co-inoculations and/or GMO (Swiegers et al., 2007).

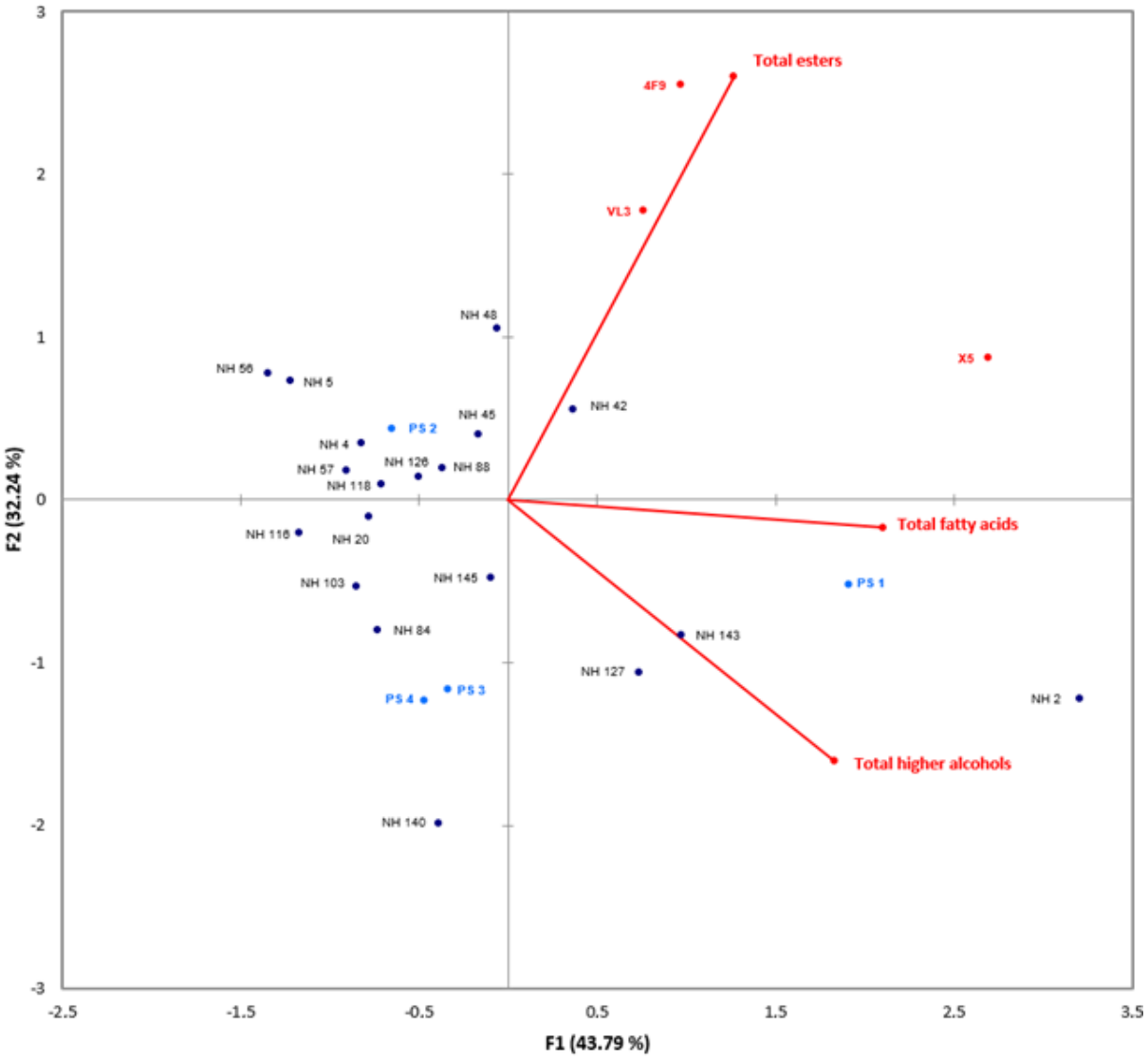

Figure 7. Biplot of aroma compounds in laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and selected hybrid yeast (NH) strains that produced wines with the fruitiest aroma. Average values of triplicate fermentations.

However, the use of GMO for wine production is currently illegal (Berrie, 2011). Therefore, it is envisioned that afore-mentioned TFPH and LVPH have a commercial role to play, since the fermentation potential of the parental strains were improved through natural occurring classical mating. Moreover, other hybrid strains (for example, NH 78, NH 46, NH 40, NH 34, NH 29, NH 28 and NH 18; and NH 136, NH 130, NH 124, NH 123, NH 92, NH 87, NH 82 and NH 77) that produced wines with pronounced vegetative and floral aromas, were also identified as LVPH. Two TFPP that is, PS 1 and PS 2 produced wines with tropical fruit and floral aromas, whilst the two LVPP that is, PS 3 and PS 4 produced wines with vegetative and floral aromas, respectively. In general, LVPH strains were evenly distributed on the sensory biplot, irrespective of wine sensory attributes.

Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry analyses were performed on wines with the most prominent fruity aromas according to the descriptive sensory evaluation to determine aroma compounds that is, esters, total fatty acids and higher alcohols (Lambrechts and Pretorius, 2000). The PCA biplot of GC-MS data showed that the commercial TRWY reference strains Zymaflore VL3 and Fermicru 4F9 produced wines with high ester levels (Figure 7). In contrast, Zymaflore X5 and PS 1 produced wines with a positive association with total acids,

amongst others, acetic acid. Three TFPH (for example, NH 56, NH 118, and NH 145) produced wines with a negative association with total fatty acids, and therefore comply with both criteria indicated in the aims. The commercial TRWY reference Fermicru 4F9 produced wines with the highest ester levels (5.58 ± 1.42 mg/L). However, NH 48 produced wines with ester levels (4.07 ± 0.17 mg/L) that were comparable to wines produced by Zymaflore VL3 (4.80 ± 0.94 mg/L) and Zymaflore X5 (4.02 ± 0.80 mg/L), respectively. It is noteworthy that aforesaid TFPH *viz.* NH 56 (48.74 ± 0.11 mg/L); NH 118 (63.75 ± 1.03 mg/L); and NH 145 (75.26 ± 2.43 mg/L) produced wines with less acetic acid, the main volatile acid than wines produced by Fermicru 4F9 (79.01 ± 1.23 mg/L). The latter produced wines with the lowest acetic acid of the all commercial references included in this study. Therefore, GC-MS complemented FTIR spectroscopy, since LVPH also produced wines with lower acetic acid.

Conclusion

Improved hybrid strains were identified compared to commercial TRWY references and TFPP (for example, PS 1 and PS 2) and LVPP (for example, PS 3 and PS 4) included in this study. These hybrids showed lower VA formation, whilst producing aromatic and/or typical Sauvignon blanc wines. Moreover, observations during this study indicate that some commercially available yeast strains can be associated with VA formation. However, VA formation is also dependant on vintage and generalisation should be avoided. This study showed that classical mating is still practical to produce novel yeast strain with desired traits, whilst maintaining the green image of wine production.

Conflict of interest

The authors have not declared any conflict of interest.

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Growth, ectomycorrhization and biochemical parameters of *Quercus suber* L. seedlings under drought conditions

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Drought conditions are the major constraint to the early establishment of *Quercus suber* species. However, drought responses of this species depend on provenances. The objective of this study was to obtain more comprehensive knowledge on the influence of drought conditions on the response of *Q. suber* L. seedlings originating from Algeria. Soil water status in soil (SWC) and relative water content (RWC) in leaves, morphological parameters for growth evaluation and physiological parameters, and ectomycorrhization were evaluated in two plots of seedlings: watered (W) and none watered (NW). Ten weeks of water starvation induced a reduction in RWC, ectomycorrhization rate and height and diameter growth of none watered seedlings. Under drought conditions, both soluble sugars and proteins were enhanced while polyphenols and %N decreased. The reduction of soil water content was negatively correlated with carbon isotope discrimination. The survival of the seedlings under these drought conditions was due to two strategies: osmotic adjustment through soluble sugars and proteins accumulations in leaves and an increase in carbon discrimination which enhances the water use efficiency (WUE).

Key words: *Quercus suber*, growth, drought, ectomycorrhization, pigments, sugars, starch, plyphenols, δ13C.

INTRODUCTION

Summer drought in the Mediterranean region is characterized by 4 months dry period with little or no precipitations, high temperatures and high irradiance, considering the major constraints for vegetation (Aussenac, 2000; Faria et al., 1999). Mediterranean species are thus, often exposed to water stress during summer (Pardos et al., 2005). Mediterranean oak species have developed mechanisms to avoid excessive loss of

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cell water and to maintain growth (Caritat et al., 2006). Increased drought severity is expected in the Mediterranean basin over the twenty-first century (Aussenac, 2002; Ramirez-Valentine, 2011) and the increase in the length of the dry season may lead to severe water deficit and tree mortality (Kurze-Besson et al., 2006). Priority should be given to ecophysiological research for the study of the adaptative plants Mediterranean behavior under these predicted summer conditions.

The cork oak (*Quercus suber*) is a western Mediterranean species, widely distributed in Mediterranean forest, thus presenting significant ecological and economic interests. It is an evergreen and sclerophyllous species growing from the sea level up to 700 m in altitude (Quezel and Médail, 2003). This plant is well adapted to summer conditions because it maintains a favorable ratio between water loss and uptake during the dry period. Also, *Q. suber* is considered as a drought tolerant species (Nardini et al., 1999; Nardini and Tyree, 1999). Maintaining a favorable water status in tissues, with a high relative water content (RWC) during summer drought, is ensured by deep roots and/or osmotic adjustment through accumulation of molecules such as proteins, sugars and proline (Kwak et al., 2011; Otieno et al., 2006; Pardos et al., 2005). A decrease in shoots and leaves biomass by reducing growth and root drop is also noted (Kurze-Besson et al., 2006; Ksontini et al., 1998).

Cork oak is an ectomycorrhizal species. Molecular and morphological approaches analysis led to the identification of fifty-five taxa of fungi forming wide diversity of ectmycorrhizas (Azul et al., 2010). Under drought conditions, controlled ectomycorrhization increases the performance of *Q. suber* L. (cork oak) nursery and field seedlings (Sebastiana et al., 2013) but little is known about the effect of drought on ectomycorrhization (Richard, 2011; Shahin, 2012).

The carbon isotope discrimination (δ13C) of foliage integrates signals resulting from environmental constraints like water availability (Warren and Adams, 2000). Therefore, δ13C may be a useful indicator of drought stress in seasonally dry climates (Warren and McGrath, 2001) because water supply affects the stomatal conductance and photosynthesis of plants, which changes 13/12C ratios in the synthesized carbohydrates (Du et al., 2015).

Although, *Q. suber* is well adapted to dry conditions, it remains that its natural regeneration is low. This seedlings recruitment limitation probably will be amplified with the predicted global changes in Mediterranean region. Muhamed et al. (2013) showed that spatial patterns of association between understory shrubs and oak seedlings are very sensitive to increasing drought under climate change, while it is known that plants neighbors can promote oak regeneration.

Many studies on *Q. suber* trees and seedlings behavior to water stress on the northern Mediterranean shore exist (Nardini and Tyree, 1999; Nardini et al., 1999; Otieno et al., 2006); however, few studies on the responses of this oak species to water stress were performed in Algeria (Acherar et al., 1992).

Understanding the responses of cork oak to actual and predicted summer conditions is essential to determine the future sustainability of cork oak woodlands. In this context, the effects of water scarcity on *Q. suber* were studied through growth, water relations, ectomycorrhization, photosynthetic pigments, sugars, starch, protein, polyphenols, δ13C and % N contents.

MATERIALS AND METHODS

The experimental setting

Acorns of cork oak (*Quercus suber*) were collected, in November 2010, from Beni Ghobri forest located in Azazga (Tizi-Ouzou, Algeria) (36°42’ to 36°47’ N; 4°22’ to 4°27’ W longitude, 620 m altitude). After one month stratification at 4°C, seeds germinated at 20°C. To limit the effect of acorn variation in the seedlings, the seedlings with similar shoot height were transplanted to plastic bags (30 x15 cm) filled with 1.5 kg of soil substrate. Soil substrate consisted of a mixture of natural soil from Beni Ghobri forest (2/3) as source of ectomycorrhizal inoculums and washed sand (1/3). The plants were grown in a greenhouse, localized at Tizi-Ouzou (140 m altitude, 36° 42' 12886" N and 4° 2' 53 3339" E) characterized by a Mediterranean climate, and were watered regularly (three fold a week) until mid-July. Then, two plots (30 seedlings and 3 replicates/ plot) of seedlings were constituted: watered (W) and none watered (NW). Ten weeks after end of September, seedlings of the two treatments (W, NW) were harvested for determination of morphological and physiological parameters.

Measurement of substrate moisture contents

Measurements of substrate moisture volumetric content were made for the plots. Soil samples of 1 g were taken from the plastic bags at a depth of 10 cm and dried at 105°C for 72 h and then water content was calculated using the formula: (FW- DW)/FW (Mathieu and Pieltain, 2003) where FW and DW are the fresh and dry weight, respectively.

Measurement of relative water content (RWC)

The RWC was measured on fully expanded leaves as described by Nardini et al. (1999). Five plants and three leaves per plants were examined. Fresh weight (FW) of the leaves was determined immediately after harvesting and then allowed to float on distilled water during 24 h at 4°C, then turgid weight (TW) of the leaves was determined. The dry weight (DW) of the leaves was determined after drying at 75°C during 72 h. The RWC were calculated as:

$$RWC = (FW - DW) / (TW - DW) \times 100$$

Morphological parameters

Morphological parameters were determined on ten seedlings per

treatment. Stem length and collar diameter were measured. Shoots, roots and leaves biomass were determined after drying at 75°C for 72 h. Then, the root to shoot ratio was estimated.

Physiological parameters

Chlorophylls, sugar, starch, proteins and total polyphenols contents were determined on five seedlings per treatment and 2 to 3 leaves/seedling.

Chlorophylls contents of fresh leaf discs (0.1 g) were determined spectrophotometrically after extraction in acetone 80% in the dark and the chlorophyll contents were calculated as proposed by Lichtenthaler and Buschmann (2001):

$$Cha+b= 7.15 \times A663 - 18.71 \times A647$$

Soluble sugars were extracted from fresh leaves in ethanol (70%), the residues was incubated in HCl (1. 1% v/v) for 30 min at 95°C for starch extraction. Then, soluble sugars and starch were quantified colorimetrically at 625 nm with anthrone reagent following Cerning-Berorard (1975) method.

Soluble proteins contents were determined following Bradford (1976) method. They were extracted from fresh leaves in distilled water and then quantified spectrophotmetrically at 595 nm after colorimetric reaction with Bioard reagent.

Total polyphenols were determined spectrophotometrically following the method described by Peñuelas et al. (1996). 2.5 g powder per sample of dry leaves were extracted with 20 ml of 70% aqueous methanol (v/v) acidified with some concentrated HCl drops. The samples were left at ambient temperature in the dark for an hour and a half. The extracts were filtered and the total polyphenols were quantified after colorimetric reaction using Folin-Ciocalteu reagent during one hour in dark at 765 nm. The total polyphenols content was calculated as gallic acid equivalent from the calibration curve of gallic standard solutions and expressed as mg gallic acid equivalent/g of dry weight.

Foliage samples for analysis of carbon isotopic discrimination δ13C, C% and N% were dried at 70°C for 72 h and ground to a fine powder. The abundance in combusted samples was performed using a mass spectrometer (Finnigan, Delta-S, Bremen, Germany) in CNRS UMR 7266 LIENSS with a precision of 0.1‰. δ13C (‰) was calculated with respect to the PDB Pee Dee Belemnite standard:

$$\delta^{13}C = (Rsample/Rstandard - 1)1000$$

where *Rsample* and *Rstandard* are the 13C/12C ratios in a sample and the standard (Pee Dee Belemnite), respectively (Warren and Adams, 2000).

Ectomycorrhizal colonization assessment

Percentage of ectomycorrhization was determined with a binocular and calculated as mycorrhizal root apex/total root apex (mycorrhizal and non mycorrhizal) of all the root systems (Parke et al., 1983).

Statistics analysis

Statistical analysis was performed using STATISTICA software (Version 7.1; StatSoft Inc.). The differences between the two lots (watered and none watered) for all recorded data were compared by the student test where the conditions of normality and equality of variances are checked. Otherwise, a Mann Whitney none parametric test was achieved. The significance level for all the tests was $P < 0.05$.

RESULTS

The seedlings of *Q. suber* were grown in irrigated and water starvation conditions for 10 weeks in greenhouse prior to morphological and physiological data measurements. At the end of the culture, significant changes were shown from the two lots in terms of substrate water contents (Figure 1a). The reduction of water availability was accompanied by a significant decrease in leaf relative water status (RWC), the RWC values were 85.43 and 70.78% in watered and none watered seedlings, respectively (Figure 1b).

The soil of Beni Ghobri forest was rich in ectomycorrhizal inoculum. Approximately eight month's cork oak seedlings were colonized by ectomycorrhizal fungi. The difference in ECM rate between the two treatments was significant. ECM decreased in drought conditions (<9%) while the well watered seedlings showed higher ECM (>39%) (Figure 1c). So, the ectomycorrhization was approximately 5-fold higher in well watered seedlings.

Length of the main stem was reduced in none irrigated lot (Figure 2a). The leaf biomass and root/shoot ratios did not differ significantly between the two batches studied (W and NW) (Figures 2b and c). The statistic analysis showed that the diameter of the stem at the base was significantly higher in the control watered plants as compared to the none watered ones (Figure 2d).

Majority of the physiological variables investigated were statistically different (Figures 3 and 4) between the two lots (W and NW). Total chlorophyll contents showed no significant difference between watered and non-watered seedlings (Figure 3a). Leaves protein content recorded in none watered samples were higher than in the well watered seedlings (Figure 3b). The levels of soluble sugars tend to increase with drought conditions. Especially, this drop was approximately 3-fold in none watered conditions as compared to the watered conditions (Figure 3c). The starch content showed a little increase in the none watered seedlings (1.026 fold higher) (Figure 3d).

On the contrary, total polyphenols synthesis and/or accumulation generally decrease. A significant decrease was observed (1.5-fold) in none watered seedlings as compared to the control individuals (Figure 3e). Non watering induced a significant increase of δ13 C contents in leaves; the δ13C were higher in none watered (-32.04) than in watered (-32.79) *Q. suber* seedlings (Figure 4a). The total nitrogen concentrations (N%) in leaves were different between the two treatments; N content was reduced in drought conditions (Figure 4b).

The C% in leaves were similar in watered conditions,

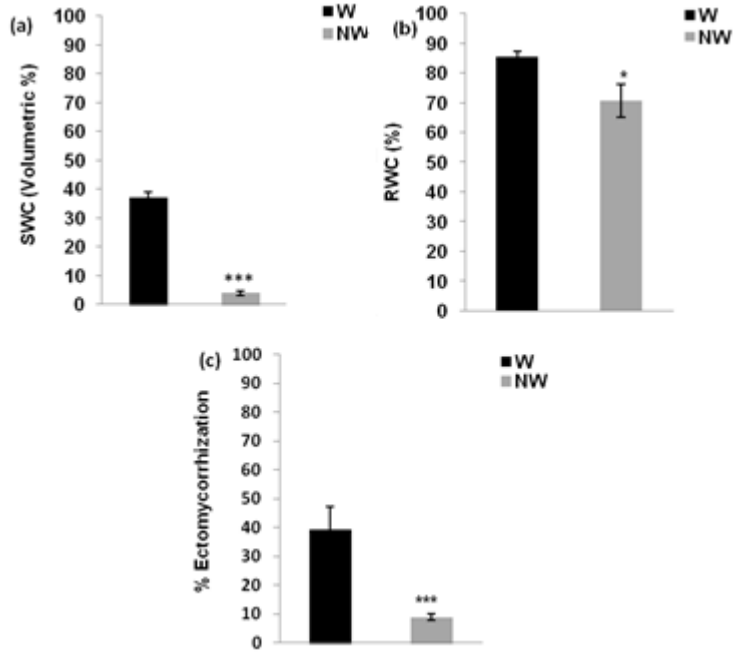

Figure 1. Effects of drought conditions on water status and ectomycorrhization of *Quercus suber* seedlings: (a) soil water content (SWC) and (b) leaf relative water content (RWC) (c) ectomycorrhization rate (ER). Means±SE. for RWC n=5, SWC n=10, and ER n=10. (*: $p<0.05$, ***: $p<0.001$).

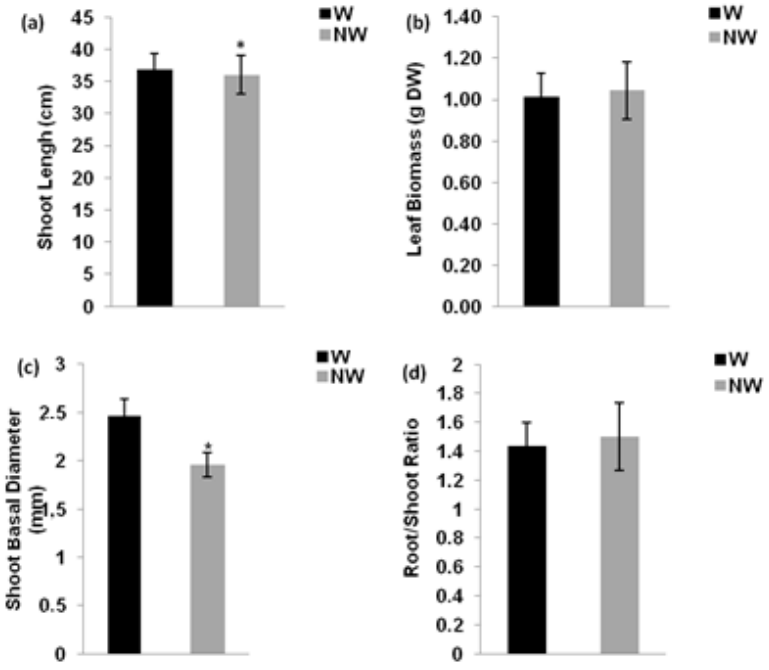

Figure 2. The effects of drought conditions on morphological traits of *Quercus suber* seedlings. (a) Shoot height, (b) Leaf biomass, (c) shoot basal diameter and (d) root/shoot ratio. Means±SE. for shoot height n=20; leaf biomass n=12; shoot basal diameter n=13 and shoot/root ratio n=10. (*: $p<0.05$).

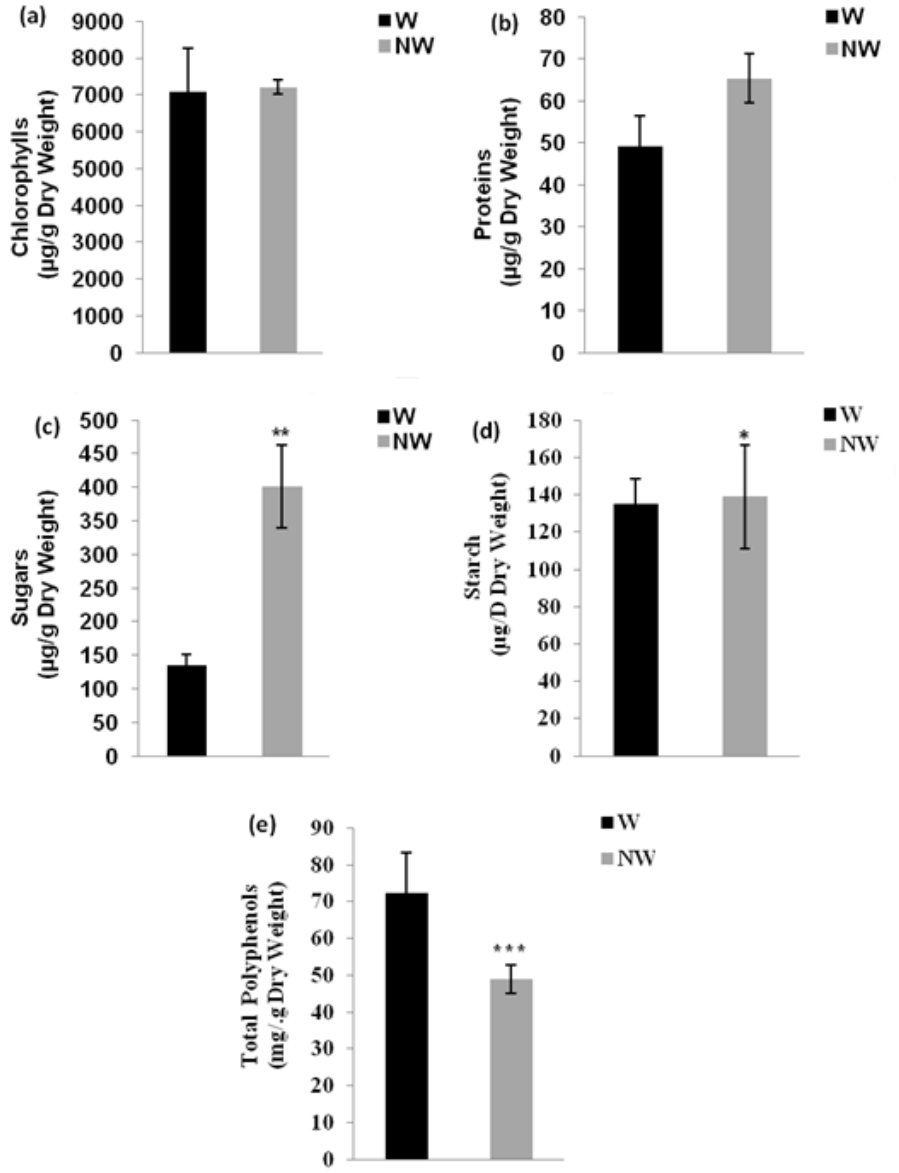

Figure 3. The effects of drought conditions on biochemical traits of *Quercus suber* seedlings. (a) Chlorophylls, (b) proteins, (c) sugars, (d) starch and (d) total polyphenols leaves contents. Means±SE. for all variables n=5. (*: $p<0.05$, ***: $p<0.001$).

thus, drought had no negative effect on this parameter (Figure 4c). The correlations between SWC and ectomycorhization, δ13C, N% and C% parameters were evaluated (Figure 5). The δ13C leaves contents showed a significant negative correlation with SWC; the reduction of SWC enhanced δ13C leaves contents ($r^2 = 0.5829$; $r = -0.7635$; $p = 0.0006$; $y = -31.918 – 0.0293*x$) (Figure 5a), while the total nitrogen (N%) and C% leaves concentration did not show correlation with the SWC ($p > 0.05$) (Figures 5b and c). A positive correlation exists between SWC and ECM rate ($r^2 = 0.3109$; $r = 0.5576$; $p = 0.0162$; $y = 8.245 + 0.7775*x$) and ECM decreases with SWC (Figure 5d).

DISCUSSION

When comparing the two treatments, soil water status in none watered pots was about 9.5-fold lower as compared to the watered substrates (Figure 1). This decrease was

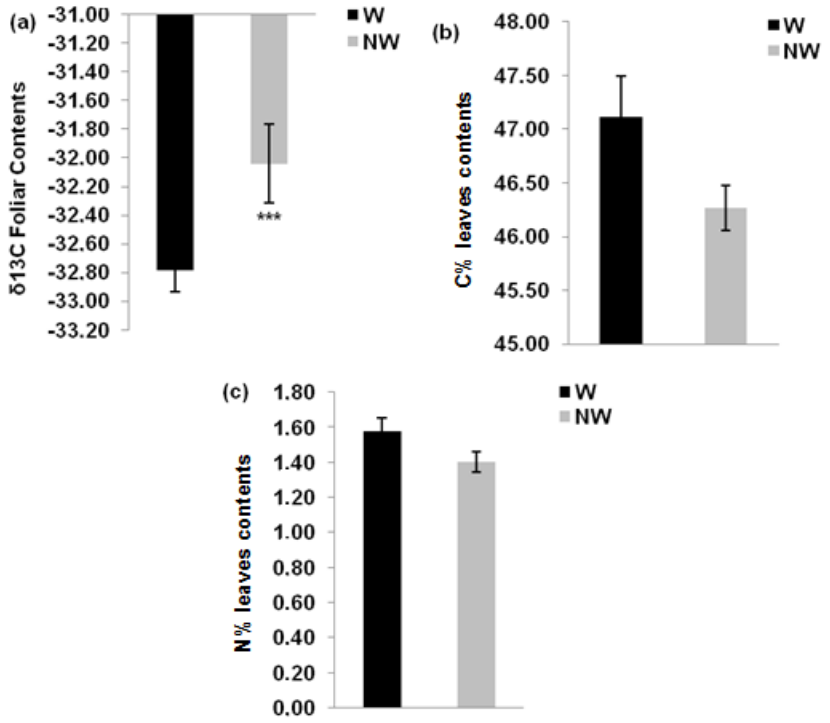

Figure 4. The effects of drought conditions on δ13C, C% and N% leaves contents of *Quercus suber* seedlings. (a) δ13C, (b) C% and N%. Means±SE. for all variables n=5 (***: p<0.001).

accompanied by a significant reduction in leaf relative water content (RWC) of the eight-month old seedlings (from 85.43 to 70.78%). In summer, the soil moisture volumetric content dropped to 5-7 % along the upper 0.6 m explored and leaf RWC decreased to nearly a constant level of about 82% that were only 8% less than those recorded in the spring (Nardini et al., 1999). The low values of RWC recorded in the current study may be due to more arid climatic conditions. Favorable tissue water status in *Q. suber* during summer drought was achieved through deep rooting which facilitate soil water uptake (Otieno et al., 2006).

The lower ECM colonization in *Q. suber* seedlings in drought conditions was previously obtained by Shahin (2012) in *Q. ilex* and *Q. pubescens* seedlings. The lower ECM colonization in dry conditions can be explained by the fact that photosynthates quantities are insufficient to both plant and fungal, thus they are preferentially allocated to plant growth or ectomycorrhizal inoculums was less abundant in none watered lot (Shahin et al., 2012). Some studies reported that seedlings inoculated with ectomycorrhizal fungi enhanced plant tolerance to water stress due to their less resistance to water flow from soil to roots, by increasing the absorbing surface and the ability of the fungus to penetrate finer pores as those operated by hairy roots (Parke et al., 1983).

Lengh and Diameter growth were reduced by drought conditions (Figure 2). While primary growth is sensitive to winter-spring warming, secondary growth is sensitive to summer-autumn warming (Camarero et al., 2015). Cambium activity is known to be negatively influenced by drought (Caritat et al., 2000). Interruption of shoot growth in the dry summer is a significant adaptation trait (Kurze-Besson et al., 2006) and thus, water stress tends to decrease the ratio of shoot biomass/root biomass (Ksontini et al., 1998).

Regarding the physiological parameters investigated, differences were shown (Figures 3 and 4). Drought generally causes decrease in photosynthetic pigments (Rajasekar and Manivannan, 2015). Decrease in chlorophyll contents was due to decrease in chlorophyll biosynthesis rather than its degradation (Jain et al., 2013). Vaz et al. (2010) showed no difference in *Q. suber* tree leaves from summer to autumn.

Osmotic adjustment is found to help cork oak seedlings to maintain turgor during moderate stress. To maintain water uptake, plants increased the water potential gradient between the plant cells and soil by increasing solute concentrations in the root cells. Like the present study results, in *Quercus* seedlings species, soluble sugars increased in drought-treated plants relative to control well watered plants and the opposite pattern was

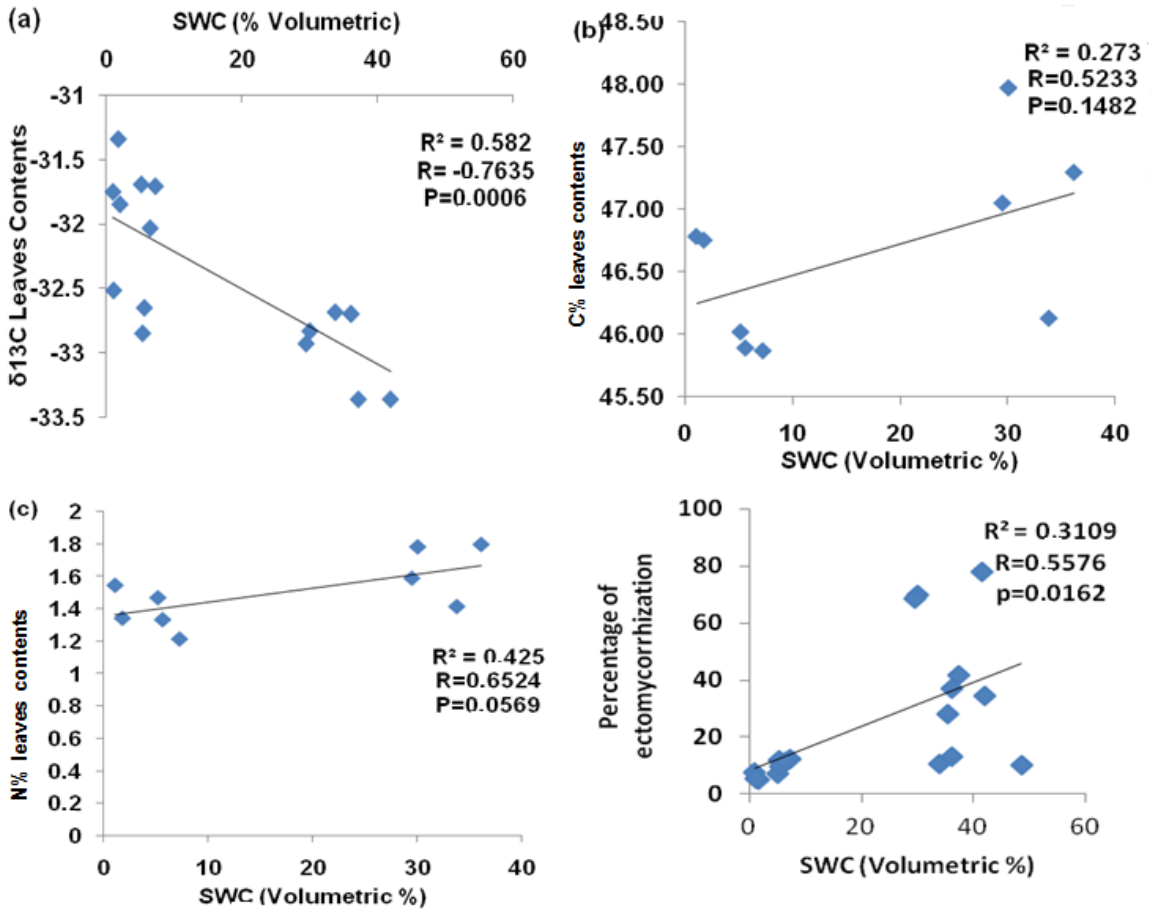

Figure 5. Correlations between SWC and δ13C, C%, N% and ectomycorrhization parameters of *Quercus suber* seedlings. (a) δ13C, (b) C%, (c) N% leaves contents and (d) percentage ectomycorrhization.

found in starch (Gonzalez-Rodríguez et al., 2011). Higher soluble sugars concentrations in cork oak seedlings grown under moderate water stress provoked a decrease of the osmotic potential and are at least partly responsible for osmotic adjustment which maintain RWC >80% (Pardos et al., 2005). Soluble proteins also contribute to osmotic adjustment (Kwak et al., 2011).

Polyphenols contents decreased in non-watered seedlings of *Q. suber*. Moderate experimental drought increased the concentrations of polyphenolic compounds with antioxidant function in the leaves of *Q. ilex* trees (Rivas-Ubach et al., 2014). Polyphenols are synthesized in large quantities during water stress, high temperatures or exposure to solar radiation, which are conditions that characterize the Mediterranean summer (Hernandez et al., 2009). Sometimes, the quantities were higher in watered plants like in loblolly pine needles (Booker and Maier, 2001).

Secondary metabolism in plants remains unclear; different regulation responses in the polyphenols pathway probably exist varying with species, development stage of the plant, intensity, duration and rate of progression of the stress (Liu et al., 2011). The increased carbon discrimination recorded in drought conditions was previously reported in *Q. suber* trees by Gouveia and Fortas (2009) which shows that trees subjected to greater water stress had δ13C enriched leaves, reflecting the trade-off between assimilation rate and water loss. The increase of carbon isotope discrimination with drought induces an increase in water-use efficiency (WUE) (Shahin et al., 2011).

These results showed that drought conditions reduced N% leaves contents while previous results have reported that leaf total nitrogen did not show a significant change in water stressed treatments (Kwak et al., 2011). In *Q. suber* trees, significant correlations were not found

between total N and rainfall (Gouveia and Fortas, 2009).

Conclusion

This study carried out in semi-controlled conditions, shows that *Q. suber* seedlings are affected by 10 weeks of summer drought. The survival of the seedlings under these conditions is due to two strategies: osmotic adjustment through soluble sugars and proteins accumulations in leaves and an increase in carbon discrimination which enhances the water use efficiency (WUE). Some responses (stability of chlorophylls contents and R/S ratios and decrease in polyphenols contents) obtained in this study with Algerian *Q. suber*, are contrary to other *Q. suber* provenances responses (Europe and Tunisia).

Further studies could involve the responses of different provenances to actual and future water stress; this will permit understanding the mechanisms of *Q. suber* tolerance to drought conditions. The success of reforestation in the perspective of Mediterranean climate aridity which is scheduled for the next decades needs provenances well adapted to these future conditions.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Coating with fungicide and different doses of fertilizer in vinhatico seeds

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Plathymenia reticulata **Benth occurs in open formations of the Brazilian Cerrado and Atlantic Forest. The seeds are affected by pathogens that reduce germination. The use of fungicides is defined for many species, but, for forest species, further studies are necessary. Thus, the aim of this study was to evaluate and identify the physical and physiological quality of mahogany seeds that are coated with fertilizer and fungicide. The treatments were: seed coating with sand + lime + fungicide with different doses of fertilizers. The seeds were evaluated in the laboratory and in a greenhouse. The experiment was conducted in a completely randomized design with four replications of 50 seeds. The treatment with 50 g of fertilizer had the highest weight out of 1000 seeds. There were no infested seedlings among the coated seeds. The coating maintained the quality of the seeds and the coating with high doses of fertilizer inhibited the germination, germination speed index (GSI), emergence and emergence speed index (ESI) of the mahogany seed.**

Key words: *Plathymenia reticulata*, germination, vigor, fungicide.

INTRODUCTION

Plathymenia reticulata Benth is popularly known as *vinhático-do-campo* or *vinhático-do-cerrado* (Brazilian mahogany). It is a forest species that has hardwood used in construction, aside from being used in the recovery of degraded areas (Lorenzi, 2008).

Studies related to the cultivation of native species have increased significantly due to the increased demand for forest deployment for various purposes, but there is still a lot to advance regarding the specificities of each botanical group in the field of seed technology or in the field of ideal nutrition for planting. According to Gonçalves et al. (2012), there are few studies on nutritional requirements of forest species, but knowledge of proper nutrition of each species plays an important role in plant development. As the understanding of the seed value and the need to protect or enhance their performance increases, it also increases the quantity of products available for the treatment of seeds, for various purposes, either for protection, with the use of fungicides and insecticides, or nutrition, with the use of nutrients, when

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the main objective is to enhance seed performance in the physiological and economic aspect.

According to Netto and Faiad (1995), one of the important aspects of forest seeds is the phytosanitary quality, because as well as causing deterioration of seeds, diseases reduce the population of seedlings, causing debilitation and epidemics. The use of fungicides for the treatment of seeds is well established for large commercial crops, but for forestry species further studies are still needed. The supply of nutrients through seeds is effective due to the fact that the required amount of nutrients and their distribution become critical in soils in which occur adverse conditions for their solubilisation, thereby affecting the growth of roots (Kirkby and Römheld, 2007).

In this context, experiments using nutrients and fungicides in seed coating are important because they take part in the development of seedlings. The objective of this study was to evaluate and identify the effect of the coating with fungicide and with fertilizer doses on the quality of Brazilian mahogany (vinhatico) seeds.

MATERIALS AND METHODS

The *Vinhático* seeds were purchased from the *Empresa Caiçara Comércio de Sementes (Rascal* Seed Trade Company). Initially, the wings, empty and malformed seeds, were removed. After this step, the selected seeds were mechanically scarified by means of 36 grit sandpaper to ensure complete soaking. For coating, the proportions between the filling materials and seeds were 3:1 (w/w). For each 50 g of seed, 150 g of filler material were used, and this material was divided into twelve portions of 12.5 g each. Every two filler portions formed a layer, totaling six layers. As adhesive solution, Polyvinyl acetate (PVA) glue diluted in water heated at the temperature of 70°C was used at a ratio of 1: 1 (v/v), according to Mendonça et al. (2007). Captan fungicide was used in the last layer. 0.25 g of fungicide + 12.25 g of limestone was added in the last two portions of 12.5 g of filler.

As filler material, sand, limestone, and increased doses of NPK 4-14-8 fertilizer + boron and zinc was used; and fungicide mixed with 10 g of boric acid + 10 g of zinc sulfate and 200 g of NPK 4-14-8 fertilizer was also used. The fertilizer was applied in the intermediate layers of the coating so as to prevent it to come into direct contact with the surface of the seed, while it was also protected by the outer layers. The arrangement of portions of fillers in the coating of the seeds was as follows:

T1: 6 portions of sand + 4 portions of limestone + 2 portions of limestone and fungicide; T2: 5 portions of sand + 2 portions of sand and fertilizer (3.125g of fertilizer + 9.375g of sand per layer) + 3 portions of limestone + 2 portions of limestone and fungicide (0.25g of fungicide + 12.25g of limestone per layer); T3: 5 portions of sand + 2 portions of sand and fertilizer (6.25g of fertilizer + 6.25g of sand per layer) 3 portions of limestone + 2 portions of limestone and fungicide; T4: 5 portions of sand + 2 portions of fertilizer + 3 portions of limestone + 2 portions of limestone and fungicide; T5: 4 portions of sand + 4 portions of fertilizer + 2 portions of limestone + 2 portions of limestone and fungicide; T6: uncoated seeds.

The coating process was carried out in a coater, model N-10, Newpack. The equipment has stainless steel chamber spinning at a speed of 40 rpm, spray at pressure of 4 bar and hot air at 40°C to dry the seeds. The seeds were placed inside the coater along with a portion of filler material. Then, the spray of adhesive solution was applied 3 times, with one-minute intervals between each application, and then another portion of filler material was put on the seeds, followed by another application of the adhesive solution. Next, the hot air blower was activated for 1 min. This process corresponded to a coating layer. After coating, the seeds were evaluated for physical and physiological characteristics: The procedures used for evaluation were as follows:

Water content (WC) and weight of thousand seeds (WTS)

Germination test (GT)

This test was performed with four repetitions of 50 seeds, sown in rolls of paper for germination, previously moistened with water equivalent to 2.5 times the mass of the paper. Then, the rolls were taken to the germinator (BOD) at a constant temperature of 25°C, light/dark for 8/16 h. The evaluations were performed on days 10 and 16, computing the number of normal, abnormal seedlings, infested and non-germinated seeds, according to the criteria set in the Rule for Seed Analysis (Brasil, 2013).

Germination speed index (GSI)

This was conducted with the germination test, and evaluations were carried out every two days from sowing to the end of test. The index was calculated based on the formula of Maguire (1962).

Emergence at house of vegetation (%E)

Four repetitions of 50 seeds were sown in perforated plastic trays containing washed sand, corresponding to each of the treatments. The trays were kept in a greenhouse and the counting of normal seedlings emerged was held on the 16th day after sowing.

Emergency speed index (ESI)

This was conducted along with the evaluation of seedling emergence in greenhouse, and the evaluations carried out every two days from sowing until the 16th day after sowing using the formula of Maguire (1962).

Shoot (SL) and root (RL) length

The length of shoot and root was measured after 60 days in the greenhouse, in four repetitions of ten washed and cut plants, separating shoot and root from the neck of the plant and measured by a millimetre ruler.

Fresh and dry mass of shoot and root

After the measurements of shoot and root length, they were weighed on an analytical scale to obtain the values of fresh mass and both parts were packed separately using paper bags and put to dry in forced-air oven at 60°C for 72 h and were then weighed on an analytical scale.

Statistical analysis

The experiments in the laboratory and greenhouse were conducted in a completely randomized design with six treatments and four repetitions of 50 seeds. The data were submitted to analysis of

Table 1. Weight of a thousand seeds (g) and Coating Increase (g) on uncoated vinhático seeds and seeds coated with fungicide and increasing fertilizer doses

Treatments	Thousand seed weight	Coating Increase
Control	5.34[e]	0
Without fertilizer	7.18[d]	1.84
6.25 g fertilizer	9.13[b]	3.79
12.5 g fertilizer	8.03[c]	2.69
25 g fertilizer	7.11[d]	1.77
50 g fertilizer	10.66[a]	5.32

Averages followed by the same letter do not differ among themselves by Duncan's test, at the 5% level probability.

variance and comparison of means was carried out by Duncan test at 5% probability, with the help of the ASSISTAT program (Silva and Azevedo, 2002).

RESULTS AND DISCUSSION

Laboratory

For the thousand seed weight (TSW), a significant increase was observed after the coating and after the increase in the TSW, in the order of 1.77 to 5.32 g in coated seeds, when compared with uncoated seeds (Table 1). The coating with 50 g of fertilizer had the highest TSW. The difference in seed weight is possibly related to the difference of granulometry of the materials used, as the fertilizer has a higher particle size in relation to sand and limestone. Some authors are unanimous in saying that one of the advantages of the coating process is the increase of the size of the seeds, in order to facilitate manual and mechanical seeding (Nascimento et al., 2009). The coated seeds showed lower water content than the non-coated seeds (Figure 1A). The values of water content in the seeds that received coating fluctuated between 7.09 and 8.69%, below the uncoated seeds, which had 10.72% of water. These results indicate that the drying process of the coater, at 40°C, was efficient and that the materials have not retained moisture from the adhesive solution.

Conceição et al. (2009) also found lower water values in coated corn seeds, and claim that the coated seeds have less water content because the water contained in the coating is not absorbed by the seed and it is quickly lost in the process. Therefore, the coated seeds tend to have lower water content, when compared to uncoated seeds. The results of the first count showed decreasing linear behavior depending on the dose of fertilizer in the coating. As the doses of fertilizer in the coating of vinhatico seeds were increased, there was a reduction of the first count germination (Figure 1B). The first count germination values were greater for coated seeds without fertilizer, surpassing even the uncoated seeds, reaching 85% of emission of radicle compared to uncoated seeds that showed 60% of issued radicles. It is also important to note that among the treatments that were coated with doses of fertilizer, the only treatment that achieved less than the uncoated seeds was treated with 50 g of fertilizer.

The reduction of first count germination with the raise of fertilizer doses could be caused by the effect of fertilizer toxicity, and reduction was observed on the first germination count. Different results were found by Tavares et al. (2013). Studying the potassium action through soybean seed coating and the effects upon physiological quality and performance, it was possible to notice that the first count germination and germination variables did not significantly differ, regardless of the source and concentration used. However, Bays et al. (2007), working with the coating of soybean seeds with micronutrient, fungicide and polymer, observed that there was damage on the plant standard development, because the final volume from the mixture surpassed the recommendation of 300 ml/50 kg seeds, reaching a final volume of 400 ml/50 kg.

Fungueto et al. (2010) analyzing the rice seed coating with source zinc, fungicide and polymer did not observe differences on germination. Derré et al. (2013) worked with *Urochloa brizantha* cv. Xaraes and *Urochloa ruziziensis* cv Kennedy seeds and confirmed that coated seeds soak more slowly. For the germination, the data also adjusted better to the decreasing linear model. As the doses of fertilizer on vinhatico seeds were increased, there was reduction of germination (Figure 1C). According to the germination test, it can be observed that coated seeds without fertilizer present 82% of germination, while uncoated seeds presented 67% of germination (Figure 1C). As the fertilizer was added to the coating, reduction was observed in germination, reaching lower values when compared to uncoated seeds. This result resulted from the toxicity of high doses of fertilizer on the coating and/or due to exacerbated delay on seed soaking, caused by the presence of fertilizer on the coating.

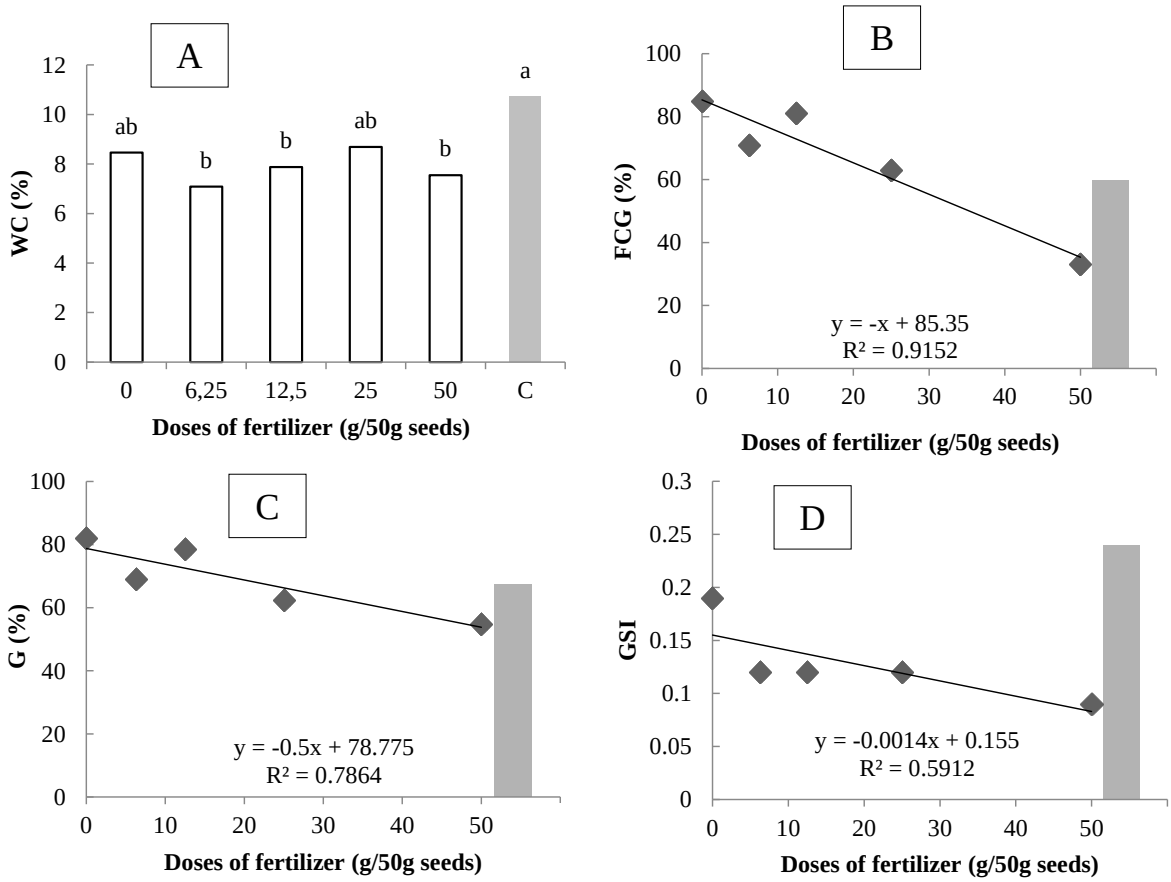

Figure 1. (A) Water content (WC %); B: first germination count (FCG %); C: germination (G %); D: germination speed index (GSI) on vinhatico seeds coated with fungicides and increasing fertilizer doses (0; 6.25; 12.5; 25 e 50 g/50 g of seeds) and uncoated seeds (gray column).

According to Xavier et al. (2015), the fertilizers interfere with the hydric potential of the coating around the seed, reducing the gradient between coating and seed surface. This limits the amount of water absorbed, reducing seed germination, which can be seen in this work, in which the nutrients added on the higher dose of fertilizer resulted in lower germination and first count germination (Figure 1). The results found on literature regarding germination of coated seeds varied. Some authors have observed reduction while others have noticed increase on germination. These results depend on the seed condition, on the studied species, on the type and amount of coating used during the process. Dutra et al. (2014), studying *maxixe* seeds observed germination and increase of strength on seeds treated with phytin, compared to the ones treated with dicalcium phosphate.

According to Soares et al. (2016), the use of soybean seeds coated with 0.6 to 0.8 g of monosodium phosphate per 100 g of seeds resulted in germination and strength increase, as well as emergence and emergence speed improvement. In relation to germination speed index, a linear behavior on the results was observed. The coated seeds presented reduction on the GSI as the doses of fertilizers were increased (Figure 1D). These results can be explained because coating, initially, reduces the speed of water soaking by the seed and can still hamper gas exchange between seed and environment. As a consequence, there is a delay of the seed respiratory process and standard metabolism, promoting reduction of germination speed index. This causes a positive effect on the germination of coated seeds without fertilizer and coated seeds with lower doses of fertilizer (Figures 1C and D).

The main responsible factor for the decrease in germination speed of coated seeds is the physical barrier imposed on the seed, which delays the root protrusion, aggravated by the increase in size and coating resistance. Pereira et al. (2011), working with pelleted *Brachiaria* treated with fungicide and insecticide, observed a higher germination speed index for seeds that were not pelleted. According to the authors, this happened because the coating layer acted as a physical barrier.

The percentage of non-germinated seeds (NGS)

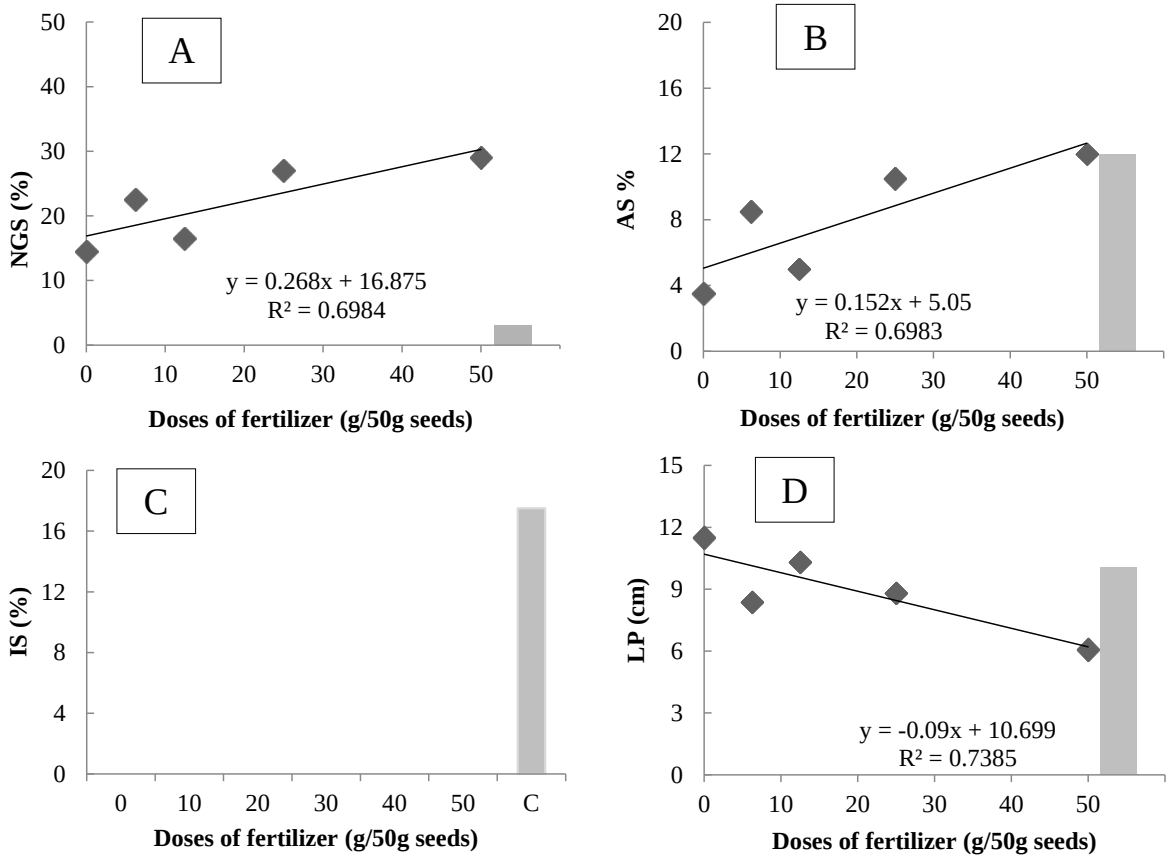

Figure 2. Non-germinated seeds (NGS %), abnormal seedlings (AS), infected seedlings (IS %) and seedling length (LP) on seedlings originated from vinhatico seeds coated with fungicides and increasing doses of fertilizer (0; 6.25; 12.5; 25 e 50 g/50 g of seeds) and uncoated seeds (gray column).

presented increasing linear behavior, that is, as the fertilizer dose was increased on the coating, there was a significant raise on the number of NSG (Figure 2A). This is another effect from higher doses of fertilizer used on seed coating, causing toxic effect added to the gas exchange restriction caused by coating. This can be proved when observing the increase of NSG as the seed is coated, reaching from 14 to 30% of NSG when the fertilizer (50 g) is added to the coating. Brites et al. (2011), working with scratched seeds and coated seeds with different tropical forage species, found a higher percentage of dead seeds after coating, comparing to the scarified seeds, reaching 73% in *Brachiaria humidicola*.

The unusual seedlings (US) results presented increasing linear behavior due to the increase of fertilizer doses on the coating (Figure 2B). For this variable, it was observed that uncoated seeds reached 12% of (US), against 3.5 and 5% of US on coated seeds with and without fertilizer, respectively, highlighting that uncoated seeds lost strength quickly while the coating protected the seeds, keeping their strength, reducing water absorption speed and reducing damages to the membranes during the soaking process. Only damages caused by high fertilizer doses on coating made the results of US stay closer to the control treatment, without coating. These results corroborate with the results found by Brites et al. (2011). Observing the germination of *Brachiaria* and *Panicum* seeds, scarified with sulfuric acid and coated, it was possible to conclude that coated seeds reduced the number of unusual seedlings compared to uncoated seeds, scarified and not scarified.

During the experiment, the presence of infested seedlings on coated seeds was observed, proving that the fungicide dose was efficient to combat fungi that were found on previous experiments. It is worth highlighting that uncoated seeds from the control treatment presented 17.5% of seedlings infested with fungi (Figure 2C). Ludwig et al. (2014), studying the strength and production of *crambe* seeds treated with fungicide, insecticide and polymer, concluded that the application of fungicide and/or insecticide associated or not to the polymer, affected the strength of the seed in laboratory tests, but in soil conditions, it did not affect germination and emergence of *crambe* seedlings.

Table 2. Percentage of emergence (%E), emergence speed index (ESI), length of aerial part length (LAP), length of root (LR), fresh and dry mass from the aerial part (FMAP/DMAP), fresh and dry mass from the root (FMR/DMR) of vinhatico seedlings in greenhouse 60 days after sowing.

Treatments	E (%)	IVE	CPA (cm)	CR (cm)	MFPA (g)	MSPA (g)	MFR (g)	MSR (g)
Control	74.5^{ab}	0.11^{a}	7.6^{ab}	7.9^{a}	1.94^{b}	0.85^{a}	1.07^{c}	0.33^{a}
Without fertilizer	79.5^{a}	0.11^{a}	7.9^{a}	7.5^{ab}	2.74^{a}	0.85^{a}	2.14^{a}	0.44^{a}
6,25g fertilizer	68.5^{ab}	0.08^{b}	7.2^{b}	6.7^{c}	2.16^{b}	0.76^{a}	1.18^{c}	0.28^{a}
12,5g fertilizer	67.5^{ab}	0.09^{b}	7.4^{ab}	7.1^{bc}	2.51^{a}	0.77^{a}	1.81^{ab}	0.35^{a}
25g fertilizer	70.5^{ab}	0.09^{b}	7.7^{ab}	7.3^{abc}	2.57^{a}	0.85^{a}	1.74^{b}	0.39^{a}
50g fertilizer	61.5^{b}	0.07^{c}	7.9^{a}	7.4^{abc}	2.53^{a}	0.77^{a}	1.60^{b}	0.27^{a}
CV (%)	11.96	6.69	4.78	4.7	7.68	7.19	15.97	32.31

Averages followed by the same letter do not differ among themselves by Duncan's test, at the 5% level probability.

Regarding the length of the seedling, the results indicated a linear behavior, with the length reduced due to the increase of fertilizer doses (Figure 2D). There is no loss to this variable when the seeds are coated, but the decrease is significant when the fertilizer is added to the coating.

Almeida et al. (2015), studying the soaking and physiological quality of white lupin seeds treated with micronutrients, observed no significant effects on the dry mass weight and on the length of the seedling between lots and pre-sowing treatments.

Greenhouse

With regard to the characteristics evaluated in the greenhouse, low correlation coefficient was verified in the regressions. After analysis of variance, the averages were compared by Duncan's test at the 5% level of probability. Analyzing the emergence in the greenhouse (Table 2), no significant reduction was observed after the increase of fertilizer doses on coating. However, it is important to highlight the benefit from the coating without fertilizer to the plant emergence (79.5%), even if not significant.

Santos et al. (2010) analyzed coated *Brachiaria brizantha* cv. Marandu seeds and concluded that the pelletization reduces both seedling and speed emergence. The presence of fertilizer on the coating reduced the emergence speed index (ESI), which did not happen to the coated seeds without fertilizer, demonstrating once more the negative effect of adding fertilizer to the coating of vinhatico seeds (Table 2).

Some authors affirm that the coating can improve seed quality when it is associated to the use of micronutrients, insecticides and fungicides as it was observed on rice (Tavares et al., 2012), carrot (Hölbig et al., 2010) and millet seeds (Peske and Novembre, 2011). However, the coating also delays the plant germination and emergence (Brites et al., 2011; Derré et al., 2013). The different responses to coating happen due to the species, to the specific material used, to the thickness of the coating layer placed on the seeds, to the way the material is applied and to the process used for the coating.

For the length of the aerial part, no treatment differed from the uncoated seeds. This indicates that until the evaluated phase, the coating treatment did not cause any damage regarding this characteristic (Table 2). The root length was reduced by intermediate doses of fertilizer on the coating, whereas higher doses was equal to the control treatment (Table 2). Soares et al. (2014) analyzed the soybean seeds coated with phosphorus and concluded that the coating caused soybean plant nodulation and growth raise.

For the fresh mass from the aerial part (FMAP) variable, significant increase was observed when the seeds were coated with higher doses of fertilizer, although it did not reflect on the dry mass from the aerial part (DMAP) (Table 2). These results corroborate with Yagi et al. (2006) who, studying the application of zinc on sorghum seeds, concluded that it does not affect the accumulation of shoot dry mass. On the other hand, Soares et al. (2014) observed that soybean seeds coated with phosphorus showed an increase in plant growth. For the fresh mass from the root (FMR) variable, the higher value (2.14 g) is noticed in coated seeds without fertilizer, which is 100% higher than the observed on uncoated seeds, demonstrating positive and significant effect of coating on FMR. Although this treatment reached a great value for dry mass from the root (DMR, 0.44 g), it was not significant compared to the other treatments, probably due to the high coefficient of variation, since the DMR value of this treatment was 33% higher than on uncoated seeds (Table 2).

These results show that the application of coating on vinhatico seeds promotes higher development of this plant until that evaluated phase, although it also delays the germination and emergence. Under greenhouse conditions, the negative effect of fertilizer on the coating is significantly reduced, probably due to the dilution of the coating compounds by the irrigation during the period of this experiment and also because of the adsorption and

leaching of part of the compounds. An option to minimize the problems could be the reduction of fertilizer doses as well as the amount of inert material that is used on the coating (Ludwig et al., 2014).

Conclusions

1. The coated seeds without fertilizer reached better results of the count of first and final germination. As the doses of fertilizer on coated seeds were increased, reduction of the first germination count, of the germination and of the germination speed index was observed.
2. The coating with fertilizer provided higher values of seeds that were not germinated and abnormal seedlings. The dosage of fungicide used on the coating was efficient to combat fungi.
3. The plants originated from seeds coated with fertilizer reached higher FMAP and FMR. The length of aerial part length (LAP), length of root (LR), DMAP, DMR and the emergence were not affected, only the ESI was reduced on coated seeds with fertilizer.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Somatic polyploidization and characterization of induced polyploids of *Dioscorea rotundata* and *Dioscorea cayenensis*

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Genetic improvement of major food yams is constrained by a number of factors, such as the scarcity of flowers and lack of synchronization between male and female flowering. Consequently, somaclonal variation including somatic polyploidization has been considered as a useful tool in yam breeding. Somatic polyploidization and its effect on phenotypic traits of *Dioscorea* species such as *D. alata*, *D. japonica* and *D. zingiberensis* has been reported; however, optimization of this method in two major yam species, *D. rotundata* and *D. cayenensis,* is yet to be achieved and the effect of polyploidization on phenotypic traits of this species yet to be elucidated. In the present study, a high rate of somaclonal polyploid variation was successfully achieved by *in vitro* colchicine treatment of *D. rotundata* and *D. cayenensis*. In most cases, except TDc 3704, the highest rate of polyploid induction appeared after 0.1% colchicine treatment. However, in triploid yellow yam accessions the induction rate was relatively low. Tetraploid variants of *D. rotundata* tended to display somewhat rounder leaves than their diploid parents. The size and shape of *D. rotundata* stoma were also affected by levels of ploidy, with tetraploid variants exhibiting larger stomata at a lower density compared to their diploid parents. The efficient method of *in vitro* polyploidy induction reported here is therefore a highly useful tool for obtaining polyploid variants for use as genetic resources in *D. rotundata* breeding.

Key words: Leaf, nodal segment culture, ploidy level, somaclonal variation, stoma, white guinea yam, yellow Guinea yam.

INTRODUCTION

In terms of production, yams (*Dioscorea* spp.) are the fourth most important tuber crop in the world (FAOSTAT, 2014), serving as a staple for millions of people in tropical regions. This is especially true in West Africa, also known as the “yam belt”, where 92% of the world’s yams are produced (FAOSTAT 2014).

Here, white Guinea yam (*Dioscorea rotundata;* hereafter "white yam"), yellow Guinea yam (*D. cayenensis;* hereafter "yellow yam") and *D. alata* (water yam) are the most important edible species and a major component of the total yam production in the region. In a short period of 20 years from 1992 to 2012, the production quantity of yams in West Africa rapidly increased from about 27 to 54 million tons, largely through the use of landraces and rapid expansion of cultivated acreage from 2.4 to approximately 4.5 million hectares (FAOSTAT, 2014). To meet this growing demand, breeding of higher-yielding cultivars is therefore becoming an important focus. However, genetic improvement of major *Dioscorea* species is constrained by a number of factors, such as the scarcity of flowers and lack of synchronization between male and female flowering. Consequently, somaclonal variation including somatic polyploidization is being considered as a useful tool in yam breeding.

A wide range of intra-specific variation in ploidy level is observed in major food yams. In most reports on ploidy level, white yam and yellow yam are treated as a *D. cayenensis/D. rotundata* complex. Dansi et al. (2001) reported diploids (2*n*=2x=40), triploids (2*n*=3x=60) and tetraploids (2*n*=4 x = 80) in *D. cayenensis/D. rotundata*, while Gamiette et al. (1999) reported only diploids and tetraploids. Meanwhile, Obidiegwu et al. (2009) reported diploids, triploids and tetraploids in a white yam collection but only triploids and tetraploids in yellow yam. Water yam reportedly displays all three ploidy levels, diploids (2*n*=2x=40), triploids (2*n*=3x=60) and tetraploids (2*n*=4x=80) (Abraham and Nair, 1991; Gamiette et al., 1999; Egesi et al., 2002; Arnau et al., 2009; Babil et al., 2010; Obidiegwu et al., 2010).

Triploid and tetraploid water yam cultivars tend to be more vigorous and higher yielding than diploid cultivars (Arnau et al., 2007). An association between higher ploidy levels and yield potential in water yam has also been reported, with diploids yielding 2 kg fresh tubers/plant and triploids and tetraploids yielding an average of 2.5 kg and 3 kg/plant, respectively (Lebot, 2009). During investigations of water yam landraces in Myanmar, Babil et al. (2010) reported that leaf size tended to be larger in triploids than in diploids and tetraploids. However, despite these findings, little is known about the effects of ploidy variation on morphological and agronomical traits of white and yellow yam.

Artificially induced polyploidy has been reported in numerous plant species belonging to at least 150 genera (Dewey, 1979). Agronomically successful examples include triploid sugar beet, tetraploid clover and tetraploid rye. Somatic polyploidization in water yam (Babil et al., 2011), *D. japonica* (Kenji et al., 2005) and *D. zingiberensis* (Huang et al., 2008) has also been reported. Artificially induced tetraploid (2*n*=4x=80) tubers of *D. japonica* were found to be shorter and thicker than diploid tubers, while viscosity, a major trait determining commercial value, was unaffected (Kenji et al., 2005). Moreover, artificially induced tetraploids (2*n*=4x=40) of *D. zingiberensis* were found to produce a higher content of diosgenin compared to the diploid (2*n*=2x=20) mother plant (Huang et al., 2008). Therefore, somatic polyploidization might also be applicable in genetic enhancement of white and yellow yam; however, to the best of our knowledge, information on the artificial induction of polyploidy is currently limited in both species. Colchicine is the most effective chemical agent for polyploid induction. However, since all plant species respond differently to colchicine treatment, optimization of colchicine concentration and application is required. The present study aimed to develop an efficient *in vitro* technique for the induction of somaclonal polyploid variation in white and yellow yam. Successfully induced polyploid variants of white yam were subsequently characterized in relation to the respective parent plants.

MATERIALS AND METHODS

Plant materials and *in vitro* multiplication

In vitro seedlings of white yam accessions TDr 2720, TDr 1793, TDr 2351 and yellow yam accessions TDc 2790, TDc 2812 and TDc 3704 maintained in the Genetic Resource Unit (GRU) of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, were used. Morphological traits of yellow yam accession TDc 2790 were similar to white yam accessions, and therefore, in the present study it was treated as a white yam. In addition, TDc 2790 was referred to as "R-20" in the present study.

In vitro multiplication was conducted from July 2013 to March 2014 at the IITA. Nodal segments excised from *in vitro* seedlings were cultured on 10 ml MS medium (Murashige and Skoog, 1962) supplemented with 30 mgl^{-1} sucrose. Prior to autoclaving at 121°C for 15 min, the medium was adjusted to pH 5.8 and solidified with 0.2% gellant gum. The cultured explants were incubated in a growth chamber at 28°C under a 16/8 h light/dark photoperiod. New shoots were then subcultured in the above medium to produce sufficient plantlets for colchicine treatment.

Colchicine treatment to induce polyploidy

Thirty-three-month-old nodal segments with a single axillary bud were planted on MS solid medium supplemented with 30 mgl^{-1} sucrose and cultured for 48 h prior to colchicine treatment. The nodal segments were then soaked in 0.1, 0.2 and 0.3% colchicine solution for 8 h. Sterilized distilled water was used as the control treatment. The colchicine solution was autoclaved at 121°C for 15

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min before use. Treated nodal segments were rinsed three times in sterilized water then replanted on MS medium as described previously. Two replications were conducted per treatment with 15 nodes per replication. The nodal segment cultures were continued for 3 months under a 16-h photoperiod at 28°C.

Flow cytometric analysis for identification of ploidy levels

Survival rates of cultured nodal segments were determined and ploidy levels of regenerated plantlets identified by flow cytometry using a Partec Ploidy Analyzer. Experiments were conducted at the Setagaya campus of Tokyo University of Agriculture (TUA) from April to October 2014. Materials were transferred from IITA to TUA after both parties signed a standard material transfer agreement and plants underwent quarantine.

For flow cytometry, portions of leaf blade (5 mm) collected from plantlets obtained *in vitro* were chopped into pieces then homogenized in a petri dish containing an extraction buffer solution to release nuclei. The homogenate was filtered through a nylon filter (50 µm pore size) and the extracted nuclei stained with 4,6-diamino-s phenylinode (DAPI). Flow cytometric analysis was performed at a rate of 5 to 20 nuclei per second. Rice plants (*Oryza sativa* L.) were used as the inter-reference. Relative amounts of DNA (the DNA index) in a sampled plant were determined in relation to that of rice to determine the ploidy level. Flow cytometry was carried out with each leaf from sample plants to confirm the ploidy level.

Mitotic chromosome observations

Chromosome numbers in control mother plants and induced polyploids, except TDc 3704, were confirmed by microscopic observation. For mitotic chromosome observation, root tips were sampled from *in vitro* seedlings and fixed in acetic acid-alcohol (1 to 3 ratio) for 24 h without pretreatment. Fixed root tips were stained with 1% aceto-carmine solution for 24 h. Preparation was conducted using the squash method and prepared samples observed under an optical microscope (BX53, Olympus) at a magnification of ×400.

Characterization of induced white yam polyploids

Artificially induced polyploid variants of white yam were characterized with reference to their parents in terms of leaf and stoma characters. The yellow yam polyploids grew abnormally and were excluded from characterization. Characterization was conducted from June 2014 to January 2015 at the Setagaya campus of TUA. Three- to four-month-old *in vitro* plants were acclimatized under a 12-h photoperiod at 25°C. Two parent plants and three induced polyploid plants were used for characterization. Five fully developed leaves obtained from the center of the vines were sampled. Observed phenotypic characters included the length and width of the leaf blade, depth and width of the sinus, and shape (length/width) of the leaf. The length, width, shape (length/width), size (length × width) and density of stomata were also observed. The same samples were used for measurements of stoma and leaf characters. Five stomata per leaf were measured. Photos of stomata were taken using a digital camera (DP71, Olympus) attached to the microscope (BX53, Olympus). Measurements of stomata were carried out using photo images with Win Roof software (Mizutani corporation, Japan). Stomata density was calculated based on the number of stomata per 64 mm^2 of a microscopic field at a magnification of ×200. To analyse statistical difference between induced tetraploid and mother plant, student's T-test was performed for all traits observed.

RESULTS

Effects of colchicine treatment on survival of the cultured nodal segments

Survival rates of the white and yellow yam accessions after colchicine treatment are shown in Tables 1 and 2, respectively. Survival of the cultured nodal segments was largely affected by the concentration of colchicine. In both species, survival rates decreased with increasing colchicine concentration. The highest survival rate was observed with 0.1% treatment except in the case of TDr 1793, the survival rate of which was higher under 0.3% treatment (46.7% compared to 43.3% under 0.1% colchicine). Overall, survival rates were higher in yellow yam accessions compared to white yam.

Effects of colchicine concentration on induction of polyploidy

DNA indexes and estimated numbers of chromosomes in control and colchicine treated *in vitro* seedlings as determined by flow cytometry and mitotic chromosome observations are shown in Table 3 and Figure 1. Based on mitotic chromosome observations and flow cytometry, ploidy levels of white yam accessions TDr 2720, 1793, 2351 and R-20 used in the present investigation were found to be diploid (2n = 40). Meanwhile, ploidy levels of yellow yam accessions TDc 2812 and 3704 were found to be triploid (2n=3x=60).

DNA indexes of nuclei at the G1 stage in the diploid white yam accessions ranged from 1.88 to 1.93, and in the induced polyploids from 3.77 to 3.86, which is twice the value of the diploid parents (Table 3). DNA indexes of nuclei of triploid yellow yam accessions ranged from 2.74 to 2.86 and that of induced polyploids from 5.31 to 5.41, twice the value of the triploid parents. Polyploid variants derived from the diploid white yam accessions were found to be tetraploid (2n=4x=80), while those from the triploid yellow yam accessions were hexaploid (2n=6x=120). The highest rate of polyploidy was induced by 0.1% colchicine in TDr 2720, 2351, R-20 and TDc 2812, and by 0.3 and 0.2% in the remaining two accessions, TDr 1793 and TDc 3704, respectively (Tables 1 and 2).

Characterization of induced polyploid variants in terms of leaf and stoma characters

Leaf and stoma characters of three- to four-month-old induced tetraploid variants of white yam were compared with the control (Tables 4 and 5). A statistically significant difference was observed in leaf length and width between the diploid parent and tetraploid variants, with tetraploid leaves appearing longer and wider than leaves in diploid. A significant difference was also observed in leaf shape

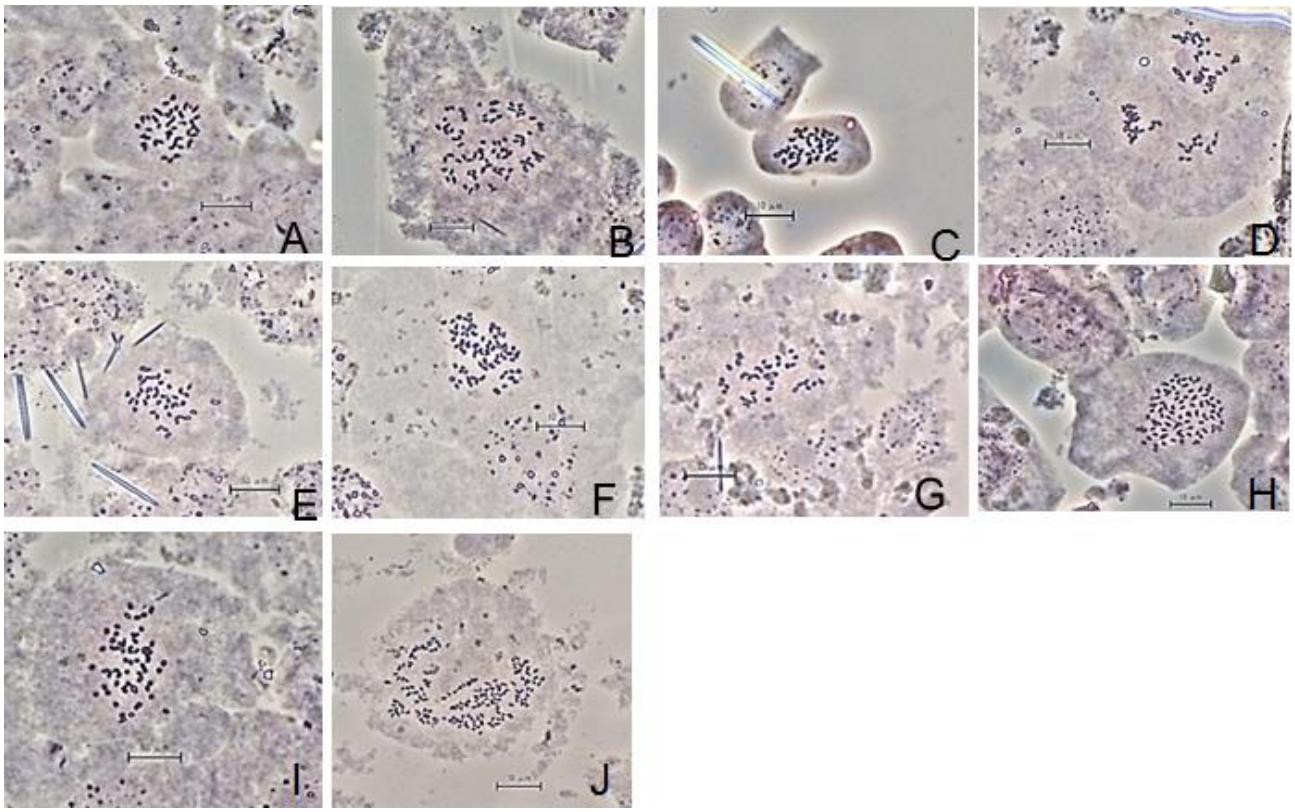

Figure 1. Chromosome images of control and induced polyploids of white yam (*Dioscorea rotundata*) and yellow yam (*D. cayenensis*). TDr 2720 control diploid (A) and induced tetraploid (B); TDr 1793 control diploid (C) and induced tetraploid (D); TDr 2351 control diploid (E) and induced tetraploid (F); R-20 control diploid (G) and induced tetraploid (H); TDc 2812 control triploid (I) and induced hexaploid (J). Scale bar: 10 µm.

Table 1. Effect of colchicine concentration on survival rate and induction of polyploids of white yam (*D. rotundata*).

Accession no.	Colchicine concentration	No. of explants	No. of survivors (%)	No. of diploids (%)*	No. of tetraploids (%)*
TDr 2720	0	30	28 (93.3)	28 (93.3)	0 (0)
	0.1	30	21 (70.0)	5 (16.7)	16 (53.3)
	0.2	30	14 (46.7)	6 (20.0)	8 (26.7)
	0.3	30	8 (26.7)	2 (6.7)	6 (20.0)
TDr 1793	0	30	27 (90.0)	27 (90.0)	0 (0)
	0.1	30	13 (43.3)	9 (30.0)	4 (13.3)
	0.2	30	10 (33.3)	6 (20.0)	4 (13.3)
	0.3	30	14 (46.7)	7 (23.3)	7 (23.3)
TDr 2351	0	30	29 (96.7)	29 (96.7)	0 (0)
	0.1	30	14 (46.7)	6 (20.0)	8 (26.7)
	0.2	30	12 (40.0)	8 (26.7)	4 (13.3)
	0.3	30	10 (33.3)	5 (16.7)	5 (16.7)
R-20	0	30	28 (93.3)	28 (93.3)	0 (0)
	0.1	30	28 (93.3)	10 (33.3)	18 (60.0)
	0.2	30	16 (53.3)	9 (30.0)	7 (23.3)
	0.3	30	11 (36.7)	5 (16.7)	6 (20.0)

*Percentage of initial explants was calculated.

Table 2. Effect of colchicine concentration on survival rate and induction of polyploids of yellow yam (*D. cayenensis*).

Accession no.	Colchicine concentration	No. of explants	No. of survivors (%)	No. of triploids (%)*	No. of hexaploids (%)*
TDc 2812	0	30	26 (86.7)	26 (86.7)	0 (0)
	0.1	30	28 (93.3)	24 (80.0)	4 (13.3)
	0.2	30	24 (80.0)	21 (70.0)	3 (10.0)
	0.3	30	20 (66.7)	17 (56.7)	3 (10.0)
TDc 3704	0	30	28 (93.3)	28 (93.3)	0 (0)
	0.1	30	29 (96.7)	27 (90.0)	2 (6.7)
	0.2	30	27 (90.0)	18 (60.0)	9 (30.0)
	0.3	30	25 (83.3)	21 (70.0)	4 (13.3)

*Percentage of initial explants was calculated.

Table 3. DNA relative values and estimated numbers of chromosomes in control and induced variants of white yam (*D. rotundata*) and yellow yam (*D. cayenensis*).

Accession no.	Treatment	DNA index	Estimated no. of chromosomes (2n)
TDr 2720	Control	1.88	40
	Variant	3.82	80
TDr 1793	Control	1.93	40
	Variant	3.83	80
TDr 2351	Control	1.92	40
	Variant	3.86	80
R-20	Control	1.80	40
	Variant	3.77	80
TDc 2812	Control	2.74	60
	Variant	5.31	120
TDc 3704	Control	2.86	60
	Variant	5.41	120

DNA index: Relative amount of DNA in nuclei of samples compared to the inter-reference (*O. sativa*).

Table 4. Leaf characteristics of diploid (2*n* = 40) and induced tetraploid (2*n*=4x=80) variants of white yam (*Dioscorea rotundata*) accessions.

Accession no.	Accession	Leaf length (cm)	Leaf width (cm)	Shape (length/width)	Width of sinus (cm)	Depth of sinus (cm)
TDr 2720	Diploid	7.57	5.19	1.45	3.38	1.49
	Tetraploid	9.16**	6.90**	1.33**	3.58	1.81
TDr 1793	Diploid	4.96	3.85	1.29	2.33	0.97
	Tetraploid	6.54**	5.37**	1.23	2.66	1.65**
TDr 2351	Diploid	5.48	4.45	1.24	2.95	0.96
	Tetraploid	6.75*	5.80**	1.16	3.18	1.26
R-20	Diploid	7.41	5.48	1.37	3.36	1.17
	Tetraploid	8.31*	7.01**	1.20*	4.25**	1.35

* and **Significant difference according to the students t-test at 5 and 1%, respectively.

Table 5. Stoma characteristics of diploid (2*n*=2x=40) parents and induced tetraploid (2*n*=4x=80) variants of white yam *D. rotundata*) accessions

Accession		Length (µm)	Width (µm)	Shape (length/width)	Size (length×width)	Density (mm^{-2})
TDr 2720	Diploid	32.4	18.5	1.8	599.0	12.0
	Tetraploid	43.3**	22.6**	1.9**	989.1**	5.8**
TDr 1793	Diploid	30.5	19.4	1.6	595.1	17.2
	Tetraploid	38.4**	23.9**	1.6	917.0**	7.9**
TDr 2351	Diploid	34.4	18.1	1.9	623.5	15.0
	Tetraploid	41.7**	20.6**	2.0**	861.3**	8.4**
R-20	Diploid	33.5	18.00	1.9	605.6	15.0
	Tetraploid	40.9**	20.2**	2.0**	829.3**	7.2**

**Significant difference according to the student's t-test at 1%.

(length/width) between TDr 2720 and R-20, leaves of tetraploid variants appearing rounder than diploid leaves. Similarly, leaves of tetraploid variants appeared thicker than diploid leaves, except in the case of TDr 1793. A slightly deformed leaf shape was observed in the hexaploid variants derived from the triploid parents of the yellow yam accession and most hexaploid variants grew abnormally until death (data not shown).

Significant differences were observed in all stoma characters between tetraploid white yam variants and their diploid parents except for stomata shape in TDr 1793 (Table 5). Stoma was significantly longer and wider in the induced tetraploids compared to the diploid parents. Stoma (length/width) in the tetraploid variants also appeared somewhat longer than in the diploid parents. Although stoma size (length × width) was larger in the tetraploid than the diploid parent, stoma density was lower in the tetraploid variants.

DISCUSSION

To this date, various efficient techniques for polyploid induction using colchicine have been proposed for different crop species. The efficiency of colchicine-induced somatic polyploidization varies among plant species and the method of application and/or concentration used. The *in vitro* induction rate in *Phlox subulata* L. reached 20% (Zhang et al., 2008), while in diploid and triploid cultivars of *Colocasia esculenta* it was low at 4.3 and 1.4%, respectively (Miyazaki et al., 1985). Huang et al. (2008) also reported a polyploidy rate as high as 36.7% in *D. zingiberensis* calli immersed in 0.3% colchicine solution for 16 h prior to culture. However, the above protocol is not applicable to white and yellow yam because of difficulties associated with plant regeneration from a callus. Other papers have reported findings in *Dioscorea* species such as *D. floribunda* (Sharma and Chaturvedi, 1988) and *D. zingiberensis* (Huang et al., 2008; He et al., 2010; Xiao et al., 2010), and recently, Babil et al. (2011) proposed an efficient *in vitro* technique for induction of somatic polyploids of water yam that is also effective in white and yellow yam. In this study, we adopted this *in vitro* technique to optimize somatic polyploidization in white and yellow yam. We also elucidated the effects of somatic polyploidization on leaf and stoma traits in white yam.

This is the first time polyploid variants of white and yellow yam have been induced at a high frequency. The highest rate was attained after *in vitro* treatment of the diploid accession R-20 with 0.1% colchicine. However, in the case of triploid yellow yam accessions, the induction rate of polyploids was relatively low, probably due to the higher ploidy level of the mother plant. In most cases, except TDr 1793 and TDc 3704, the highest rate of polyploid induction appeared after 0.1% colchicine treatment.

Nodal segment culture of white and yellow yam is an easy reproduction method for growth of shoots. In fact, the shoot survival rate was as high as 96.7 and 93.3% in a control plot of white and yellow yams, respectively.

Tetraploids (2*n*=4x=80) were successfully induced from the diploid parent (2n=2x=40) white yam and hexaploids (2n=6x=120) from the triploid parent (2n=3x= 60) yellow yam accessions. The induced tetraploid variants of white yam grew almost normally; however, the hexaploid variants of yellow yam grew abnormally until death. A similar phenomenon was observed in spontaneous hexaploid (2n=6x=120) polyploid mutants redifferentiated from cultured calli of triploid (2n=3x=60) water yam accessions (Iijima, unpublished) and in colchicine induced somatic polyploids of triploid water yam (Babil et al., 2011). These results suggest that in yellow yam tetraploidy may be the highest level of ploidy at which

normal growth and survival are observed.

Somaclonal polyploidization is thought to affect various phenotypic characters such as cell size, chlorophyll content, fertility and organ size. Over the past few decades, a considerable number of studies have reported the effects of somaclonal polyploidization on phenotypic characters of various plant species (Speckmann et al., 1965; Miyazaki et al., 1985; Liu et al., 2007; Zhang et al., 2008). In water yam, tetraploid variants tend to have rounder leaves than their diploid parents. Similar relationships were also recognized between diploid and tetraploid variants of water yam accessions collected in Myanmar (Babil et al., 2010).

However, information about the effects of ploidy level on phenotypic characters and practically important traits such as tuber yield and quality are limited for white and yellow yam. In the present report, the relationship between ploidy level and leaf and stoma characters was therefore investigated in white yam. Significant differences were observed in leaf length and width, with tetraploid variants tending to have rounder leaves than their diploid parents.

Stomatal traits such as size (length × width), shape (length/width) and density are considered reliable indicators of ploidy in various plant species (Speckman et al., 1965; Daniel and Yao, 1996). In cassava (*M. esculenta*), for example, stomata were less dense and their size larger in polyploids because cells were enlarged as a result of polyploidization (Hahn et al., 1992). In the present study, artificially induced tetraploid variants displayed larger stomata at a lower density compared to their diploid parents. A similar trend was observed between diploid and tetraploid landraces of water yam collected in Myanmar (Babil et al., 2010) and between diploid and artificially induced tetraploids of water yam (Babil et al., 2011). Accordingly, this observation confirms that stoma traits are reliable indicators of polyploidization in white yam. As well as reduced stomatal density is also known to increase drought tolerance without decreasing photosynthesis rate (Yu et al., 2008). Thus it could be a trait to explore in terms of inheritability to breed yam for dry conditions derived from climate change.

The efficient method of *in vitro* induction of polyploidy reported here is a useful tool for obtaining polyploid variants for use as genetic resources in white yam breeding. However, utilization of somatic polyploidization as a tool to enhance genetic improvement of yellow yam remains limited due to the low induction rate and abnormal growth of induced hexaploid variants. Investigations are ongoing with the aim of elucidating the effect of polyploidization on important agronomical traits such as yield and tuber quality.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Callus induction, direct and indirect organogenesis of ginger (*Zingiber officinale* Rosc)

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The present study aimed to induce callus, direct and indirect organogenesis of ginger (*Zingiber officinale* Rosc) by using Murashige and Skoog (MS) medium fortified with different concentrations and combinations of growth regulators. Shoot tip, *in vitro* leaf and root segments were used as explants to induce callus by MS medium containing (0.00 as control, 0.5, 1.00, 2.00 and 3.00 mg/L) of 2,4-dichloro-phenoxyacetic acid (2,4-D). Callus induced was subcultured on MS+2,4-D at different concentrations (0.5, 1.00, 2.00 and 3.00 mg/L) and one concentration 0.5 mg/L of 6-benzyl amino purine (BAP) was used. The sprouting buds (about 1 to 1.5 cm) were used as explants for direct shoots and roots induction by MS medium + 2.00, 3.00 and 4.5 mg/L of BAP. Callus induced by 1.00 mg/L 2,4-D was regenerated on MS + 0.5 mg/L 2,4-D to obtain a green callus, this callus was transferred to MS medium with combinations of 0.5 mg/L 1-naphthaleneacetic acid (NAA) with different concentrations of BAP (1.00, 2.00,3.00 and 4.00 mg/L) for indirect organogenesis. The results reveals that, for callus induction, callus was only induced from shoot tip explant in all concentrations of 2,4-D. The highest callus fresh weight was obtained by 1.00 mg/L of 2,4-D (1.302 ± 0.09) g than that induced by other treatment ($p < 0.05$). In the case of callus induced by subculture, the highest callus fresh weight initiated was 1.509 ± 0.00 g by 0.5 mg/L 2,4-D. For direct organogenesis, 4.5 mg/L BAP showed the highest number of *in vitro* shoots and roots, 4 ± 0.35 shoots and 15 ± 0.46 roots per explants. For indirect organogenesis, the best shoots and roots initiated were 2 ± 0.21 shoots and 22 ± 0.33 roots by combination of 1.00 mg/L BAP+0.5 mg/L NAA.

Key words: Callus induction, growth regulators, *Zingiber officinale* Rosc, organogenesis.

INTRODUCTION

Plant tissue culture is the *in vitro* aseptic culture of any part of plant or whole plant under controlled nutritional and environmental conditions. This technique depends mainly on the concept of totipotentiality of plant cells,

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Table 1. MS medium supplemented with different concentrations and combinations of plant growth regulators.

Type of culture	Treatment	Phytohormone	Concentration mg/L
Callus induction	1	2,4-D	0, 0.5, 1, 2, 3
Callus subculture	2	2,4-D	0, 0.5, 1, 2, 3
	3	BAP	0, 0.5, 0.5, 0.5, 0.5
		2,4-D	0, 0.5, 1, 2, 3
Direct organogenesis	4	BAP	0, 2, 3, 4.5
Indirect organogenesis	5	NAA	0, 0.5, 0.5, 0.5
		BAP	0, 2, 3, 4

which refers to the ability of a single cell to express the full genome by cell division (Hussain et al., 2012). *In vitro* culture techniques provide an alternative means of plant propagation and a tool for crop improvement (Guo and Zhang, 2005). In many tissue culture, experiments prior to successful agri-biotechnological research on crops, reliable callus induction and efficient *in vitro* regeneration system is urgently required (Thingbaijam and Huidrom, 2014). Through tissue culture, diseases-free clones could be obtained with a rapid multiplication rate (Guo and Zhang, 2005).

Ginger (*Zingiber officinale* Rosc) is a slender, perennial rhizomatous herb (Kathi, 1999). The rhizome of *Z. officinale* Rosc has long served culinary and medicinal uses (Afzal et al., 2001). *Z. officinale* Rosc normally propagates by underground rhizomes, with a low proliferation rate, easily infected by soil-borne pathogens such as bacterial wilt (*Pseudomonas solanacearum*), soft rot (*Pythium aphanidermatum*) and nematodes (*Meloidogyne* spp.); these pathogenic factors are readily transmitted through traditional cultivation practices, which cause heavy losses in yield (Guo and Zhang, 2005). Therefore, it was deemed important to overcome these problems through cultivated this crop under favourable conditions. Plant tissue culture technique can be used as a solution for these problems and a proper alternative to produce disease-free clones of ginger plant by direct and indirect organogenesis.

The present study aimed to investigate effect of growth regulators on callus induction, direct and indirect organogenesis of *Z. officinale* Rosc by different explants.

MATERIALS AND METHODS

This investigation was conducted in Plant Tissue Culture Laboratory at Department of Biology and Biotechnology, Faculty of Science and Technology, AL-Neelain University, Khartoum, Sudan.

Plant

The healthy fresh rhizome of *Z. officinale* Rosc were acquired from the local market of Khartoum city.

Preparation of explants

Healthy and clean *Z. officinale* rhizomes were incubated in the dark at 25±2°C for three weeks for sprouting buds, buds about 1 to 1.5 cm were excised and used as explants for direct shoots and roots induction, while shoot tip about 0.5 cm was used as explant for callus induction. All explants were washed with soap and water several times, then dipped in 70% alcohol for 60 second. This was followed by soaking in 20% clorox (0.5% free chlorine) with two to three drops of Tween-20 for 15 min, and rinsed three to five times in sterile distilled water. Finally, the explants were treated with 0.1% ($HgCl_2$ W/V) for 10 min and washed thoroughly 5 to 6 times with sterile distilled water.

Culture medium

The sterilized explants were planted in (MS) medium (Murashige and Skoog, 1962) supplemented with different concentrations and combinations of plant growth regulators as follows in Table 1. All cultures were incubated at 25±2°C, photoperiod of 16/8 h light/dark for two months for callus induction and three months for direct and indirect shoots and roots formation. All cultures were subcultured at four weeks intervals.

Data analysis

The data were expressed as mean ± Standard deviation of three replicates. Statistical analysis was performed with SPSS software (Griffth, 2007). Means with significant differences were performed using Duncan least significant difference (LSD) at $p<0.05$ probability level of significance.

RESULTS AND DISCUSSION

Callus induction

The shoot tip, *in vitro* leaf and root segments were implanted on MS medium with 0.00 as control, 0.5, 1.00, 2.00 and 3.00 mg/L of auxin 2,4-D for callus induction. Callus was initiated from shoot tip explants, this callus was initiated by meristematic dome after the fourth week

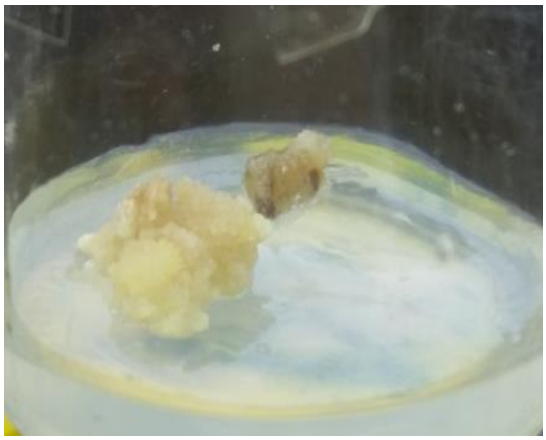

Figure 1. Callus induced by shoot tip explant after eight weeks.

Table 2. Effect plant growth regulators on callus induced from shoot tip explants of *Z. officinale* Rosc after 8 weeks using MS medium.

Type of culture	PGR. in basal MS medium mg/L		Parameter		
	2,4-D	BAP	Fresh weight of callus (g)	Color of callus	Texture of callus
Callus induction	0.00	0.00	-	-	-
	0.5	0.00	0.601±0.04	Creamy	Compact
	1.0	0.00	1.302±0.09	Creamy	Compact
	2.0	0.00	0.370±0.05	Brown	Friable
	3.0	0.00	0.319±0.01	Brown	Friable
Callus subculture	0.00	0.00	-	-	-
	0.5	0.00	1.509±0.00	Creamy	Friable
	1.0	0.00	1.05±0.07	Creamy	Friable
	2.0	0.00	0.70±0.28	Creamy	Friable
	3.0	0.00	0.59±0.09	Creamy	Friable
	0.00	0.00	-	-	-
	0.5	0.5	0.594±0.60	Creamy	Friable
	1.0	0.5	0.5±0.65	Creamy	Friable
	2.0	0.5	0.47±0.65	Creamy	Friable
	3.0	0.5	0.48±0.67	Creamy	Friable

*PGR= Plant growth regulator, mean ± standard deviation.

of culture; no callus was observed when the leaf and root segments were used as explants (Figure 1). These results agree with El-Nabarawy et al. (2015), they used different type of explants (shoot tip, leaves and rhizome segments) to induce callus from *Z. officinale* Rosc. They found that callus was only observed from shoot tip explants after 30 day of culture. Seyyedyousefi et al. (2013) found that, callus induction is hard and time consuming in many monocotyledons like *Alstroemeria* sp.

The results in Table 2 show the mean of fresh weight of callus induced by different concentrations of 2,4-D. The highest mean of fresh weight of callus was induced by 1.00 mg/L 2,4-D (1.302 ± 0.09 g) (Figure 2C) followed by 0.5 mg/L (0.601 ± 0.04 g) (Figure 2B) and 2.00 mg/L (0.370 ± 0.05 g) (Figure 2D). 3.00 mg/L of 2,4-D induced lowest amount of fresh weight of callus (0.319 ± 0.01 g) (Figure 2E). Significant differences ($p<0.05$) were detected among the effect of different concentrations of 2,4-D on callus induction. Resmi and Shylaja (2010) showed that 1.00 mg/L 2,4-D recorded the highest callusing (56.37%) and callus growth by *Z. officinale* Rosc shoot tip explants, also Ma and Gang (2006) reported the presence of 2,4-D at 0.5 to 1.5 mg/L in the culture medium resulted in callus growth for *Z. officinale*

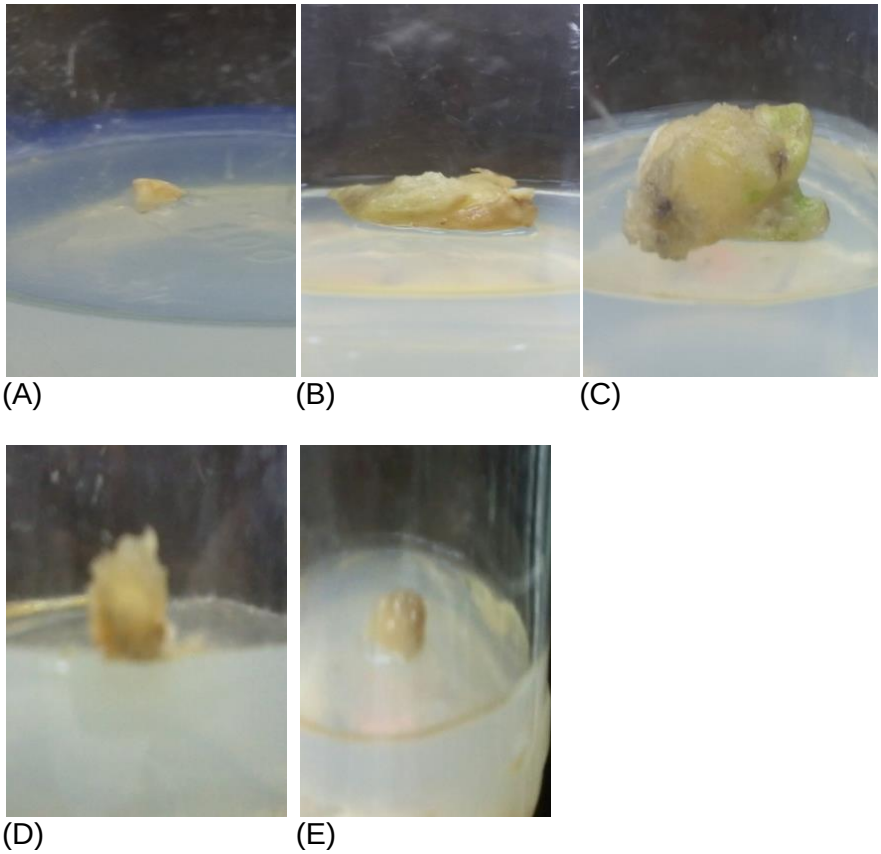

Figure 2. Callus induced from shoot tip explants by MS medium supplemented with 2,4-D hormone, (A) 0.00 mg/L as control, (B) 0.5 mg/L, (C) 1.00 mg/L, (D) 2,00 mg/L and (E) 3.00 mg/L.

Rosc explants, moreover Malamug et al. (1991) and Babu et al. (1992) reported that 2,4-D is the most effective auxin for callus induction in ginger and turmeric.

Callus of *Z. officinale* was compact and showed creamy colour by 0.5 and 1.00 mg/L of 2,4-D, while the callus induced by concentration 2.0 and 3.00 mg/L 2,4-D was friable and showed brown colour. These results are similar to that obtained by Anasori and Asghari (2008); they found some callus of *Z. officinale* turned brown in colour. Cream coloured callus was subcultured after four weeks in MS medium with four concentrations of 2,4-D (0.5 , 1.00, 2.00 and 3.00 mg/L) and one concentration of BAP (0.5 mg/L) was used in combination with 2,4-D concentrations. The highest mean of fresh weight initiated by subculture among different treatments was 1.509 ± 0.00 g by MS + 0.5 mg/L 2,4-D (Figures 3 and 5). Combinations of 0.5 mg/L BAP with different concentrations of 2,4-D induced lower fresh weight of callus compared to callus initiated by subculture on medium supplemented with 2,4-D only. The best fresh weight of callus initiated by combinations of 0.5 mg/L BAP with different concentrations of 2,4-D was 0.594 ± 0.06 g by MS medium containing 0.5 mg/L BAP + 0.5 mg/L 2,4-D (Figure 4).

Direct organogenesis

The sterile sprouting buds (about 1 to 1.5 cm) were used as explants to induce shoots and roots on MS medium with 2.00, 3.00 and 4.5 mg/L of BAP. *In vitro* shoots and roots were successfully induced in all treatments (Figure 6). *In vitro* roots were initiated after 15 to 20 days from culture time earlier than shoots which were initiated after 30 to 40 days. The shoots and roots number/explant and shoots, roots length were measured for three months *in vitro* plants (Figures 8, 9 and 10). BAP at concentration 4.5 mg/L showed the highest number of *In vitro* shoots (4 ± 0.35) and roots (15 ± 0.46) per explant (Figures 6B and 7). These results agree with Abbas et al. (2011), they found that augmentation of MS medium+4.5 mg/L BAP recorded the highest shootlets multiplication percentage from *in vitro* propagation of *Z. officinale* using sprouting buds. Also, Nkere and Mbanaso (2010) investigated the optimizing concentrations of growth regulators for *in vitro* ginger propagation, they found combination of 0.05 mg/L NAA and 4.0 mg/L BAP gave the highest shoot regeneration rate.

Two shoots and 10 ± 0.8 roots per explants were initiated by 3.00 mg/L BAP (Figure 6C). MS medium +

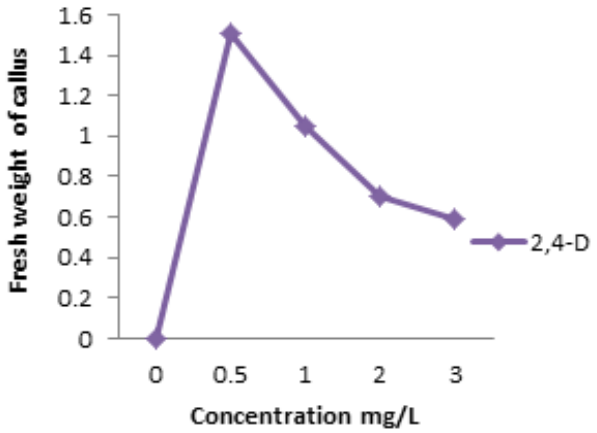

Figure 3. Effect 2,4-D on fresh weight of callus by subculture.

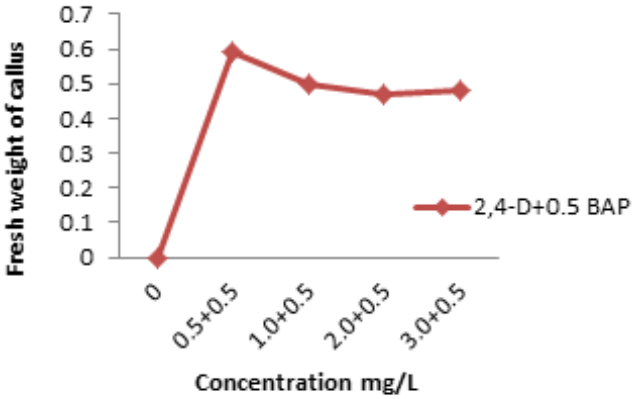

Figure 4. Effect combination of 0.5mg BAP+2,4-D concentrations on fresh weight of callus by subculture.

Figure 5. Callus subcultured on MS medium + 0.5 mg/L of 2,4-D.

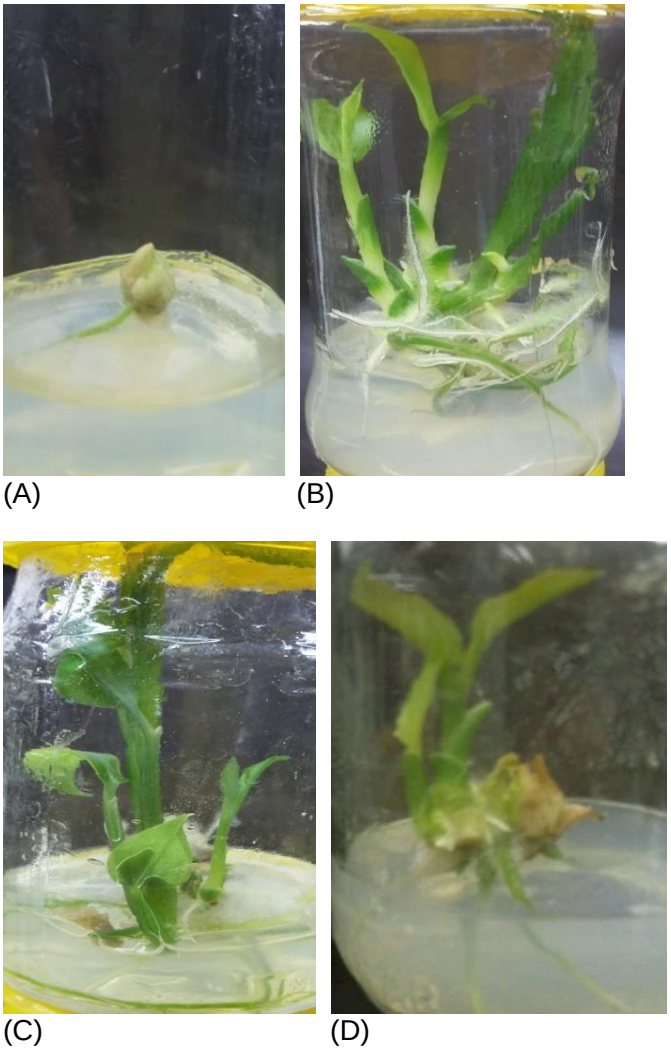

Figure 6. Direct organogenesis(*In vitro* shoots and roots initiation) by MS medium supplemented with, A (0.00) as control, B (4.5 mg/L BAP), C (3.00 mg/L BAP) and D (2.00 mg/L BAP).

2.00 mg/L BAP induced lowest number of *in vitro* shoots (2 ± 0.67) and roots (8 ± 0.35) per explant (Figure 6D). Shoots length is shown in Table 3 and Figure 9 and roots length is shown in Figure 10.

Indirect organogenesis

Callus induced by 1 mg/L 2,4-D after six weeks was subcultured on MS fortified with 0.5 mg/L 2,4-D for six weeks to obtain a green coloured callus, then this callus was transferred to MS with 1.00 mg/L BAP+0.5 mg/L NAA, 2.00 mg/L BAP+0.5 mg/L NAA and 4.00 mg/L BAP+0.5 mg/L NAA to induce shoots and roots from callus (indirect organogenesis). Combination of 1.00 mg/L BAP+0.5 mg/L NAA induced the best shoots and roots initiated from callus, 2 ± 0.21 shoots and 22 ± 0.33

Figure 7. *in vitro* shoots and roots initiated by MS medium supplemented with 4.5 mg/l BAP.

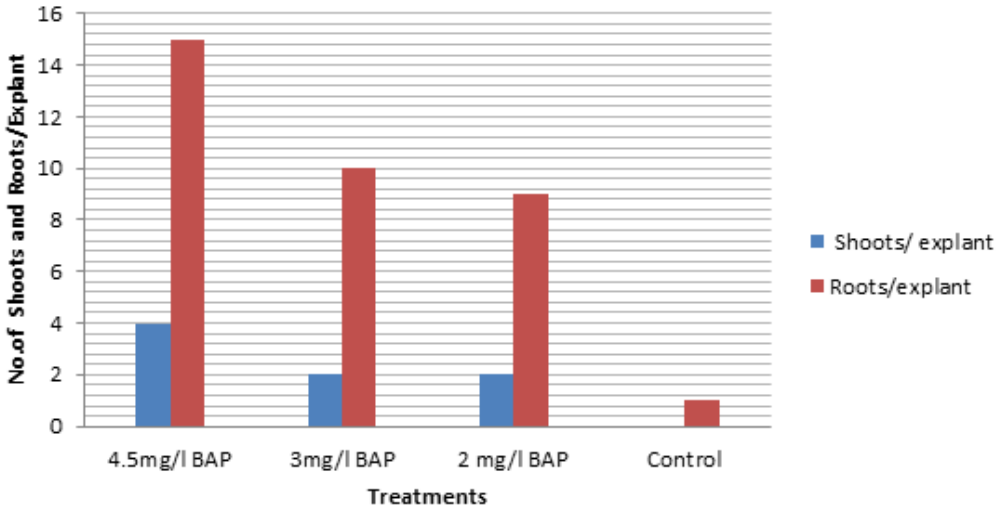

Figure 8. Effect of growth regulator (BAP) added to MS medium on shoots and roots induced/explant of *Z. officinale* for three months of culture.

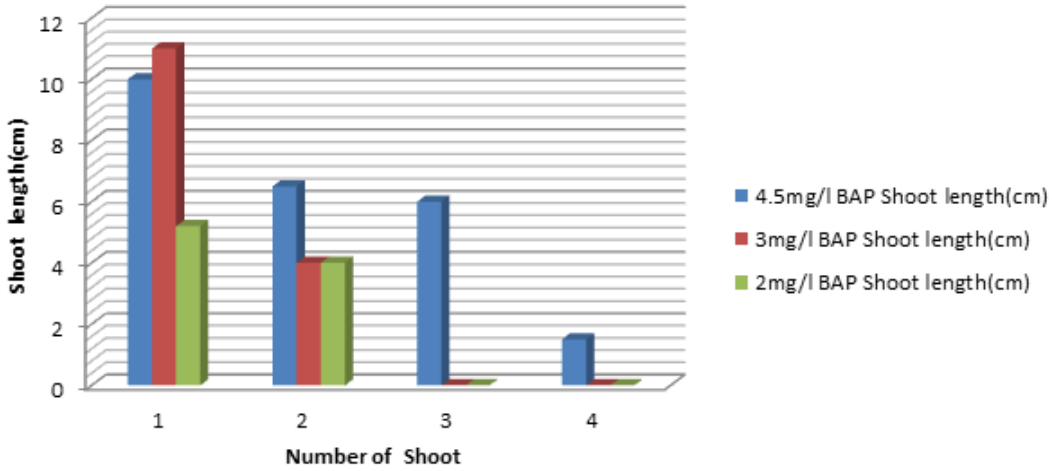

Figure 9. Effect of BAP on shoots length (cm) of *Z. officinale* for three months of culture.

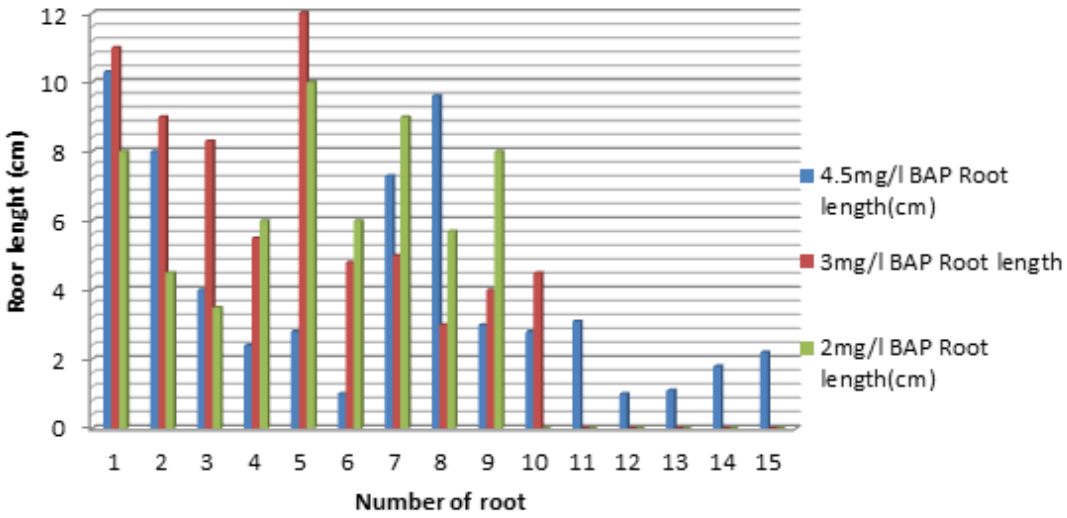

Figure 10. Effect BAP on roots length (cm) of *Z. officinale* for three months of culture.

Table 3. Effect BAP on shoot length (cm) of *Z. officinale* for three months of culture.

PGR	No. shoot	Shoot length (cm)
2 mg/l BAP	1	5.2
	2	4
3 mg/l BAP	1	11
	2	4
4.5 mg/l BAP	1	10
	2	6.5
	3	6
	4	1.5

roots (Figure 11A and 12), compared to other treatments, which were one shoot and 7 ± 0.00 roots by combination of 2.00 mg/L BAP+0.5 mg/L NAA (Figure 11B), one shoot and 20 + 0.15 roots were initiated by 3.00 mg/L BAP + 0.5 NAA (Figure 11C), while the combination of 4.00 mg/L BAP + 0.5 mg/L NAA induced 21 roots and no shooting was observed during three months of culture (Figure 11D). Our findings are in harmony with Solanky et al. (2013) who investigated *in vitro* regeneration of ginger through callus culture, they found that maximum shoot regeneration per gram of callus was achieved from the MS media containing 1 mg/L BAP with 1 mg/L Kin 82.24% shooting with 8.62 number of shoots under 2000 lux light intensity. Taha et al. (2013) reported that the highest number of regenerated shootlets of callus derived from ginger leaves were (1.67) by MS+1 mg/L BAP, and the lowest number of regenerated shootlets (0.4) was recorded with 4 mg/L BAP.

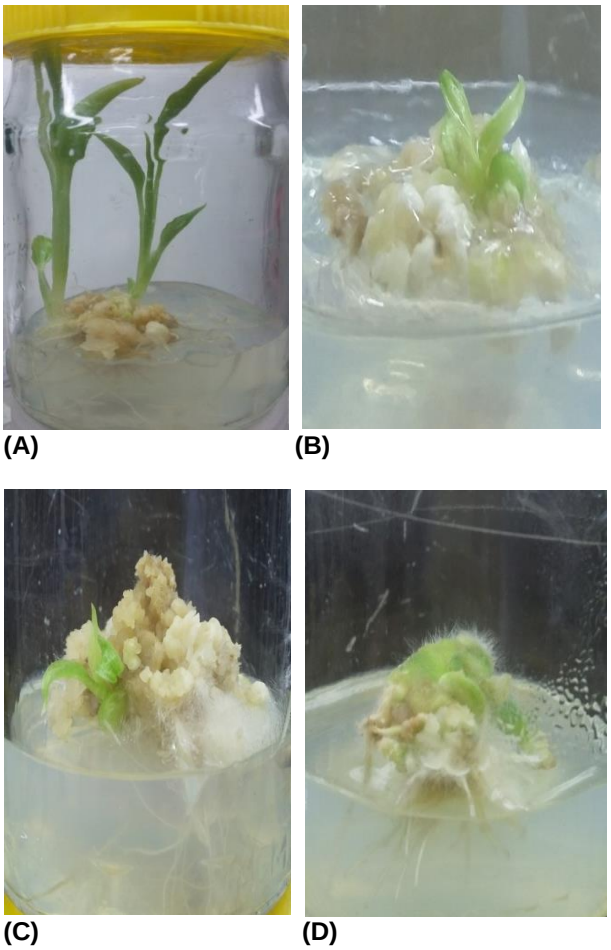

Figure 11. Indirect organogenesis (*In vitro* shoots and roots initiation) on MS medium supplemented with combinations of: (A)1.00 mg/L BAP+0.5 mg/L NAA, (B)2.00 mg/L BAP+ 0.5 mg/L NAA, (C) 3.00 BAP+0.5 mg/L NAA and(D) 4.00 BAP+0.5 mg/L NAA.

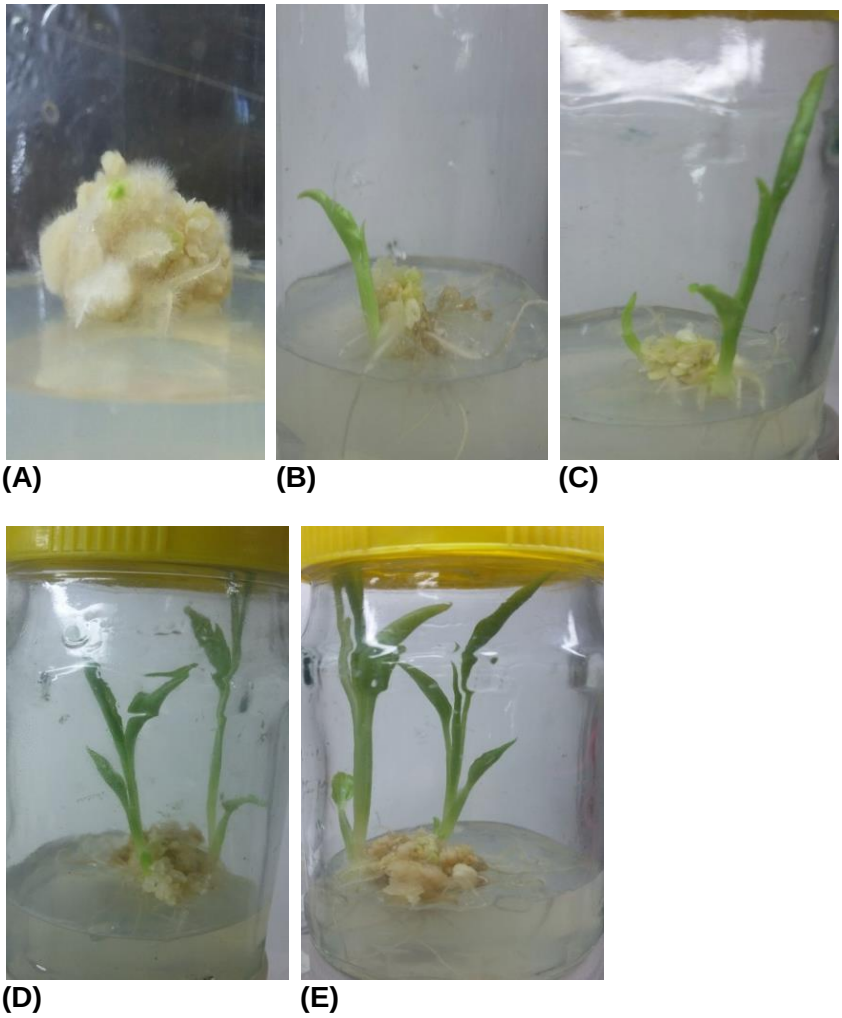

Figure 12. Development the best indirect organogenesis (*In vitro* shoots and roots initiation) on MS combined with 1.00 mg/L BAP+0.5 mg/L NAA during three month of culture. (A) Callus subcultured on MS + 0.5 mg/L 2,4-D for six weeks (B, C, D and E), *in vitro* shoots and roots initiation during three months of culture.

The medium containing BAP+NAA enhanced roots formation. Similar finding was reported by Nkere and Mbanaso (2010), they investigated optimizing concentrations of growth regulators for *in vitro* ginger propagation; they revealed that combination of BAP+NAA supports root formation.

Conclusion

From the study, we can conclude that callus was only induced from shoot tip explant. The highest significant value of callus fresh weight was induced from shoot tip explants by 1.00 mg/L of 2,4-D. A suitable concentration to regenerate callus by subculture was 0.5 mg/L 2,4-D. Direct organogenesis showed highest number of *in vitro* shoots and roots by 4.5 mg/L BAP, and the best shoots and roots from indirect organogenesis were induced by combination of 1.00 mg/L BAP+0.5 mg/L NAA.

Conflict of Interests

The authors have not declared any conflict of interests.

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African Journal of Biotechnology

Full Length Research Paper

Antibacterial potential of silver nanoparticle synthesized by marine actinomycetes in reference with standard antibiotics against hospital acquired infectious pathogens

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Multi resistance to antibiotics is a serious and disseminated clinical problem, common to several new compounds that block the resistance mechanism. The present study aimed at the comparative study of silver nanoparticles synthesized through actinomycetes and their antimicrobial metabolites with standard antibiotic. Marine actinomycetes collected from Gulf mannar costal region, Kayalpatnam, located at Tuticorin district, Tamil nadu, India. Totally, five actinomycetes were isolated and identified based on their spore formation and biochemical studies. Three isolates belonged to the genera of *Streptomyces* sp and two were M*icromonospora sp*. *Streptomyces* sp KPMS3 showed potent antibacterial activity against Gram negative pathogens. Gram positive isolates are sensitive to *Micromonospora* sp (KPMM2). Among the five isolates, isolate *Micromonospora* sp (KPMM2) was found to be an effective silver nanoparticle synthesizer. The obtained silver nanoparticles were characterized using UV-Vis spectroscopy, FTIR and TEM. The morphology of nanoparticle is found to be spherical and an average size of ranges between 38 to 52 nm. The antimicrobial activities of silver ion against test pathogens were found to be superior to cephalosporin antibiotic. The *in vitro* hemolytic assessment of silver nanoparticles were found to be non-hemolytic at maximum of 20 µg/ml. It was found that smaller silver nanoparticles synthesized by microbial route had a greater antibacterial activity and less hemolytic in nature.

Key words: ESBL, cefotaxime, drug resistant and hemolysis.

INTRODUCTION

Hospital acquired infection are a cause of prolonged hospital stay and contagious which may lead to high morbidity and mortality throughout the hospital patients. Hospital acquired infection constitute an economic

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worries on health care. It is estimated that 80% of all hospitals death is directly or indirectly linked to Hospital acquired infection (Hughes et al., 2005). In India, 10 to 30% patients are admitted in hospitals or nursing homes are associated with hospital acquired infection. The failure of this infection due to the development of drug resistance organisms and the rate is 3.4% (Khoiee et al., 2008). Antibiotic resistant hospital infection can be especially deadly because antibiotics are used intensely in hospitals compared with the community and frequent use drives the development of multi drug resistance bacteria. Microbes are known to fit on inanimate source such as touch surface for extending period of time. This can be making troublesome in hospital environments where patients with weakened immunity are at increased risk for contracting hospital acquired infection (Hidron et al., 2008). The spreading of drug resistant bacteria is stopped by using the application of nanotechnology in order to synthesis nanoparticle. Nanoparticles (Nps) are focused as fundamental building blocks of nanotechnology, particularly silver nano particles. Indeed over a past several years, silver nano particle are synthesized by a potential new antibiotics developer actinomycetes from marine sediments (Absar et al., 2003).

Actinomycetes are free living gram positive saprophytic bacteria and it has a major source for production of numerous natural biological metabolites especially antibiotics. Silver nanoparticle play an important role in many field such as nano crystalline silver dressings, creams, gel effectively reduce bacterial infections in chronic wounds (Ip et al., 2006) and also used to control bacterial infectious disease (Smriti et al., 2012). Silver is the metal of interest and it provides a most durable antimicrobial protection against microorganism. Nano sized silver particle is the most promising source to kill microbes very effectively because that act on both intra and extracellularly. Silver nanoparticle exhibit as a strong bactericidal agent and depicts activity against both multi drug resistant gram positive and negative bacteria (Zeng et al., 2007; Roe et al., 2008). Marine actinomycetes will develop a valuable resource for novel metabolites of pharmaceutical and medicinal interest, including antimicrobial agents (Mitsuiki et al., 2002). The objective of this work was to synthesize characterize and evaluate the efficacy of biosynthesized Ag Nps against nosocomial pathogens.

MATERIALS AND METHODS

Sample collection

Systematic screening of actinomycetes from marine sediments was done by random sample collection between 200 m distance at a depth of 5 m using core sampler from Gulf mannar costal region, Kayalpatnam, located at Tuticorin district, Tamil nadu, India. The central portion of the marine sediments were aseptically transferred to the sterile bottles during June 2013 and brought up to laboratory with help of ice bag. The sediments sample was blackish brown colour and of a sandy texture.

Isolation of actinomycetes

All the marine sediments were air dried to minimize bacterial contaminants. One gram sediments were serially diluted up to 10^{-6} dilution. One ml of diluted sample was permitted in to the Petri plate followed by Starch casein nitrate agar (SCNA) medium supplemented with cyclohexamide 50 µg/ml and nystatin 50 µg/ml. After solidification, all the plates were incubated at 28°C for 7 to 15 days until the colonies were developed.

Spore morphology

All the isolates were identified by slide culture method. A drop of 0.1% tryphan blue stain was placed over the glass slide and then cover slip was placed over the stain gently. The slide was examined under bright field microscope and the spore was noted.

Antibacterial activity of secondary metabolites

Antimicrobial activity of actinomycetes culture filtrate was analyzed with agar well diffusion method. The 24 h nutrient broth culture of multidrug resistant pathogens such as ESBL *Escherichia coli*, ESBL *Klebsiella pneumonia, Enterococcus faecium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Proteus mirablis* and *Proteus vulgaris* were isolated from hospital acquired infection and bio assayed against standard antibiotics. 100 µl of actinomycetes culture filtrate were used as a test sample against clinical isolates. Inoculated plates were incubated at 37°C for 24 h. After incubation, all plates were examined for the presence of zone of inhibition around the wells.

Bio synthesis and characterization of silver nanoparticles

The isolated actinomycetes culture was inoculated in to ISP IV medium and incubated at 28°C for 7-15 days. Actinomycetes broth medium was centrifuged at 5000 rpm for 10 to 20 min to remove cell debris. 10 ml of actinomycetes cultured filtrate was permitted in to the Ag NO_3 aqueous solution and incubated at room temperature in a shaker under dark condition. Bio reduction was monitored by colour change and UV spectrum analysis. Further the nanoparticles were collected at 15, 000 rpm centrifugation and characterized by FTIR and SEM analysis.

Antibacterial activity of silver nanoparticles

Antimicrobial activity of biosynthesized nano particle was analyzed with agar well diffusion method. The multidrug resistant pathogen such as ESBL *E. coli*, ESBL *K. pneumonia, E. faecium*, *S. aureus*, *P. aeruginosa, P. mirablis* and *P. vulgaris* were isolated from hospital acquired infection and inoculated into nutrient broth and incubated at 37°C for 24 h. After incubation, the test pathogens were inoculated on Muller Hinton agar (MHA) by using sterilized cotton swabs. In each of these plates, wells were cut out using a sterilized gel borer and 50 µl of biosynthesized Ag NPs were used as a test sample against clinical isolates. Inoculated plates were incubated at 37°C for 24 h. After incubation, all plates were examined for the presence of zone of inhibition around the wells.

Haemolysis assay of Ag NPs

The cytotoxic effect of silver nanoparticles was studied by

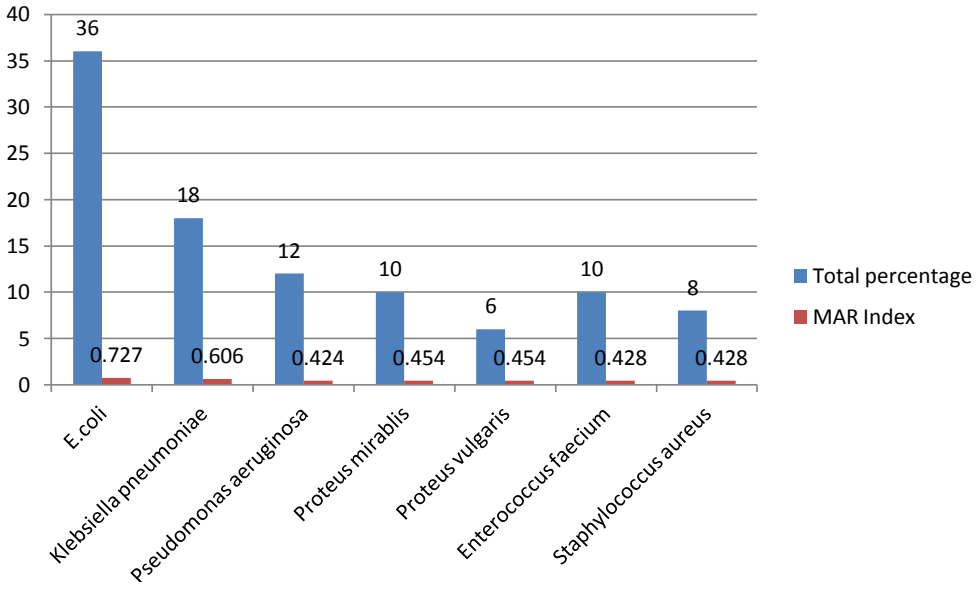

Figure 1. Distribution of isolated pathogen and its MAR index.

performing hemolytic test (Krajewski et al., 2013).Two milliliters (2 ml) of blood was mixed with 0.2 M PBS (pH 7.0) and centrifuged at 10000 rpm for 10 min. The pellet of RBC was collected and washed three times by PBS at 10000 rpm for 3 min. The obtained RBC was diluted with PBS at 1:10 ratio. 10 µL of nanoparticles in tyrode (1, 5, 10, 25 and 50 µg/ml), tyrode (negative control) and triton X-100 (positive control) were added to 290 µL of washed RBC. The suspension was incubated at room temperature by shaking plate for 12 h. After incubation, the suspension was centrifuged at 10000 rpm for 5 min. Supernatant was recorded in spectrophotometer at 550 nm. The haemolysis (H) was calculated as:

H (%) = (OD sample – OD tyrode) / (OD Triton X-100 1% - OD tyrode)*100.

RESULTS AND DISCUSSION

Isolation of marine actinomycetes

The present work was used to carry out the synthesis of silver nanoparticle by marine actionomycets collected from Gulf Mannar Costal Region, Kayalpatnam, located at Tuticorin district, Tamil nadu, India. Based on the cultural characterization of Actinomycetes were identified as *Streptomyces* sp(KPMS) and *Micromonospora* sp (KPMM). All the isolated Actinomycetes strains were Gram positive but differ morphologically by producing different mycelium and spore. *Streptomyces* sp showed white coloured aerial mycelium and dark brown substrate mycelium with refractile spiral spore. The colors of the substrate mycelium were yellowish white to dark ash after sporulation. The morphological characteristics of these isolates were consistent with their classification in the genus *Micromonospora* sp (Kawamoto, 1989). Currently, taxon actinomycetes are accommodates spore forming gram positive bacteria that from extensive branching substrates and aerial mycelia. Based on this developments, the actinomycetes strains were recorded (Waksman, 1961).

Isolation of drug resistant pathogens

Out of 34 samples, 28 showed poly microbial infection and six were found to be negative. Totally, 50 isolates were recorded and identified as *E. coli, K. pneumoniae, P. aeruginosa, P. mirablis, P. vulgaris, E. faecium* and *S. aureus.* The highest numbers of patients with nosocomials were found within the age range of 40 to 50 followed by the age range 50 to 60. Figure 1 represents the frequency and MAR index of tested isolates against standard antibiotics. The percentages of frequency of isolated pathogens were 36 ≥ 18 ≥ 12 ≥ 10 ≥ 06 ≥ 10 ≥ 8. The most common nosocomial pathogens in our study were *E. coli* (36%) and *K. pneumonia* (18%). *E. coli* was the predominant bacteria found in hospitalized patients (Manikandan et al., 2011). The antimicrobial sensitivity of isolated clinical pathogens reveals that 76 percent of isolates were found to be MDR. Among the tested genera's *E. coli* and *K. pneumonia* showed high degree of resistance against all tested antibiotics. The MAR indices of indigenous bacterial isolates ranges was found in 0.727 to 0.424 (Figure 1). The highest multiple antibiotic resistant indices (MARI) for *E. coli* were 0.727. Mandal et al. (2001) determined that the overall rate of resistance against *E. coli* was worldwide reported which was similar with this study. Multiple antibiotics resistance (MAR) index is a tool that reveals the spread of bacteria resistance in a given population (Krumpermann, 1983). A

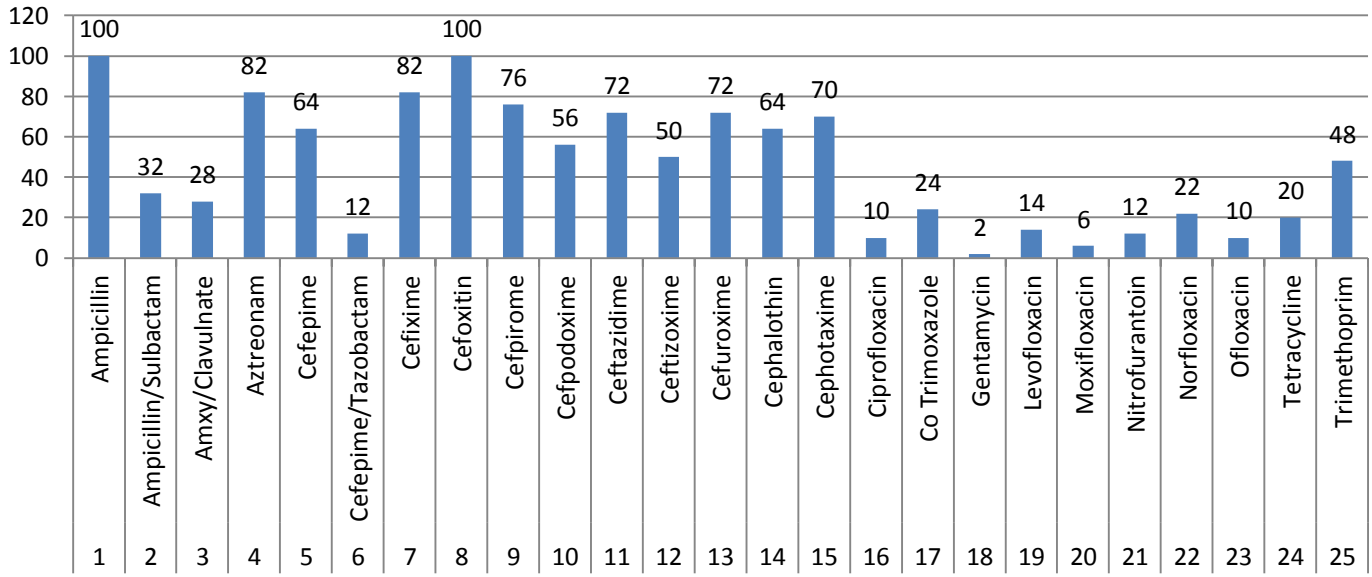

Figure 2. Percentage of Gram negative Resistant Isolates against tested Antibiotics.

MAR index greater than 0.20 implies that the strains of such bacteria originate from an environment where several antibiotics are used. The MAR indices obtained in this study is a possible indication that a very large proportion of the bacteria isolates have been exposed to several antibiotics. These bacteria are common environmental organisms which act as opportunistic pathogen in clinical cases were the defense system of the patient is compromised to broad spectrum antibiotic resistance particularly penicillin and cephalosporins (Obritsch et al., 2004).

Out of 32 broad spectrum tested antibiotics, 13 antibiotics were come under cephalosporin groups, two were aminoglycosides (amikacin and gentamycin), five antibiotics come under fluoroquinolones (ciprofloxacin, ofloxacin, norfloxacin, moxifloxacin and levofloxacin), four penicillin derivatives (ampicillin, ampicillin/sulbactam, amoxy/clavulnate and piper tazobactam), two of sulfonamides (trimethoprim and cotrimoxazole), three of carbapenems and one of tetracycline, nitrofurans and monobactam (aztreonam) were tested against 50 isolates. Antibiotic sensitivity reveals that the high degree of resistance (50 to 100%) was reported against cephalosporin, monobactam and ampicillin. The 100% of ampicillin resistance were significantly reduced into 28% by clavalunate (Figure 2). Significant resistance (≤ 50%) was observed against fluoro quinolones derivatives. Among the tested antibiotics seven of antibiotics belong to amikacin, carbapenems, tazobactam and cepahotoxime clavulnate showed potent antibacterial activity against tested pathogens. No resistance was observed against these antibiotics. It has been reported that amikacin is the most effective antibiotic against *E. coli* and *K. pneumonia* (Schaeffer et al., 2001). Our result was further supported by another study where the susceptibility rate of *E. coli* to amikacin remained 93 to 100%. Out of four tested Gram positive antibiotics against *S. aureus* and *E. faecium* 100% of resistance was isolated against penicillin and methicillin (Figure 3). *S. aureus* showed 33% of resistance against doxycycline and no resistance was found against vancomycin. *E. faecium* showed 33% of resistance against vancomycin and no resistance was observed against doxycycline. As described previously, methicillin resistance was associated with resistance to other antibiotics (David and Daum, 2010). In the present study, high prevalence of MRSA infection have showed sensitive to vancomycin encourages the usage of vancomycin than doxycycline (Appelbaum, 2007).

Antibacterial activity of marine actinomycetes isolates

The antimicrobial activity of culture filtrate reveals that *Streptomyces* sp KPMS 3 showed potent antibacterial effect (18 mm) against ESBL *K. pneumoniae* and *E. coli,* moderately active against *P. aeruginosa and P. vulgaris* but less significantly active against *P. mirablis. S. aureus* and *E. faecium* were not sensitive against *Streptomyces* sp but highly sensitive against *Micromonospora* sp. Of these two isolates of *Micromonospora* sp KPMM1 showed antibacterial activity against *S. aureus* and *E. faecium* (Table 1). It was found that amikacin was more effective than actinomycetes due to the productivity and purity of metabolites during fermentation. *Micromonospora* and *Streptomyces* sp the common inhabitants and have proved to be a continuing source of

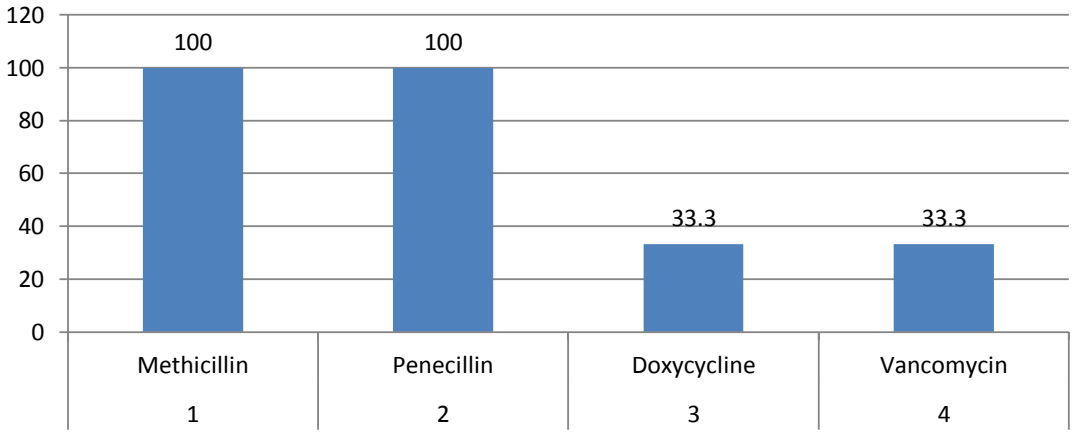

Figure 3. Percentage of gram positive resistant isolates against tested antibiotics.

Table 1. Antibacterial activity of Marine Actinomycetes against Multi drug resistant pathogens.

Strain code	*ESBL E coli*	*ESBL Klebsiella pneumoniae*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Proteus mirablis*	*Staphylococcus aureus*	*Enterococcus faecium*
KPMS 1	-	-	-	-	-	-	-
KPMS 2	-	-	-	-	-	-	-
KPMS3	18±0.04	18±0.036	15±0.02	16±0.04	12±0.4	-	-
KPMM1	-	-	-	-	-	14±0.04	16±0.02
KPMM2	-	-	-	-	-	-	-
Amikacin	18±0.02	18±0.02	18±0.1	18±0.04	20±0.1	20±0.2	20±0.04

novel bioactive compound having great structural and functional diversity including antibacterial, antifungal, antiviral, anticancer agents (Koehn and Carter, 2005). Actinomycetes that produce secondary metabolites often have the potential to produce various compounds from a single strain (Schiewe and Zeeck, 1999).

Bioreduction of silver nitrate

The culture filtrate of *Micromonospora* sp showed pale yellow color before the addition of silver ions and converted to brownish colour. The appearance of pale yellow color to brownish color in the solution containing biomass was indication of the formation of silver nanoparticles (Ag NPs) (Sastry et al., 2003) further confirmed at 450 nm UV- Visible surface Plasmon absorption peak (Figure 4). The homogenous Ag Nps are known to produce the surface Plasmon resonance band at the range of 420 to 450 nm (Duran et al., 2007). The absorption peak at 450 nm by *Streptomyces glaucus* was observed by one another researcher (Tsibakhashvili et al., 2011). FT-IR measurements was carried out to identify possible interaction between silver and protein molecules which may be responsible for synthesis, stabilization and well dispersed silver nanoparticles in the reaction mixture (Navin et al., 2011). FT-IR revealed a spectrum at 3467. 19 and 3435. 06 cm^{-1} which is assigned to the hydrogen bonded OH stretch, peak such 2386 cm-1 corresponding to carboxylic acid group. Similarly, peak 1635 is assigned to the primary or secondary amines (N-H) (Figure 5). The FT-IR studies confirmed the fact that COOH derivative have strong ability to bind silver and to stabilize the synthesis of nanoparticles (Mervat et al., 2012).

Antibacterial activity of biosynthesized silver nanoparticles

Antibacterial study of silver ions showed broad spectrum antibacterial activity against all tested pathogens. The maximum zone of inhibition was 24 mm against ESBL *E. coli* and ESBL *K. pneumoniae* with 75% of relative zone of inhibition (RIZD*).* 22 mm zone of inhibition was recorded against *P. aeruginosa* and *E. faecium* with 55 and 66% RIZD (Table 2). These result suggested that the biosynthesized silver nanoparticles are highly potent than amikacin and cephalosporin antibiotic. The antimicrobial activity was reported to be due to the penetration of Ag

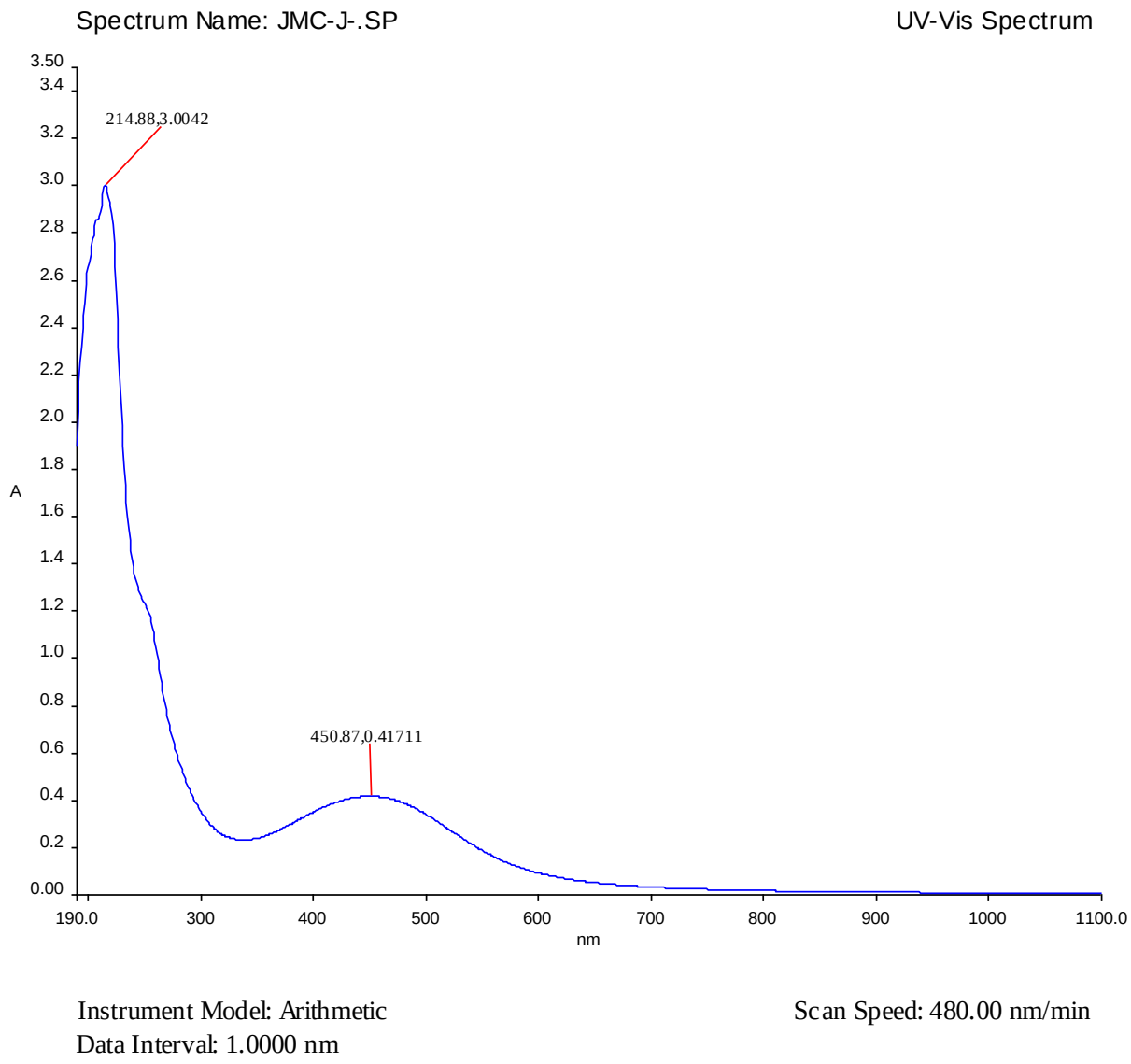

Figure 4. Characterization of silver synthesized nanoparticle by U-vis spectroscopy.

Nps into the drug resistant bacteria, damaging the cell membrane and released cell contents (Panacek et al., 2006). Antimicrobial property of silver nanoparticles depends on the size of nanoprticles synthesized (Richard et al., 2012). Smaller size of nanoparticle effectively penetrates to cell due to its larger surface availability for interaction and interfere the metabolism of cell. In this study, the TEM analysis reveals that (Figure 6) size of nanoparticles was found to be 38 and 52 nm, respectively. The biomolecules present in the surface of nanoparticles leads to agglomeration structure. The similar results were observed by *Bacillus licheniformis* mediated silver nanoparticles (Kalimuthu et al., 2008). The application of silver in combination with microbial system would be effective in enhancing its antimicrobial activity.

Hemolytic effect of silver nanoparticles

Figure 7 shows the synthesized nanoparticles showed no hemolytic activity at 10 and 20 µg/ ml but less significantly at 30 µg and have significant hemolytic activity at 50 µg/ml after 12 h incubation. 90% Haemolysis was observed at 50 µg/ml after 12 h incubation. A lower hemolytic activity was observed at 25 µg can lead to adverse health effects. Result showed that the concentration of Ag Nps is safe at the range of 10 to 20 µg/ml. Negative control (tyrode) and positive control (triton X-100 1%) induced 0 and 100%, respectively of hemolysis. The determination of hemolysis is based on hemoglobin absorbance at 550 nm, with subtraction of the interference of Ag NPs. Although, various studies evaluated the hemolytic activity of Ag NPs, our results

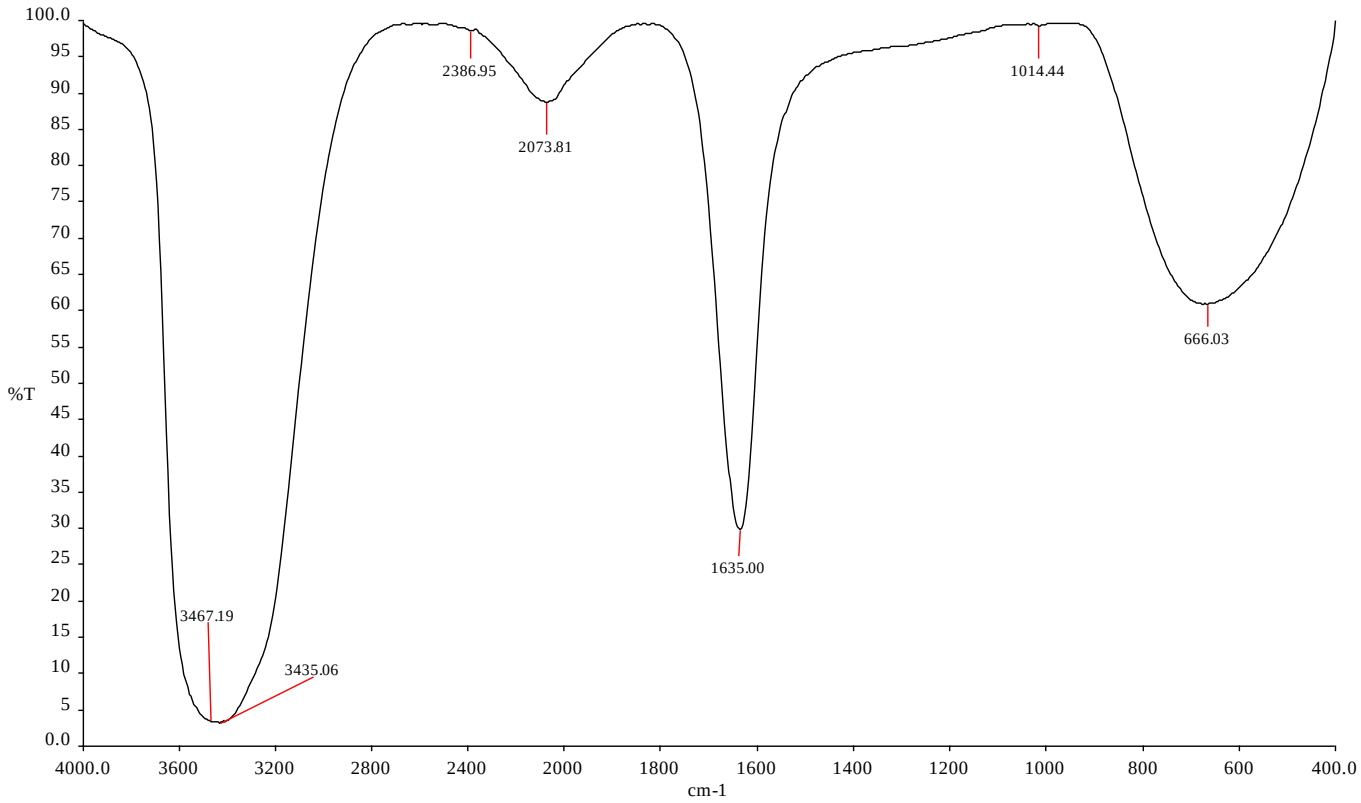

Figure 5. Characterization of silver synthesized nanoparticle by FTIR analysis.

Table 2. Antimicrobial activity of biosynthesized silver nanoparticles against MDR pathogens.

Sample	*ESBL E. coli*	*ESBL K. pneumoniae*	*P. vulgaris*	*P. aeruginosa*	*P. mirablis*	*S. aureus*	*E. faecium*
AgNP B	24	24	20	22	20	18	22
Cephotaxime	16	16	16	18	16	16	18
Negative control	12	12	12	12	12	12	10
RIZD	75	75	44	55	44	37	66

are in agreement with previous studies that concluded to the prohemolytic properties of Ag NPs (Choi et al., 2011). These results may have a clinical impact at high concentration of Ag NPs, since a release of hemoglobin can lead to adverse health effects such as anaemia.

Conclusion

Many of these secondary metabolites are potent antibiotics, which have been made by marine *Streptomyces* sp, the primary antibiotic-producing organisms exploited by the pharmaceutical industry. It is suggestive that the exploitation of *Streptomyces* sp. in nanotechnology has recently received considerable attention. Marine source provides a promising source of Actinomycetes that can ruin the multidrug resistant pathogens. Silver nanoparticles have an important advantage over conventional antibiotics in that they kill all pathogenic microorganisms, no organisms has ever been reported to readily develop resistance to it because of their high reactivity that is due to the large surface to volume ratio. The synthesis of nanomaterials of specific composition and size is a burgeoning area of materials science.

Conflict of interest

The authors have not declared any conflict of interest.

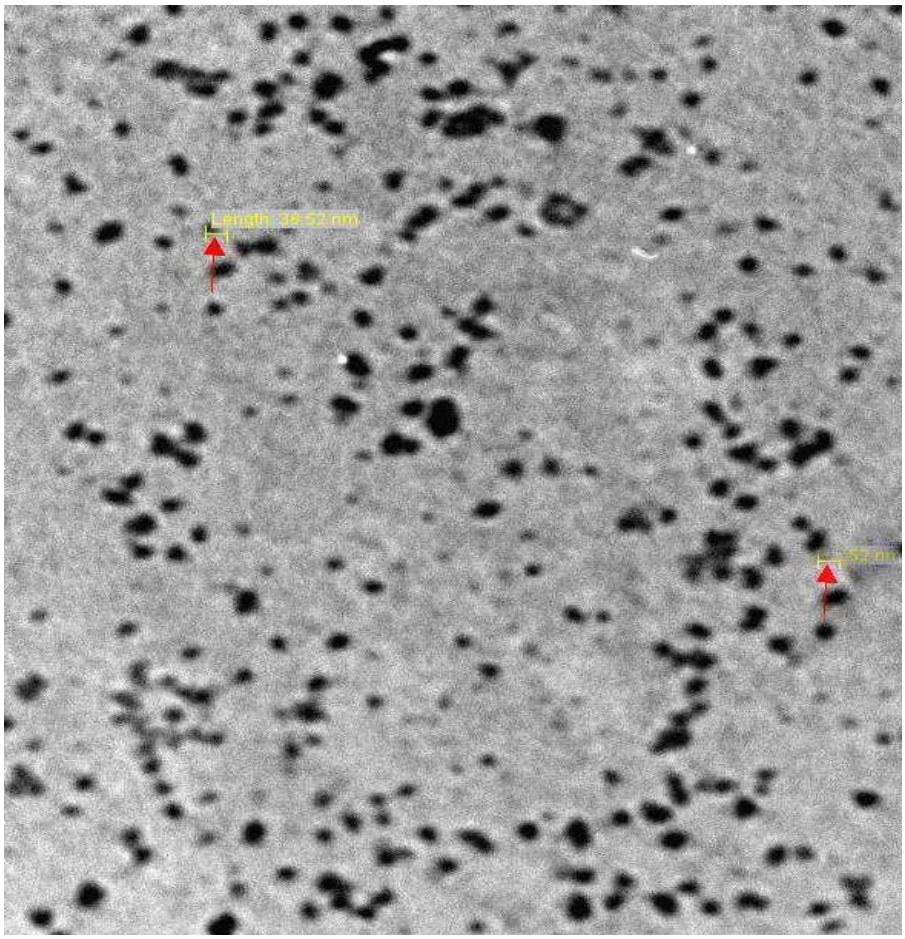

Figure 6. TEM image of microbial and chemically synthesized silver nanoparticle.

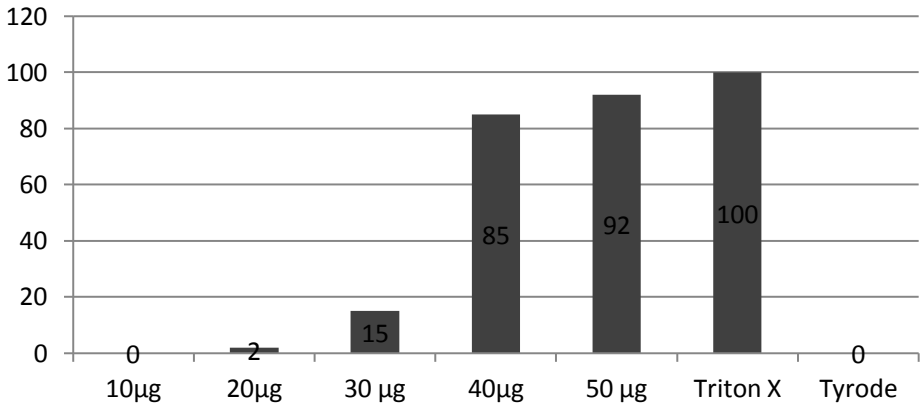

Figure 7. Hemolytic effect of synthesized silver nanoparticles.

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