The impact of silver nanoparticles on plant physiology

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ABSTRACT

Engineered nanoparticles (ENPs) are increasingly being exploited in a whole range of applications and products due to their novel physicochemistry. Hence it is inevitable that ENPs will enter the environment at an increasing rate over the coming years. The consequential impact following interaction between ENPs with plants and soil microbial communities is of great concern given that they play fundamental roles in the environment and food production.

In this study, the impact of capped silver nanoparticles (cAg NPs) on terrestrial (*Arabidopsis thaliana* and *Vicia faba*) and aquatic (*Lemna minor*) plants was investigated. In addition, due to the important role of bacteria in plant survival and growth, this study also assessed the effect of cAg NPs on plant-associated soil microbial community structure.

cAg NPs demonstrated varied toxicity towards plants and the associated soil microbes. Whilst the aquatic plants and soil microbial communities investigated in this study were not affected by cAg NPs up to 100 mg/L, for the terrestrial plants evaluated here, cAg NPs above 12 mg/L (specifically 50 and 100 mg/L) demonstrated differential toxic responses. Based on the results of this study, it is clear that concentration, exposure method, released ions, plant species, light intensity and growth mediums are key factors that influence the toxicity of cAg NPs.

Although the cAg NP concentrations applied in this study are not yet environmentally relevant, with continued and uncontrolled commercial production of Ag NPs and/or in the event of spillage, such concentrations could occur in the environment in the future.

Chlorophyll fluorescence and gas exchange are valuable techniques for analysing the toxicity of ENPs on plants, due to their rapid and reliable results. Further studies in the interactions between plants and Ag NPs are urgently needed and would benefit from the use of different application methods such as aerosolization.

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ABBREVIATIONS

A	assimilation rate
AFM	atomic force microscopy
$AgNO_3$	silver nitrate
AR	ampliflu (amplex) red
cAg NPs	capped silver nanoparticles
DGGE	denaturing gradient gel electrophoresis
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DW	distilled water
ENPs	engineered nanoparticles
FIFFF	flow field-flow fractionation
F_v/F_m	maximum quantum efficiency of PSII photochemistry
F_q'/F_m'	operating efficiency of PSII photochemistry
g_s	stomatal conductance
GP	germination percentage
HNO_3	nitric acid
H_2O_2	hydrogen peroxide
ICP-MS	inductively coupled plasma – mass spectrometry
mPEG	methoxy PEG
mRNA	messenger RNA
NPs	nanoparticles
PCR	polymerase chain reaction
PEG	polyethylene glycol
PPFD	photosynthetic photon flux density
ROS	reactive oxygen species
rRNA	ribosomal RNA
TAE	tris-acetate-EDTA
TEM	transmission electron microscopy
UHP	ultra-high purity

UNITS

°C	degree Celsius
g	gram
h	hour
kg	kilograms
L	litre
µg	micrograms
µL	microliter
mg	milligrams
min	minutes
%	percentage
s	second
v	volts

Chapter 1. Introduction

1.1. Nanotechnology and nanoparticles

"Nanos" which in Greek means "a dwarf", is the origin of the prefix "nano". Scientifically, this prefix has been used to refer to one billionth; therefore, one billionth of a meter equals one nanometer (Whatmore, 2006). In 1974 the term "nanotechnology" was first used by Norio Taniguchi (Taniguchi, 1996). Today, the term refers to those technologies that deal with materials at the level of nanometers (Whatmore, 2006).

The European Union has defined a nanomaterial as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm" (Rauscher *et al.*, 2015).

Nanoparticles (NPs) can be divided into three main classes: natural, incidental, and engineered. Since the beginning of the history of the earth, natural NPs have been presented in the environment as a result of natural sources such as volcanoes (Handy *et al.*, 2008). The second class, incidental NPs, result from human industrial activities, for example, burning coal and diesel exhaust (Monica and Cremonini, 2009). Of particular interest is the third class which represents engineered nanoparticles (ENPs). These have unique physicochemical properties such as conductivity, reactivity, and optical sensitivity which show different characteristics compared to their bulk form (Lin and Xing, 2007). This ENPs class has been divided into five subclasses: carbonaceous nanoparticles, metal oxide nanoparticles, semiconductors, metal nanoparticles, and nanopolymers (Handy *et al.*, 2008; Monica and Cremonini, 2009; Ma *et al.*, 2010; Bhatt and Tripathi, 2011). Figure 1.1 summarizes the different types of nanoparticles.

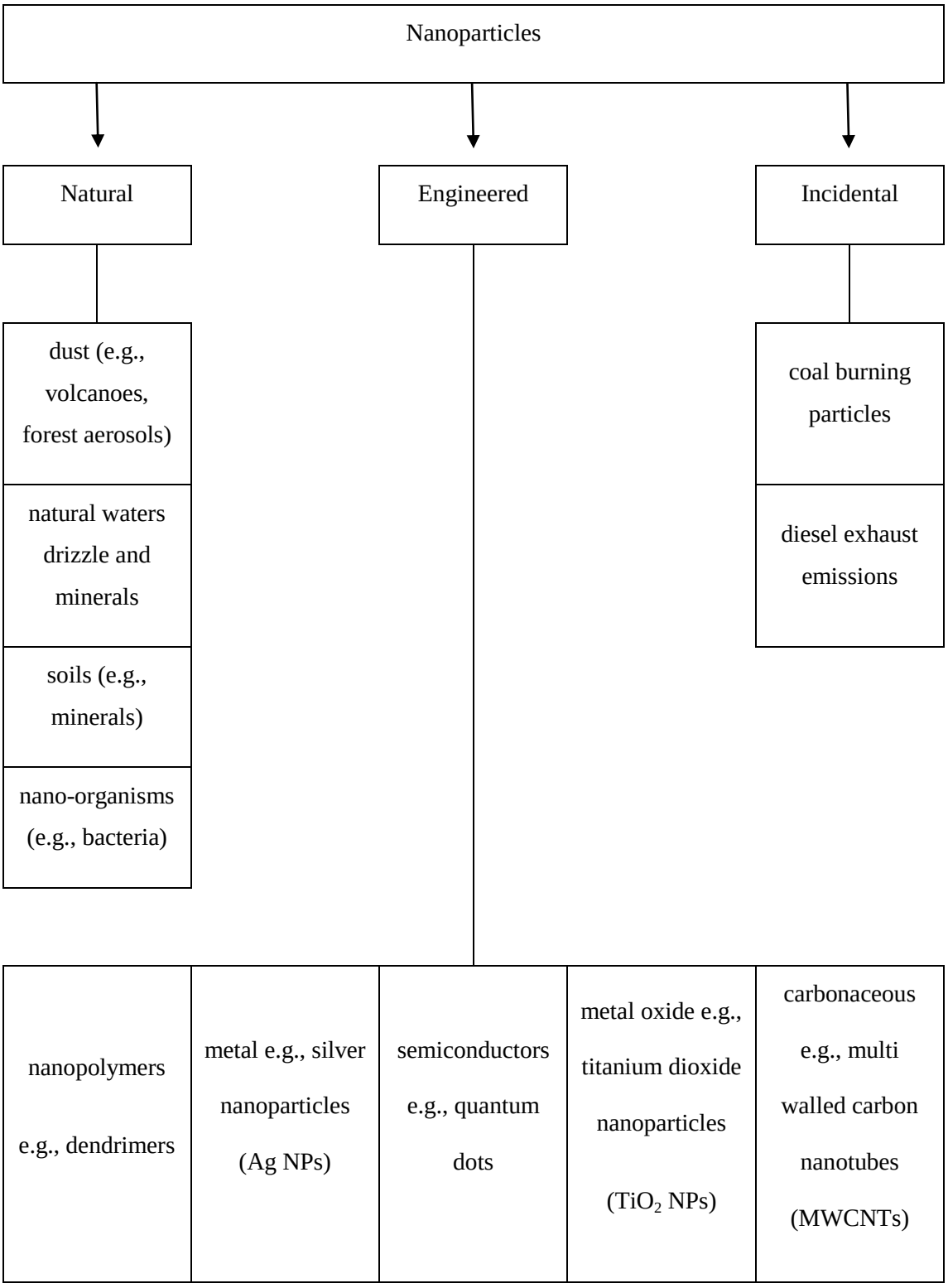

Fig. 1.1. Different types of natural, engineered, and incidental nanoparticles.

The emergence of nanotechnology can be described as an industrial revolution (Kurwadkar *et al.*, 2014) and it is one of the advanced technologies that has been invested industrially in order to improve products and meet preferable properties. Such use of nanotechnology has shown many advantages; for example, improved energy efficiency, and better performance of the nanotechnological products and applications (Brar *et al.*, 2010; Keller *et al.*, 2013).

Since the emergence of nanotechnology, many disciplines have employed ENPs due to their novel or enhanced properties which contribute to unique functions. As a result, ENPs are used widely in a diverse range of products and applications (Petersen *et al.*, 2014); for example, as antimicrobials and photocatalytic agents. Today, nanotechnology is used in areas that include: consumer products such as household chemicals, sports equipment, textiles, cosmetics, medicine, personal care products and electronics, in addition to environmental and energy applications (Dowling *et al.*, 2004; Woodrow Wilson database, 2015). The international nanomaterials market was estimated to be $125 million in 2000 and expected to reach $30 billion by 2020 (Barceló and Farré, 2011). The annual production of products containing ENPs globally was estimated to be 2000 tons in 2004, however, by 2020, it will increase above 58,000 tons (Gubbins *et al.*, 2011) and this predicted to grow (Navaaro *et al.*, 2008). More importantly, with this continuing growth, a shift towards the production of ENPs rather than their corresponding bulk material may occur (for example, with titanium (TiO_2)). Assuming such a scenario, a complete shift towards the production of ENPs would be achieved by 2025 leading to the production of around 2.5 million metric tons of ENPs per year (Robichaud *et al.*, 2009). As a result, nanotechnology and its applications have become a key point of discussion in many different societies for more than two decades (Peralta-Videa *et al.*, 2011).

1.2. Engineered silver nanoparticles and their use in industrial products

Silver is one of the heavy metals whose abbreviation ‘Ag’ comes from the Latin word argentum. Due to its well-known and valuable antimicrobial properties, humankind has used Ag widely for many centuries in everyday life, such as in cutlery, jewellery, and currency with little, if any, toxic effects on human health (Quadros and Marr, 2010). Thus the idea of using silver in industrial applications is already well accepted (Kedziora *et al.*, 2013). However, due to the novel physicochemical properties of Ag NPs (Nowack *et al.*, 2011), they are the most significant ENPs used in industrial products (as for example, antimicrobial and antifungal agents) (Sun *et al.*, 2014) and are increasingly entering the market (Quadros and Marr, 2011). According to the Woodrow Wilson database (2015), 1827 products that contain ENPs are currently available on the market and those that contain Ag NPs make up around one third of this. Ag NP-containing products include textiles, medical equipment, paints, cosmetics, washing machines, and others.

The accurate global production volume of Ag NPs is unknown and studies that have attempted to predict this volume have reported a wide range of estimations. Piccinno *et al.* (2012) predicted the production of Ag NPs worldwide to be ranged from 5.5-550 tons/year. Keller *et al.* (2013) reported that 452 metric tons/year was globally produced in 2010. It was estimated that whilst Europe produced 0.6-55 tons/year (Piccinno *et al.*, 2012), the U.S. produced 2.8-20 tons/year (Hedren *et al.*, 2011). In 2012, the total manufactured Ag NPs in Europe was reported to be more than 30 tons (Sun *et al.*, 2014). From the above mentioned estimations, the production volume of Ag NPs ranges from less than 1, to 550 tons/year and the variation is large. Knowing the exact volume of ENPs and particularly Ag NPs would greatly contribute to establishing the basis for nanotoxicological investigations.

1.3. Engineered nanoparticles in the environment

Nano-environmental studies provide growing evidence that ENPs can be found in the environment and their quantities are predicted to increase in the future (Gottschalk and Nowack, 2011; Nowack *et al.*, 2012; Sun *et al.*, 2014). Yet, the current concentrations of ENPs in the environment are not well known (Sun *et al.*, 2014) due to the lack of tools that are available to monitor and quantify their release and concentration in the environment (Gottschalk and Nowack, 2011).

Direct (for example, from manufacturing points and spills (Gottschalk and Nowack, 2011)) and indirect (for example, from nanotechnological products (Nowack *et al.*, 2012)) release of ENPs into all environmental compartments (i.e. air, soil, and water) are possible. Release could occur during manufacture, use, disposal, and recycling of ENPs (Gottschalk and Nowack, 2011) (see Fig. 1.2).

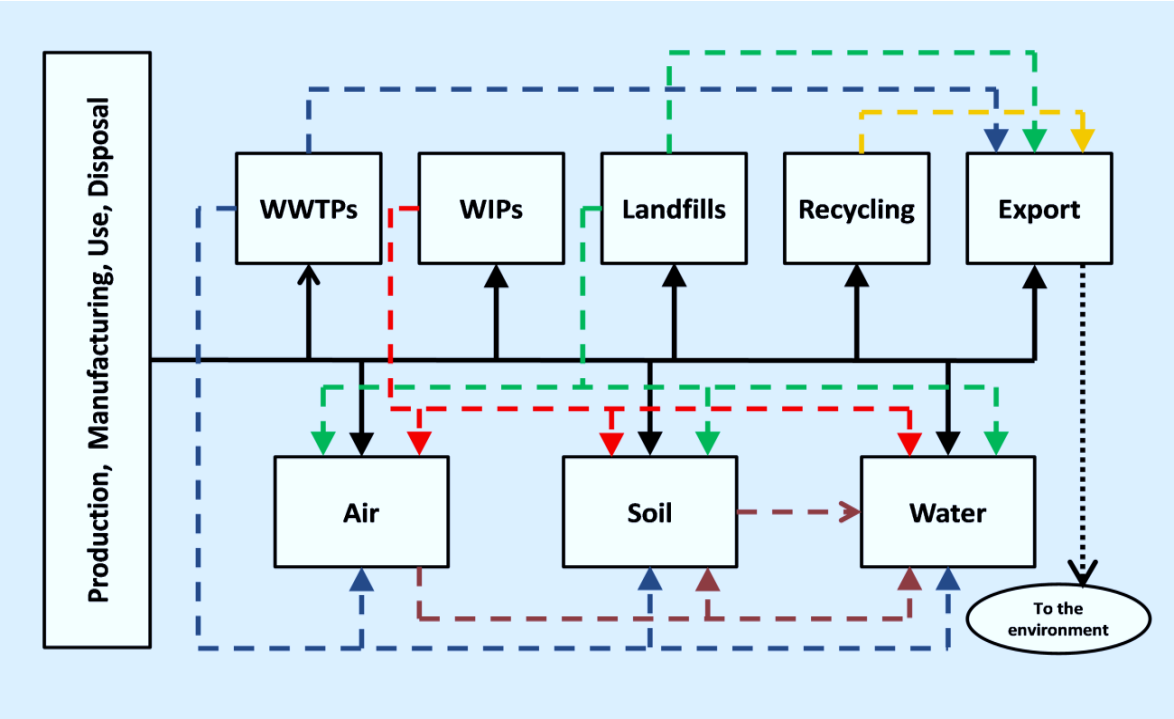

Fig. 1.2. Schematic lifecycle of ENPs in the environment. The lifecycle begins from the release points which include production, manufacture, use, and disposal. Following release, ENPs enter both environmental (air, soil, and water) and technical (wastewater treatment plants (WWTPs), waste incineration plants (WIPs), landfills and recycling unites) compartments.

Release may occur over short or long time periods, and as single or large particular forms. Once released, ENPs can be in their original form (Whatmore, 2006) or may undergo changes in their physicochemical characteristics, leading to unique behaviours (Bour *et al.*, 2015) and possible toxic effects in the environment.

Considering ENPs sources and emissions is one way by which the environmental concentrations of nanomaterials can be estimated (Markus *et al.*, 2015). Although estimated concentrations of ENPs in the environment are provided in the literature, they are not entirely accurate as the values are built upon predictive calculations (Sun *et al.*, 2014). Such predictions are

the only way to quantify ENPs in the environment (Whiteley *et al.*, 2013; Sun *et al.*, 2014) and are valuable substitutes for unavailable measurement studies (Gottschalk *et al.*, 2009). It is worth highlighting that alongside the increase in the use of ENPs, their release will also increase, leading to rising concentrations of ENPs in the environment (Sun *et al.*, 2014). However, the wide variation in estimating and measuring the concentrations of ENPs in the environment provided in the literature is clear, and is compounded by differences in synthesizing of ENPs and products into which ENPs were incorporated (Keller *et al.*, 2013), and the fast growth in production and application of ENPs (Sun *et al.*, 2014). Therefore, improved or developed methods are required for better detection and quantification of ENPs in the environment (Handy *et al.*, 2008; Bour *et al.*, 2015).

The majority of studies that have considered the environmental concentration of ENPs have focused on certain countries or regions; for example, the United Kingdom (Whiteley *et al.*, 2013), Switzerland (Caballero-Guzman *et al.*, 2015), Germany and Sweden (Wigger *et al.*, 2015), America (Gottschalk *et al.*, 2009) and Europe (Sun *et al.*, 2014). However, in other countries and regions such considerations of environmental ENPs concentrations have not yet been conducted. Exporting waste containing nanotechnological products to other countries for further recycling processes is a potential route for environmental release of ENPs (Caballero-Guzman *et al.*, 2015; Wigger *et al.*, 2015). For example, 95% of textiles (one of the main products in which ENPs are incorporated) used in Switzerland are sent abroad (Caballero-Guzman *et al.*, 2015). Therefore, the environmental concentration of ENPs should be considered in a global perspective and consequently the recent predicted concentrations would significantly increase.

1.3.1. Engineered nanoparticles in water

As the use of ENPs in consumer and industrial products is increasing, their release into the aquatic environment is inevitable (Markus *et al.*, 2015). Direct (for example, from cosmetics) and indirect (for example, from WWTPs) release into water bodies are possible (Keller *et al.*, 2013) and has been considered and investigated in different studies. Paints are one of the highest risk points from where ENPs can be released into water (Keller *et al.*, 2013). Under weathering conditions of sun and rain, the release of TiO_2 NPs from exterior facades of old and new paints into water was studied. Analysis showed between 8 and 16 μg/L of TiO_2 NPs in the runoff. The authors concluded that such leaching of TiO_2 may represent a significant release into surface water (Kagi *et al.*, 2008). Processes such as abrasion, photochemical, and oxidation reactions are likely contribute to this release (Nowack *et al.*, 2012). Such studies under normal weather conditions represent realistic scenarios (Bour *et al.*, 2015). In a study by Gottschalk et al. (2009), the authors modelled the environmental concentrations of some ENPs. The results showed that in 2008, European, American, and Swiss surface waters received 0.002-0.021 ng/L of TiO_2 NPs, 0.001-0.013 ng/L of zinc oxide nanoparticles (ZnO NPs), 0.001-0.004 ng/L of carbon nanotubes (CNTs) and 0.003-0.04 ng/L of fullerenes. In a another study, Keller *et al.* (2013) considered information on market research of ENPs production volumes and applications, and those provided by the literature, to estimate the potential release of ENPs into water. The study showed that water bodies received 1,100-29,200 metric tons, which equates to 0.4-7% of the total global ENPs (260.000-309,000 metric tons) produced in 2010. In a probabilistic model for environmental flows of ENPs in Europe, water bodies were reported to receive 28,825 tons in 2012 through direct or indirect release (Sun *et al.*, 2014).

The concentration of Ag NPs in the aquatic environment has also been estimated and shown to be varied. In a study by Gottschalk *et al.* (2009), the authors estimated that in 2008, European, American, and Swiss surface waters received between 0.116 and 0.764 ng/L of Ag NPs. WWTPs receive a significant amount of some ENPs (Whiteley *et al.*, 2013) and may further release ENPs into the aquatic environment (Brar *et al.*, 2010). In a pilot WWTP, Kaegi *et al.* (2011) confirmed the release of Ag NPs into effluent samples. The amount of Ag NPs that pass into WWTPs was estimated to be 3.3 (Mueller and Nowack, 2008) and 8.8 (Whiteley *et al.*, 2013) tons per year in Switzerland and the UK, respectively. Of that value, 0.4 tons in UK WWTPs will be passed into natural waters. Furthermore, the total concentration of Ag NPs in northern and southern hemisphere waters is estimated to range between 0.6-73 kg and 1-35 kg, respectively (Whiteley *et al.*, 2013). The release of Ag NPs from washing nine textiles under real conditions was investigated and results indicated that leaching of Ag particles of different sizes ranging between 1-450 nm occurs (Geranio *et al.*, 2009). Maximal and minimal scenarios of the release of Ag NPs from two clothing textiles (polyester and cotton) into German wastewater were considered. In the maximal scenario, 143.8 and 167.8 kg of Ag NPs were discharged into wastewater. However, in the minimal scenario 51.4 and 76 kg were discharged. In general, washing was highlighted as an activity which leads to Ag NP release (Wigger *et al.*, 2015). Despite these valuable estimates, the actual concentration of ENPs and particularly Ag NPs in aquatic environment will be variable and dependent on many factors such as product type, using process, and region.

1.3.2. Engineered nanoparticles in soil

ENPs can enter soil through one of several scenarios including agricultural protection products such as plant-disease control, sludge deposition, and remediation (Boxall *et al.*, 2007). WWTPs receive a significant amount of ENPs (Whiteley *et al.*, 2013; Sun *et al.*, 2014) from where

the majority of ENPs are incorporated into sewage sludge (Blaser *et al.*, 2008; Sun *et al.*, 2014). The majority of sludge (which may contain high concentrations of ENPs) in America and most of Europe ends up on agricultural soils as biosolids (Gottschalk *et al.*, 2009). It was estimated that 44-47% of ENPs could end up in these soils as a result of the sludge application (Keller *et al.*, 2013). Principally, these biosolids are considered to be an important factor in the interaction between the environment and ENPs (Judy *et al.*, 2011). Once ENPs enter soil, they can migrate unless they are incorporated into soil components (Brar *et al.*, 2010).

The entry of ENPs into soil in 2010 was reported to range from 22,000-80,000 metric tons, which equates to 8-28% of the total global ENP production of 260,000-309,000 metric tons (Keller *et al.*, 2013). In 2012, soil received approximately 30% of the total volume of ENPs released into the environment through sludge application in Europe (Sun *et al.*, 2014). One study predicted that sludge treated soil in Europe and the U.S. receives between 42 and 89.2 μg/kg TiO_2 NPs, 1.99 -3.25 μg/kg ZnO NPs, 31.4-73.6 of ng/kg CNTs, and 1.01-2.2 ng/kg fullerenes per year (Gottschalk *et al.*, 2009).

The concentration of Ag NPs in WWTP sludge was estimated to be 17 μg/kg for the UK (Whiteley *et al.*, 2013) and 1.68 mg/kg for Europe (Gottschalk *et al.*, 2009). In the latter study, sludge treated soil in Europe and the U.S. was estimated to receive between 662-1581 ng/kg Ag NPs per year (Gottschalk *et al.*, 2010). The same authors also estimated an increase of three to five folds in the concentration between 2008 and 2012 in U.S. Mueller and Nowack (2008) reported an input of 1 μg Ag NPs into soil per year. Such varied results, however, represent the difficulty of measuring the accurate concentration of ENPs and particularly Ag NPs in soil.

1.3.3. Engineered nanoparticles in air

ENPs can enter the air via different routes, either directly or indirectly. The manufacture, use, and incineration in WIPs, of nanotochnological products are all sources of aerial pollution of ENPs (Keller *et al.*, 2013). Real time monitoring of ENPs emissions during manufacturing processes showed that ENPs <100 nm were released into the air (Ogura *et al.*, 2011). Incineration of waste is one of the main processes in recycling systems in some countries; for example, Switzerland (Caballero-Guzman *et al.*, 2015) and therefore it is considered as a source of ENPs in air environment (Gottschalk *et al.*, 2009). It is possible, for example, that when CNTs composites are subjected to high temperatures, CNTs will be released into the air (Nowack *et al.*, 2012). Indeed, waste incineration plants were reported to be a significant location from where more than 23 tons/year of ENPs will enter air (Caballero-Guzman *et al.*, 2015). Paints, coatings, pigments, and sludge are also considered as sources of ENP release into the atmosphere. For example, as much as 1.8% of the ENPs present in sludge have been reported to be emitted into the atmosphere during application (Keller *et al.*, 2013).

Ag NPs could find their way into air and therefore their concentrations have been estimated in a number of different studies. It was reported that from the incineration of nanotechnological products containing Ag NPs, 1.6 tons of Ag NPs were released into the environment (Whiteley *et al.*, 2013). Quadros and Marr (2010) reported that 14% of Ag NPs incorporated into consumer products may be released into the atmosphere. Under a minimal release scenario, the release of Ag NPs into air from two clothing textiles (polyester and cotton) was reported to be 38 and 99.9 kg in Germany (Wigger *et al.*, 2015). Generally, wearing and drying clothes were highlighted as major contributors to this release and air was highlighted to be the greatest recipient of released Ag NPs. On a larger scale, Sun *et al.* (2014) predicted that approximately 0.5231 tons of Ag NPs were released into the

European atmosphere in 2012. From three spray products alone (an anti-odour spray for hunters, a surface disinfectant and a throat spray) that claimed to contain nanoparticular and ionic silver, the release of Ag NPs of up to 10 μm was reported (Quadros and Marr, 2011). From these results, it is clear that the concentration of ENPs and particularly Ag NPs in air is varied.

Taken together, although the predicted environmental concentration of ENPs are high, such concentrations could be found especially if we bear in mind that high concentrations of concentrated ENPs would intentionally and/or unintentionally be discharged into the environment (Klaine *et al.*, 2008; Wang *et al.*, 2013a; Holden *et al.*, 2014). Consequently, it is likely that the released ENPs into the environmental systems could directly and inevitably interact with plants with potentially toxic effects. Therefore, understanding the toxicity of ENPs on plants is of great importance.

1.4. Impact of engineered nanoparticles on plants

Plants are a vital component of the environment and play fundamental roles; for example, in providing food for heterotrophic organisms such as humans and animals. Any disruptions to food production could lead to an imbalance between rising human population and increasing food requirements. According to John Beddington, England's chief scientific advisor, by 2030 the population will increase from 6.8 to 8.3 billion and will require the production of 50% more food (Population Institute, 2015).

Although the volume of natural NPs released into the environment is significantly greater than that of ENPs (Handy *et al.*, 2008), the latter could pose real dangers to the environment (Gottschalk and Nowack, 2011) and living organisms (Huang *et al.*, 2014) due to the presence of toxic chemical components that do not exist in natural NPs (Handy *et al.*, 2008). Moreover, ENPs could react with organic pollutants and hazardous metals present in the environment, leading to

further toxicity (Brar *et al.*, 2010). Many living organisms are already adapted to natural NPs (Handy *et al.*, 2008), but whether or not they could adapt to ENPs is still a challenging question. Clearly, there is a large gap in our understanding about the behaviour and effects of ENPs in the environment (Nowack *et al.*, 2012).

Due to the novelty of ENPs as environmental pollutants, considerable concerns have been raised over their potential release and the consequent effects on plants and plant productivity (Zhao *et al.*, 2015). As a result, some studies have recently been conducted in order to investigate the potential effects of ENPs on different plants. However, the database of nanotoxicity on plants is still limited (Lee *et al.*, 2012; Vannini *et al.*, 2013; Larue *et al.*, 2014), and better information in this area is critically important.

When ENPs emitted into the environment, they can enter plants through several routes including seeds, roots, and leaves (Fig. 1.3) (Lin and Xing, 2007; Khodakovskaya *et al.*, 2009; Cifuentes *et al.*, 2010; El-Temsah and Joner, 2012; Wang *et al.*, 2012a; Wang *et al.*, 2013a; Hong *et al.*, 2014). Following entry, ENPs can be transported inside plants through vascular pathways (Wang *et al.*, 2012b).

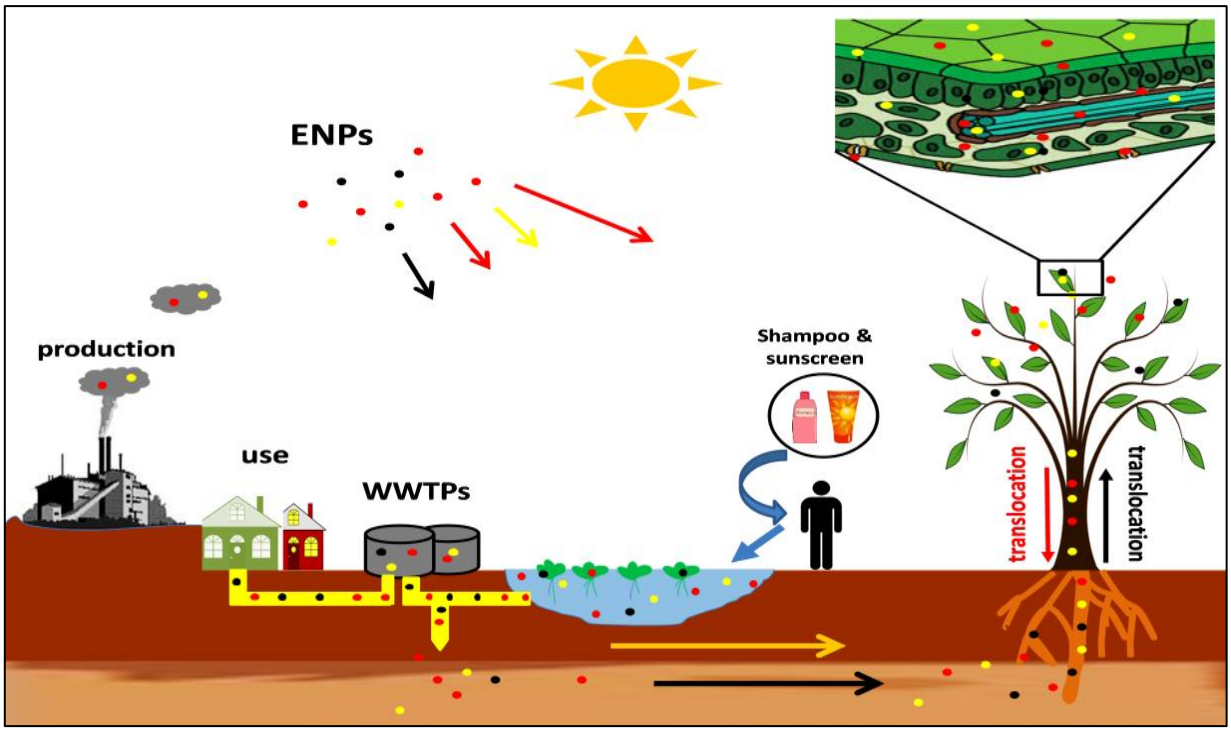

Fig. 1.3. Scenarios following the release of ENPs from different sources into the environment, including consequential interactions with aquatic and terrestrial plants through seeds, roots, and leaves. ENPs may be released directly from industrial sources, during and following the use of nanotechnological products, or indirectly through WWTP discharge or the application of sludge. Exposure to plants and other organisms inevitably follows the release of ENPs.

Once interacting with plants, ENPs have been shown to release ions, which in turn can lead to the generation of reactive oxygen species (ROS) (Wang *et al.*, 2015). It is worth mentioning that the toxicity of ENPs on plants does not necessarily require penetration into the plant, as aggregation on the root surface, for example, alone leads to toxic effects by causing physical damage (Asli and Neumann, 2009).

Metal oxide-based ENPs have shown different effects on plants. Exposing four edible plant species (alfalfa, corn, tomato, and cucumber) to cerium oxide nanoparticles (CeO_2 NPs) at concentrations of

0-4000 mg/L caused varied effects on seed germination, root growth, stem length, and biomass (López-Moreno *et al.*, 2010). Hong *et al.* (2014) reported changes in ascorbate peroxidase (APX) enzyme and cell structure, indicating toxicity on cucumber exposed aerially to CeO_2 NPs at concentrations ranged between 0 and 320 mg/L. In contrast, no effects were reported on pumpkin and wheat when exposed to CeO_2 NPs at 100 mg/L, although the NPs were detected in pumpkin shoots (Schwade *et al.*, 2013). Under realistic conditions, CeO_2 NPs at low concentrations (up to 1 g/kg) caused inhibitory effects on growth and pod biomass of soybean (Priester *et al.*, 2012). In contrast, in the same study, ZnO NPs at the same concentrations showed slight stimulatory effects on plant growth. However, the authors did not consider the ionic form, and clear explanations about the mechanisms of either enhancement or inhibition were not provided. Of four different ENPs, ZnO NPs at 2000 mg/L were one of the most toxic ENPs, which significantly diminished the root length of six plants investigated and inhibited seed germination of corn. The authors excluded Zn ions as directly involved in these effects (Lin and Xing, 2007), although ZnO NPs are known to be highly soluble (Du *et al.*, 2011). In comparison, Stampoulis *et al.* (2009) showed no effects of ZnO NPs at 1000 mg/L on cucumber root length when cucumbers were germinated in Petri dishes, and attributed that to factors including low ENPs concentration and the plant species used. Importantly however, when the authors changed the application method to cucumber seedling growth in hydroponic solution, a significant decrease in biomass occurred. Clearly, such results are a direct indication that the application method affects the behaviour of ENPs and their consequent effects on plants. The exposure of pea seeds to ZnO NPs (up to 1000 mg/L) showed no effects on seed germination, but significantly decreased root length by 50 to 60%, particularly at high concentrations of 250-1000 mg/L. Additionally, chronic exposure of pea to ZnO NPs at 750 mg/L resulted in a significant decrease in the number of first order lateral roots, and progressive decrease in the number of second

order lateral roots, by 75% and 55% respectively (Huang *et al.*, 2014). The same authors also highlighted the sensitivity of the stem length compared to the average surface area per leaf, whereby ZnO NPs decreased them by 67% and 25%, respectively. In the same study, respiration of plants was also significantly decreased. The authors concluded that the toxicity was mainly due to the free Zn^{+2}, thought the attachment of ZnO NPs on the root surface stressed the plants and consequently impacted plant development. Considering the effects of ZnO NPs and CeO_2 NPs on the physiological function of corn, it was reported that while CeO_2 NPs were not toxic, in the same experimental conditions ZnO NPs at different concentrations (up to 800 mg/kg) decreased net photosynthesis, stomatal conductance, and relative chlorophyll content by 12%, 15%, and 10%, respectively (Zhao *et al.*, 2015). Such different results for various ENPs under the same conditions emphasize the role of ENPs type in affecting plants in varied ways. Moreover, these differences make investigations into ENPs toxicity to plants complicated. Under field conditions, 5 g of ZnO NPs applied to 110 kg of soil affected the biomass of wheat and this toxicity was attributed to the dissolved ions due to the high solubility of ZnO NPs (Du *et al.*, 2011). In the same study, 10 g of TiO_2 NPs similarly affected the biomass, as a result of the dissolved ions. Studying the effects TiO_2 NPs at different concentrations (up to 50 mg/L) on broad beans, revealed no effects on plant growth (Foltęte *et al.*, 2011). The effects of ENPs do not necessarily result from chemical reactions, physical interactions could also affect plants. In this manner, TiO_2 NPs (up to 1 g/L) accumulated on root surfaces demonstrated inhibitory effects on cell wall pore size, water transport capacity, leaf growth, and transpiration of corn (Asli and Neumann, 2009). The accumulation of copper oxide nanoparticles (CuO NPs) at different concentrations (up to 100 mg/L) in corn was reported to inhibit seedling growth and this was mainly attributed to CuO NPs themselves rather than the ionic forms (Wang *et al.*, 2012b). To clarify the toxicity mechanism of ENPs, Wang *et al.* (2015) evaluated the

effect of CuO NPs at a concentration of 5 mg/L on ROS generation alongside root growth. Significant decreases in the elongation and biomass of roots were observed which attributed to the greatest ROS generation. In parallel, cell structure was also influenced.

ENPs can also lead to positive effects on plants, including an increased supply of plants nutrients, enhanced seed germination and seedling growth, facilitation of water and fertilizer absorption, increased activity of antioxidants such as superoxide dismutase and catalase, and increased photosynthetic pigments (Morteza *et al.*, 2013). Uptake of essential elements in addition to ENPs by plants, may increase photosynthetic performance through a mechanism that provides energy to soil microbes, which in turn facilitate a greater volume of nutrients to be taken up by plants (Wang *et al.*, 2013b). Morteza *et al.* (2013) found that in comparison with controls, spraying TiO_2 NPs with the concentration of 0.01% and 0.03% on corn leaves significantly increased photosynthetic pigments which was related to the role of TiO_2 NPs in stabilizing the integrity of chloroplast membrane and protecting the chloroplast from aging. However, it was concluded that such an increase in pigment levels would consequently increase crop yield.

Other, less well-studied ENPs such as $CoFe_2O_4$ NPs, Fe_2O_4 NPs, and SiO_2 NPs have also demonstrated varied positive and negative impacts on plants (Bao-shan *et al.*, 2004; Sheykhbaglou *et al.*, 2010; Ursache-oprisan *et al.*, 2011). The results of previous research on the effect of metal-oxide ENPs on plants are summarized in Table 1.1.

Table 1.1. Impact of metal-oxide ENPs on plants

ENPs	Size	Concentration	Application method	Plant	Effect	Reference
CeO_2 NPs	7 nm	0, 500, 1000, 2000, 4000 mg/L	aqueous suspension in Petri dish	corn, tomato, cucumber	reduced significantly seed germination	López-Moreno *et al.* (2010)
				alfalfa, tomato	reduced root growth	
				cucumber, corn	increased root growth	
				alfalfa, corn	reduced significantly the biomass	
	8 nm	0, 40, 80, 160, 320 mg/L	aerial application	cucumber	changes in stress enzymes (increased in catalase activity and decreased in ascorbate peroxidase activity) and cell structure (increased vacuole space)	Hong *et al.* (2014)
	between 17 and 100 nm	100 mg/L	Hoagland solution	pumpkin and wheat	no effects on growth	Schwade *et al.* (2013)

	8 nm	0, 400, 800 mg/kg	soil	corn	no effects on net photosynthesis, stomatal conductance, and relative chlorophyll content	Zhao *et al.* (2015)
	8 nm	0.1, 0.5, 1 g/kg	soil	soybean	reduced growth and pod biomass	Priester *et al*. (2012)
ZnO NPs	10 nm				stimulated plant growth	
	20 nm	2000 mg/L	aqueous suspension in Petri dish	corn	inhibited significantly seed germination	Lin and Xing (2007)
				radish, rape, corn, cucumber, lettuce, ryegrass	inhibited significantly root length	
	<5 nm and <10 nm	1000 mg/L	aqueous suspension in Petri dish	cucumber	no significant effect on seed germination and root length	Stampoulis *et al.* (2009)
			25% Hoagland solution		reduced the biomass significantly by	

					(87-90%)	
	<50 nm	100, 250, 500, 750, 1000 mg/L	agar	pea	no effect on seed germination, but significantly affected root length	Huang *et al.* (2014)
		0, 250, 500, 750 mg/L	hydroponic solution		decreased the number of the first- and second-order lateral roots, stem length, leaf surface area, and the transpiration	
	24 nm	0, 400, 800 mg/kg	soil	corn	reduced net photosynthesis, stomatal conductance, and relative chlorophyll content by 12%, 15%, and 10%, respectively	Zhao *et al.* (2015)
	≤ 100 nm	5 g	soil	wheat	reduced the biomass	Du *et al.* (2011)
TiO_2 NPs	20- 100 nm	10 g				
	> 700	5, 25, 50	aqueous	broad	no effect on plant	Foltęte *et al.*

	nm and < 10 µm	mg/L	suspension	bean	growth	(2011)
	30 nm	0.3, 1 g/L	hydroponic solution	corn	inhibited cell wall pore size, water transport capacity, leaf growth, and transpiration	Asli and Neumann (2009)
	--	0.01%, 0.03%	soil	corn	increased significantly chlorophyll contect (a and b), total chlorophyll (a + b), chlorophyll a/b, carotenoids, and anthocyanins	Moreza *et al.* (2013)
CuO NPs	20-40 nm	10, 100 mg/L	hydroponic solution	corn	inhibited seedling growth, chlorotic symptoms	Wang *et al.* (2012b)
	<50 nm	5 mg/L	hydroponic solution	rice	inhibited elongation and biomass of root	Wang *et al.* (2015)
$CoFe_2O_4$	8.5 nm	0, 20, 40, 60, 80, 100 µl/L	watered porous paper in Petri dish	sunflower	affected negatively chlorophyll contents	Ursache-oprisan *et al.* (2011)

Fe_2O_4	_	0, 0.25, 0.5, 0.75, 1 g/L	soil	soybean	increased pod and leaf dry weight	Sheykhbaglou *et al.* (2010)
SiO_2 NPs	_	62, 125, 250, 500, 1000, 2000 µl/L	aqueous suspension	changbia larch	affected growth positively	Bao-shan *et al*. (2004)

The effects of metal-based ENPs have been investigated in several studies. In one study, the germination of cucumber seeds in the presence of silicon nanoparticles (Si NPs) at a concentration of 1000 mg/L was completely inhibited. Similarly, copper nanoparticles (Cu NPs), at the same concentration, in the presence of the surfactant sodium dodecyl sulfate (SDS) decreased the germination and significantly the biomass by 90% (Stampoulis *et al.*, 2009). The authors highlighted the important role of surfactants in inducing phototoxicity, as they are well known in facilitating the dispersion of ENPs. In another study, Cu NPs caused a decrease in seedling growth of mung bean and wheat which was related to the Cu NPs (up to 1000 mg/L) themselves rather than the cupric ions released. Meanwhile, Cu NPs bioaccumulated in cells and increased with increasing concentration, suggesting that transportation from roots to shoots during the uptake of water and nutrients occurs (Lee *et al.*, 2008). In a comparison between five different types of ENPs, Zn NPs at 2000 mg/L were one of the most toxic which significantly inhibited root length of the six plants investigated and seed germination in ryegrass. However, Zn ions were not found to be directly involved in these effects (Lin and Xing, 2007). Being intensively used in remediation processes (Nowack, 2008), iron nanoparticles (Fe NPs) in aqueous suspension showed inhibitory effects on ryegrass, flax, and barley at a concentration of 250 mg/L. As expected, higher concentrations were

required to affect the three plants when germinated in soil (El Temash and Joner, 2012). In the latter study, soil type was highlighted to play a role in ENPs toxicity, whereby sandy soil showed a more toxic effect compared to clay soil due to the differences in surface area of soil particles and their interactions with Fe NPs. In one of the first and least investigated exposure-methods, Corredor *et al.*, (2009) applied aerially Fe NPs and detected subcellular changes in pumpkin plants as a result of the presence of these ENPs inside the cells. The results of previous research on the effect of metal ENPs on plants are summarized in Table 1.2.

Table 1.2. Effect of metal ENPs on plants

ENPs	Size	Concentration	Application method	Plant	Effect	Reference
Si NPs	100 nm	1000 mg/L	aqueous suspension in Petri dish (with 0.2% SDS)	cucumber	inhibited seed germination completely	Stampoulis *et al.* (2009)
Cu NPs	50 nm	1000 mg/L	aqueous suspension in Petri dish (with 0.2% SDS)		affected germination and root length negatively	
			25% Hoagland solution		reduced significantly biomass by 90%	
	–	0, 200, 400, 600, 800, 1000	agar	mung bean,	inhibited seedling growth	Lee *et al.* (2008)

		mg/L		wheat		
Zn NPs	35 nm	2000 mg/L	aqueous suspension in Petri dish	radish, rape, corn, cucumbe, lettuce, ryegrass	inhibited significantly root length	Lin and Xing (2007)
				ryegrass	inhibited significantly seed germination	
nZVI	1-20 nm	0, 100, 250, 500, 1000, 2000, 5000 mg/L	aqueous suspension	ryegrass flax, barley	inhibited completely seed germination	El-Temsah and Joner (2012)
		above 1500 mg/L	sandy and clay soil	ryegrass flax, barley	no germination	
Fe NPs	_	__	aerial application	pumpkin	changes in the subcellular organisation of the parenchymatic cells	Corredor *et al.* (2009)

The effects of CNTs on plants are varied. For example, multi walled carbon nanotubes (MWCNTs) at 1000 mg/L showed no effects on seed germination of cucumber, although plant biomass was significantly reduced by 38% compared to the bulk material (Stampoulis *et al.*, 2009).

The authors suggested that standard phototoxicity tests like seed germination and root elongation are insensitive for the evaluation of nanoparticle toxicity on plants. Even at higher concentrations of 2000 mg/L, MWCNTs exposed to different plants showed no effects on seed germination, but significantly affected the root length of one species (Lin and Xing, 2007). The authors attributed the lack of toxicity to the permeability of seed coats. Conversely, MWCNTs at different concentrations (up to 40 μg/mL) penetrated the thick seed coat of tomatoes and significantly enhanced seed germination and plant growth by creating new pores and consequently promoting water uptake (Khodakovskaya *et al.*, 2009). In a different study, the same authors demonstrated the ability of MWCNTs at different concentrations (0.1, 5, 100, and 500 μg/mL) to enhance the growth of tobacco by 55-64% compared to controls (Khodakovskaya *et al.*, 2012). Exposing a different plant (wheat) to MWCNTs (up to 160 μg/mL) also resulted in significant root growth and vegetative biomass, but no enhancements were recorded on seed germination or stem length (Wang *et al.*, 2012a). The authors attributed this to the uptake of MWCNTs which increases cell elongation in the root system of wheat and improves root dehydrogenase activity which in turn enhances water uptake. Single walled carbon nanotubes (SWCNTs) have also shown differing effects on plant species. In one study, Cañas *et al.*, (2008) noticed that while SWCNTs at 104, 315, and 1750 mg/L significantly enhanced root elongation in onion and cucumber, inhibition occurred in tomato root. Neither carrot nor cabbage was affected. As ENPs have been reported to enter water bodies, the effects of ENPs on aquatic plants have also been considered. Results showed that after 7 days, exposure to C_{60} NPs at 1-10 mg/L inhibited the growth of *Lemna gibba* by 25% by decreasing chlorophylls *a* and *b*, and the production of chloroplast oxygen which consequently inhibited photosynthesis (Santos *et al.*, 2013). The results of previous research on the effect of CNTs on plants are summarized in Table 1.3.

Table 1.3. Effect of CNTs on plants

ENPs	Size	Concentration	Application method	Plant	Effect	Reference
MWCNTs	13-16 nm	1000 mg/L	aqueous suspension in Petri dish	cucumber	no effect on germination	Stampoulis *et al.* (2009)
			25% Hoagland solution		reduced the biomass by 38%	
	10-20 nm	2000 mg/L	aqueous suspension in Petri dish	radish, rape, corn, cucumber, lettuce, ryegrass	no significant effect on seed germination, but reduced significantly root elongation of ryegrass	Lin and Xing (2007)
	_	10, 20, 40 μg/mL	Murashige and Skoog	tomato	enhanced significantly seed germination rate, and the biomass	Khodakovskaya *et al.* (2009)
	20 nm	0.1, 5, 100, 500 μg/mL	Murashige and Skoog	tobacco	enhanced growth	Khodakovskaya *et al.* (2012)
	length 50-630	10, 20, 40, 80, 160 μg/mL	aqueous suspension in	wheat	no effects on seed germination and	Wang *et al.* (2012a)

	nm		Petri dish		stem length, but increased significantly root length by 32% and the vegetative biomass by 30-40%	
SWCNTs	8 nm	104, 315, 1750 mg/L	aqueous suspension	onion, cucumber	enhanced significantly root elongation	Cañas *et al.* (2008)
				carrot, cabbage, lettuce	no effect on root elongation	
C_{60} NPs	29-38 nm	1-10 mg/L	Hutner's medium	*Lemna gibba*	decreased growth rate, oxygen production, and significantly the contents of chlorophyll *a* and *b*	Santos *et al.* (2013)

Many governments and organizations have classified Ag NPs as potentially toxic. The UK Government, for example, identified a priority list of reference nanomaterials for which toxicity should be investigated and this list included silver (Aitken *et al.*, 2007). Similarly, in a revised list

provided by the Working Party on Manufactured Nanomaterials (WPMN), Ag NPs have been identified to cause risks and toxicity on the environment (OECD, 2010).

Since commercial use of Ag NPs has significantly increased (Sun *et al.*, 2014), Ag NPs have found their way into the environment (Mueller and Nowack, 2008; Geranio *et al.*, 2009). Previous studies have shown that ENPs could affect plants, and that Ag NPs especially could be highly toxic (Kim *et al.*, 2011). However, little information is available about the impact of Ag NPs on plants (Patlolla *et al.*, 2012; Thuesombat *et al.*, 2014) and consequently studying the overall environmental effect of ENPs should include plants (Patlolla *et al.*, 2012).

The impact of Ag NPs on plants has been investigated in different studies. For example, Barrena *et al.* (2009) studied the effect of Ag NPs (at 100 µg/mL) on the seed germination of cucumber and lettuce and observed little or no effects. In another study, Stampoulis *et al.* (2009) considered the effect of Ag NPs at 1000 mg/L on cucumber and reported a reduction in the biomass by 69%. Seed germination also decreased by 54%, but when SDS was removed no effect was observed. The observed toxicity was mainly due to the presence of SDS. It is not in all cases that surfactants or coating agents impact the toxicity of ENPs, because in one study, both the surfactant and control showed similar results (Lee *et al.*, 2012). However, in the latter study, a reduction in the growth of mung bean and sorghum was shown to be medium-dependent due to the changeable behaviour of Ag NPs between different mediums, agar and soil. Comparing the results of Ag NPs and their bulk forms, the authors attributed this toxicity to either Ag NPs or to the released ions, depending on the medium. Assuming the toxicity of Ag NPs is size and concentration dependent, seeds of three crop plants: flax, barley, and ryegrass were exposed to three different sizes of Ag NPs: colloidal silver nanoparticles (Ag_{coll}, size range between 0.6-2 nm), 5 nm, and 20 nm at

different concentrations (0-100 mg/L). While seed germination of ryegrass was inhibited with Ag_{coll} at 10 and 20 mg/L, barley seed germination was inhibited with 5 nm and 20 nm at 10, 20, and 100 mg/L (El Temash and Joner, 2012). Despite this toxicity attributed to the released ions, no evidence was provided. Although the study showed Ag NPs toxicity is not size and concentration dependent, other studies, in contrast, report the toxicity of Ag NPs depends on size and concentration. For example, the effects of Ag NPs at different sizes (20, 30-60, 70-120, and 150 nm) and at different concentrations (0.1, 1, 10, 100, and 1000 mg/L), on seed germination and seedling growth of rice, indicated a clear correlation between toxicity and increasing Ag NPs size and concentration. The results showed that larger sizes and the higher concentrations caused a greater inhibitory effect on both parameters (Thuesombat *et al.*, 2014). The effects of Ag NPs do not necessarily result from chemical activity, as physical interactions could also affect plants. In this manner, Ag NPs at concentrations up to 1000 µg/mL damaged cell wall and vacuoles in rice and consequently changed cell structure (Mazumdar and Ahmed, 2011). This toxicity was the result of large particles penetrating the small cell pores. As Ag NPs have been reported to enter water bodies, the effects on aquatic plants have also been considered. In one study, Ag NPs at different concentrations (0.1-200 ppm) caused growth inhibition in *Lemna paucicostata* and other visible effects such as chlorosis and frond disconnection which all were related to Ag NPs themselves rather than the released ions (Kim *et al.*, 2011).

Despite the toxicity of Ag NPs, few studies have shown a positive impact of Ag NPs on plants. For example, Ag NPs at concentrations ranged between 0.1 and 100 mg/L have been shown to stimulate root elongation of rocket (Vannini *et al.*, 2013). Surprisingly, this improvement was related to the released ions, although they have been highlighted to be a source of toxicity in a

number of studies (Yin *et al.*, 2011; Jiang *et al.*, 2014). The results of previous research on the effect of Ag NPs on plants are summarized in Table 1.4.

Table 1.4. Impact of Ag NPs on plants

ENPs	Size	Concentration	Application method	Plant	Effect	Reference
Ag NPs	2 nm	100 µg/mL	aqueous suspension	cucumber, lettuce	low to zero toxicity	Barrena *et al*. (2009)
	100 nm	1000 mg/L	25% Hoagland solution	cucumber	affected the biomass significantly by 69%	Stampoulis *et al*. (2009)
			aqueous suspension (with SDS) in Petri dish		decreased seed germination	
	5-20 nm	0, 5, 10, 20, 40 mg/L	agar	mung bean and sorghum	affected seedling growth	Lee *et al.* (2012)
		0, 100, 300, 500, 1000, 2000 mg/kg	soil		no or little effect	
	0.6-2 nm	10, 20 mg/L	aqueous suspension in Petri dish (with 0.1% of the surfactant	ryegrass	inhibited seed germination and root length	El-Temsah and Joner (2012)
	5,and 20 nm	10, 20, 100 mg/L		barley	inhibited seed germination	

	0.6-2,5,5, and 20 nm	10, 20, 100 mg/L	tween 20)	flax	no effect	
	20, 30-60, 70-120 and 150 nm	0.1, 1, 10, 100 1000 mg/L	soil	rice	decrease in seed germination and seedling growth	Thuesombat *et al.* (2014)
	25 nm	50, 500, 1000 µg/mL	Hoagland solution (with 0.1% of the surfactant tween 20)	rice	damaged cell wall and vacuoles	Mazumdar and Ahmed (2011)
	50 nm	0.1, 1, 10, 20, 40, 50, 100, 200 ppm	APP (algal assay procedure)	*Lemna Paucicostata*	inhibited growth and caused chlorosis and disconnection	Kim *et al.* (2011)
	10 nm	0.1, 1, 10, 20, 100 mg/L	aqueous suspension in Petri dish	rocket	increased root elongation	Vannini *et al.* (2013)

It is clear that seed germination and root elongation are the most widely studied endpoints to assess the toxicity of ENPs on plants. However, whilst chlorophyll fluorescence is a powerful and non-invasive tool that has been used widely to detect perturbations of leaf metabolism (Barbagallo *et al.*, 2003), it has been applied less frequently in nanotoxicological studies. In one study, the effect of TiO_2 NPs at concentrations up to 100 mg/L on the green alga *Chlamydomonas reinhardtii* was

investigated, and the results showed that although cellular oxidative stress caused lipid peroxidation and ultimately growth inhibition occurred, no significant toxicity on the maximum quantum efficiency of PSII photochemistry (F_v/F_m) was observed (Wang *et al.,* 2008). In contrast, another study showed that TiO_2 NPs themselves at different concentrations (up to 100 mg/L) sharply decreased F_v/F_m of the same algae *C. reinhardtii* in the first 10 h (Chen *et al.*, 2012). However, when the same algae were exposed to SWCNTs and MWCNTs at 2 µg/mL, F_v/F_m considerably and insignificantly decreased respectively (Matorin *et al.*, 2010). While the authors hypothesised that the toxicity was due to physical damage to the cell wall and other cell membranes that ultimately led to failure in cell metabolism, no evidence was provided. CuO NPs at 0.1-0.4 g/L caused a significant decrease in F_v/F_m in *L. gibba,* which showed a dose dependent response since the toxicity increased with increased concentration (Perreault *et al.*, 2010). Since the toxicity of ENPs could either come from the particles themselves and/or the released ions, the authors investigated the effect of copper ions and reported slight toxicity in F_v/F_m compared to that of CuO NPs. Thus, it is clear that the toxic effects of ENPs cannot only be attributed to the released ions, and ENPs themselves can affect organisms (Perreault *et al.*, 2010, Yin *et al.*, 2011). In contrast to these results, the effect of ENPs was attributed to ionic forms of nickel oxide nanoparticles (NiO_2 NPs) when *Lemna gibba* was investigated, with damage to PSII structure and function (Oukarroum *et al.*, 2015). It is therefore not yet conclusive whether ENPs toxicity comes from the NP themselves, the released ions, or both.

As an indicator of photosynthetic performance, particularly the operating efficiency of photosynthetic electron transfer, F_q'/F_m' has been considered in nanotoxicological studies. While no obvious impact on F_q'/F_m' was reported when *C. reinhardtii* exposed to TiO_2 NPs at 1-100 mg/L (Wang *et al.*, 2008), a decrease in this parameter in the presence of SWCNTs and MWCNTs at a concentration 2 µg/mL was observed (Matorin *et al.,* 2010). Although it was difficult to reach a

conclusion on how these ENPs influence photosynthesis processes, the authors proposed damage to cell wall as well as other cell membranes which caused failure of cell metabolism. In another study, the same algae were exposed to core-shell copper oxide nanoparticles (CS-CuO-NP) at three different concentrations. The results showed that at high concentrations (0.01 and 0.02 g/L), these ENPs inhibited F_q'/F_m' which was attributed to the formation of ROS (Saison *et al.*, 2010).

When ENPs are released into the environment, changes in their properties are highly likely to occur. However, even modified ENPs could negatively affect living organisms. Such a scenario was reported when *C. reinhardtii* was exposed to Ag NPs at concentrations up to 10 µmol/L, resulting in rapid agglomeration of the ENPs and an inhibitory effect on F_v/F_m by reducing the active PSII reaction centres (RCs) (Dewez and Oukarroum, 2012). Similarly, a significant and time-dependent decrease in F_v/F_m was observed when *Spirodela polyrhiza* was exposed to both Ag NPs and ionic Ag at concentrations up to 10 mg/L, whereby a correlation was observed between increasing Ag content and a significant decrease in the content of Chl *a* (Jiang *et al.*, 2012). Although the authors excluded free Ag ions from the toxicity of Ag NPs, inferring the exact toxicity mechanisms of Ag NPs and their released ions is a complex matter (Dewez and Oukarroum, 2012).

It was reported that Ag NPs at concentrations up to 10 µmol/L inhibited F_q'/F_m' in *C. reinhardtii* and this effect was demonstrated to be dose-dependent, whereby F_q'/F_m' decreased with increasing ENPs concentration (Dewez and Oukarroum, 2012). In contrast, Juhel *et al.* (2011) reported a significant increase in F_q'/F_m' when *Lemna minor* were exposed to alumina oxide nanoparticles (Al_2O_3 NPs) at 10 and 1000 mg/L. While no direct enhancement on PSII was suggested, the authors hypothesized an increase Rubisco activase activity had occurred. Table 1.5 summarises the effects of different types of ENPs on chlorophyll fluorescence parameters.

Table 1.5. Effect of ENPs on photosynthetic parameters

ENPs	Size	Concentration	Application method	Organism	Effect	Reference
TiO_2 NPs	21 nm	0.001, 0.01, 0.1, 1, 10, 100 mg/L	hydroponic solution	*C. reinhardtii*	no significant effect in F_v/F_m and F_q'/F_m'	Wang *et al*. (2008)
	21 nm	0.1, 1, 10, 20, 100 mg/L	hydroponic solution	*C. reinhardtii*	decreased sharply F_v/F_m	Chen *et al*. (2012)
MWCNTs	60-80 nm	2 µg/mL	hydroponic solution	*C. reinhardtii*	decreased F_v/F_m and F_q'/F_m'	Matorin *et al*. (2010)
SWCNTs	1,2-1,4 nm				decreased F_v/F_m and F_q'/F_m'	
CuO NPs	particles 81/ shell/ 14	0.1, 0.2, 0.4 g/L	hydroponic solution	*L. gibba*	strong inhibition in F_v/F_m	Perreault *et al*. (2010)
NiO NPs	30 nm	0, 1, 10, 100, 1000 mg/L	hydroponic solution (AAP)	*L.gibba*	inhibitory effect on F_v/F_m	Oukarroum *et al.* (2015)
CS-CuO-NP	particles 81 nm/ shell/ 14 nm	0.004, 0.01, 0.02 g/L	hydroponic solution	*C. reinhardtii*	strong inhibition in F_q'/F_m'	Saison *et al.* (2010)
Ag NPs	6 nm	0, 0.5, 5, 10 mg/L	hydroponic solution	*Spirodela polyrhiza*	decreased significantly	Jiang *et al.* (2012)

					F_v/F_m	
Ag NPs	50 Nm	1, 5, 10 µmol/L	hydroponic solution	*C. reinhardtii*	decreased F_q'/F_m' and F_v/F_m	Dewez and Oukarroum (2012)
Al_2O_3 NPs	20 nm	10, 1000 mg/L	hydroponic solution	*L. minor*	no effect on F_v/F_m significant increase in F_q'/F_m'	Juhel *et al.* (2011)

1.5. Effect of engineered nanoparticles on soil microbes

Soil microbes are of great importance in the environment as they are involved in a number of important processes such as establishing rhizobium-legume associations. These associations require a host plant and bacteria. Any disruptions to soil microbial community could lead to other effects, firstly on soil quality and then on plant productivity. Therefore, maintaining the health of soil and its associated microbes is required in order to produce vegetation of good quality.

As ENPs are already in the environment, they could affect microorganisms and that is of concern (He *et al.*, 2011). Consequently, understanding the potential effect of ENPs on soil microbes is important. However, very little information about the impact of ENPs on the plant-soil system is currently known (Fan *et al.*, 2014).

Exposure of soil microbes to ENPs could occur directly through the application of sludge onto soil (Chunjaturas *et al.*, 2014) or indirectly through root exudation of ENPs taken up by plant leaves (Wang *et al.*, 2012b). With the aim of evaluating and understanding the interactions between

ENPs and soil microbes and the resulting consequences, studies have reported varied results. In these studies, the effects on factors such as abundance, growth, and morphological changes to the microbes were documented. One study considered the potential effect of ZnO NPs at concentrations up to 750 mg/L on *Rhizobium leguminosarum bv. viciae* 3841. The results showed morphological changes and damage to the bacterial surface (Huang *et al.*, 2014). In the same bacteria (*R. leguminosarum bv. viciae* 3841), concentrations up to 750 mg/L of TiO_2 NPs caused morphological changes to the bacterial cells (Fan *et al.*, 2014). Conversely, the presence of C_{60} up to 15 mg/L did not affect the growth of *Bacillus stearothermophilus*, nor its morphology (Santos *et al.*, 2013). Likewise, under field conditions, three different ENPs: cobalt (Co NPs), nickel (Ni NPs), and Fe NPs showed no toxicity to *Bradyrhizobium* and *Rhizobium* involved in nitrogen fixation (Shah *et al.*, 2014). In contrast to these studies showing toxic effects of ENPs on bacteria, Fe_3O_4 NPs and *y*-Fe_2O_3 NPs at different concentrations (up to 1.26 mg/g) had a positive effect on soil microbial structure by stimulating the growth of some bacteria (He *et al.*, 2011). However, due to their important roles, more information on the toxic effects of ENPs on soil microbes is of importance and urgently needed.

Ag NPs are well known for their antimicrobial and antibacterial activities (Falco *et al.*, 2015). However, the potential effect of Ag NPs on beneficial soil microbes is of concern, as they could affect them adversely (Brar *et al.*, 2010). It was reported that Ag NPs at low concentrations up to 500 μg/g were toxic to many nitrifying bacteria that play a critical environmental role by supplying plants with their need of nitrogen after converting ammonia in soil. The results showed a decrease in the bacterial community with increasing Ag NPs concentration, and the authors concluded that the effect of Ag NPs is dependent on concentration, exposure time, and soil type (Chunjaturas *et al.*, 2014). Alongside this, another study showed that the addition of Ag NPs at 1

and 3 mg/L into sandy and loamy soils lead to loss of culturability of *Pesudomonas chlororaphis* O6 in the sandy soil, whilst no cell death was observed in the loamy soil (Calder *et al.*, 2012). When comparing the effects of Ag NPs and silver nitrate ($AgNO_3$), Colman *et al.* (2013) reported that under low exposure concentrations (0.14 mg/kg), the effect of Ag NPs was as large as or larger than the $AgNO_3$, which resulted in changes to many parameters including microbial community, composition, biomass, and extracellular enzyme activities. Ag NPs at concentrations up to 26 mg/L were also shown to be toxic to nitrifying bacteria by inhibiting growth, which was correlated to the intracellular generation of ROS (Choi and Hu, 2008). As Ag NPs demonstrated an even greater toxicity than Ag ions, the authors suggested other factors were involved, such as direct interaction with key enzymes on the cell membrane that are responsible for ammonia oxidation, due to the ability of Ag NPs to pass easily through the membrane. In contrast, under field conditions Ag NPs showed no toxicity on *Bradyrhizobium* and *Rhizobium* involved in nitrogen fixation (Shah *et al.*, 2014). Noticeably, different strains of bacteria have different responses to ENPs. Thus, the type of bacteria is also an important factor which must be taken into consideration when investigating the impact of ENPs on microbes. The mechanism of how Ag NPs affect microbes is in general poorly understood, but is reported to include damaging cell membranes, production of ROS, damaging DNA, and other effects (Parbhu and Poulose, 2012). Whether these effects result from the ENPs themselves and/or from the released ions is difficult to determine. The previous results of the effects of ENPs on microbes are summarized in Table 1.6.

Table 1.6. Impact of ENPs on microbes

ENPs	Size	Concentration	Organism	Effect	Reference
ZnO NPs	<50 nm	0, 250, 500, 750 mg/L	*R. leguminosarum bv. viciae* 3841	morphological changes and damaging in the bacterial surface	Huang *et al.* (2014)
TiO_2 NPs	35 nm	100, 250, 500, 750 mg/L	*R. leguminosarum bv. viciae* 3841	morphological changes in the bacterial cells	Fan *et al.* (2014)
C_{60}	ranged from 29 to 38 nm	2, 4, 6, 8, 10, 12, 14, 15 mg/L	*B. stearothermophilus*	no effects on bacterial growth	Santos *et al.* (2013)
Co NPs	ranged from 2-60 nm with average size (28 nm)	550 mg	*Bradyrhizobium* and *Rhizobium*	no toxicity	Shah *et al.* (2014)
Fe NPs	ranged from 2-58 nm with average size (25 nm)				
Ni NPs	average size 20 nm				
Fe_3O_4 NPs and *y*-Fe_2O_3 NPs	10.5 and 10.2 nm	0.42, 0.84, 1.26 mg/g	*Duganella, Streptomycetaceae,* and *Nocardioides*	stimulated growth of some bacteria	He *et al.* (2011)

Ag NPs	__	0, 50, 100, 250, 500 μg/g	_	affected bacterial community structure	Chunjaturas *et al.* (2014)
	10 nm	1, 3 mg/L	*Pesudomonas chlororaphis* O6	affected culturability in the sandy soil with no cell death was observed in the loamy soil	Calder *et al.* (2012)
	21 nm	0.14 mg/kg	_	changed microbial community, composition, biomass, and extracellular enzyme activities	Colman *et al.* (2013)
	9-21 nm	3, 14, 24, 25, 26 mg/L	nitrifying bacteria	inhibited growth	Choi and Hu (2008)
	2-50 nm and garage size 35 nm	550 mg	*Bradyrhizobium* and *Rhizobium*	no toxicity	Shah *et al.* (2014)

Importantly, the presence of ENPs could lead to further effects, for example a delay in the onset of nitrogen fixation and early senescence of nodules (Huang *et al.*, 2014). Likewise, CeO_2 NPs have been shown to decrease nitrogen fixation by more than 80% compared to controls (Priester *et al.*, 2012). Additionally, inhibition to soil enzyme activities; for example, protease, catalase, and peroxidase, which are bioindicators of soil health and quality (Du *et al.*, 2011), and a decrease in CO_2 emissions (Chunjaturas *et al.*, 2014) have been shown. Together, these impacts are likely to in turn affect plant physiology.

It could be concluded from the above data that the toxicity of ENPs on plants and microbes relates to four main factors; 1) type, characterization, and concentration of ENPs; 2) released ions; 3) species and which parts of an organism are targeted; 4) experimental procedures such as method of exposure, time, medium, and more. It is also important to highlight the difficulty in comparing between available data due to wide variations in ENPs types and properties, organisms, and experimental conditions and procedures. Moreover, extensive information about the properties of ENPs is not provided in some studies which makes this even more complicated. These difficulties, indeed, are due to the lack of a uniform protocol. Despite the existing challenges, it is of great importance to extensively investigate and understand the environmental impacts of ENPs on organisms before the effects of ENPs on the environment decline further.

1.6. The rationale of the project

Nanotechnology and its applications are growing very fast and as a consequence the release of ENPs into the environment is inevitable. Ag NPs have already been released into the environment and significant concentrations are predicted to be found alongside increasing growth in the application of Ag NPs in industrial sectors. To date, there are great uncertainties regarding whether or not Ag NPs are toxic and therefore many concerns have been raised over their effects on plants and associated soil microbes. Furthermore, it is unknown whether the mechanism of toxicity corresponds to the released ions or to the Ag NPs themselves. It is therefore extremely important to investigate the potential toxicity of Ag NPs in order to contribute to current knowledge on the environmental effects of ENPs, particularly on plants and associated soil microbes. Although previous studies have constructed the fundamental basis for nanotoxicological research, no study so far has considered the toxicity of the same type of ENPs in different media which represent the three environmental compartments; air, water, and soil, using different plants. It is well documented that

ENPs behave differently depending on the environmental compartments (Handy *et al.*, 2008; Peralta-Videa *et al.*, 2011; Smita *et al.*, 2012). Thus, a comprehensive study that combines all mimic environmental scenarios (air, water, and soil) and interactions of a single type of ENPs with different plants is more appropriate for highlighting the factors that are responsible for ENPs toxicity, leading to better understanding.

Ag NPs are the most significant ENPs used in industrial products today (primarily as antimicrobial and antifungal agents) (Sun *et al.*, 2014) and new products containing Ag NPs are increasingly entering the market (Quadros and Marr, 2011). As a result, this study was designed to investigate the effects of Ag NPs on different plants and their associated soil microbes, whilst considering the environmental scenarios that cover all possible exposure pathways of plants to ENPs. The aim of this is to draw a valuable conclusion regarding the potential impacts of Ag NPs on a whole ecosystem.

1.7. Aims and hypotheses

It is hypothesised that capped silver nanoparticles (cAg NPs) will behave differently according to exposure procedures and consequently will show different levels of toxicity to plants. Moreover, the toxicity of cAg NPs is thought to be dependent on plant species and is hypothesized to increase with increasing concentration. In addition, it is predicted that the different assessment techniques for cAg NP toxicity will show varied sensitivities. It is also hypothesised that cAg NPs may influence soil microbes which in turn will affect the photosynthetic performance of plants.

The overarching aim of this project is to determine the impact of cAg NPs on plants and associated soil microbes. Specifically, the aims of this project are to:

1) Evaluate the impact of cAg NPs on *Arabidopsis thaliana* seed germination, and assess any downstream effects on plant growth.
2) Elucidate soil microbial responses to cAg NPs applied directly to the soil, and investigate any related effects on the photosynthesis of *Vicia faba*.
3) Assess the impact of foliar injection of cAg NPs on the photosynthetic performance of *V. faba*.
4) Determine the role of light in increasing or decreasing the toxicity of cAg NPs in *V. faba*.
5) Obtain a mechanistic understanding of the role of ROS generation in cAg NP toxicity towards the photosynthetic performance of *V. faba*.
6) Investigate the impact of foliar spray and deposition of cAg NPs on the photosynthetic performance of *A. thaliana* and *V. faba*.
7) Investigate whether or not cAg NPs are toxic to aquatic plants by monitoring changes in the photosynthetic parameters of *L. minor*.

Chapter 2. Materials and Methods

2.1. Nanoparticles characterization

Information about ENPs characteristics should be included in nanotoxicological studies as such information will assist accurate comparisons between different investigations (Handy *et al.*, 2008). Additionally, characterization of ENPs will allow improved understanding about characteristics that are responsible for ENPs toxicity. In the present study, different characteristics were targeted and verified using a range of instruments. A stock solution of methoxy-polyethylene glycol (mPEG) capped silver nanoparticles (cAg NPs) at a concentration of 900 mg/L was provided by Dr. Paul Christian (University of Manchester, UK). Detailed information about cAg NPs synthesis is provided in Poole (2013). cAg NPs were chemically synthesised by reducing $AgNO_3$ (2.3 g) in methanol (2.3 L), in the presence of mPEG (11 g), using a solution of sodium borohydride (4.4 g) and methanol (300 mL). The solution was stirred for 1 h followed by evaporation at 50°C. The solution was dissolved again in methanol (50 mL) and precipitated by the addition of diethyl ether (250 mL). Isolation of the precipitate was performed by decantation and the solid powdered was dried overnight in a vacuum at room temperature. The dried solid powder was dissolved in ultra-high purity (UHP) water and then scrubbed with a mixed bed ion exchange resin (Dow Marathon 3R). Core and hydrodynamic diameters of the cAg NPs were measured by transmission electron microscopy (TEM) and dynamic light scattering (DLS) which showed them to be 13 nm ± 7 nm and 58 nm, respectively. Further characterisations were performed by Poole (2013) using DLS, TEM, flow field-flow fractionation (FIFFF), and atomic force microscopy (AFM) and the results are presented in Table 2.1.

Table 2.1. Caracterisation of size and charger of cAg NPs

Characterisation	Method	cAg NPs
Zeta potential (mV)	DLS	-37 ± 0.3
Mean hydrodynamic diameter (nm)	DLS	35 ± 0.2
Mean diameter (nm)	TEM	27 ± 1
Size distribution (nm)	TEM	6 – 118
Mean diameter (nm)	FIFFF	28 ± 0.6
Size distribution (nm)	FIFFF	8 – 60
Mean diameter (nm)	AFM	34 ± 4
Size distribution (nm)	AFM	7 – 125

The cAg NPs were diluted to 5, 12, 50, and 100 mg/L according to the experimental requirements. Assuming that factors such as light and temperature could affect the characteristics of cAg NPs during storage, the stock solutions were kept in the dark under ambient temperature. Such maintenance processes were later advised to be considered due to the possibility of dissolution, and the release of coatings from ENPs which influencing their behaviours (Petersen *et al.*, 2014). As the cAg NPs were capped, sonication was not performed in order to avoid damaging the coating, which could lead to changes to the properties of cAg NPs and subsequently their behaviour (Poole, 2013).

Although nitrate supply has been reported to have stimulatory effects on photosynthesis and plant growth (Simier *et al.*, 2006; Vannini *et al.*, 2013), $AgNO_3$ has been used in many studies as a source of Ag ions (Stampoulis *et al.*, 2009; Colman *et al.*, 2014). Therefore, to differentiate whether toxicity was related to cAg NPs or to Ag ions or to capping agent, $AgNO_3$ (Sigma Aldrich, UK) and mPEG were prepared and used at the same concentrations as the cAg NPs.

2.2. Plants material and growth conditions

2.2.1. *Arabidopsis thaliana*

Wild type (WT) seeds of *A. thaliana* (Wassilewskija) (Ws-0) were sown in pots containing compost (Everris Limited, Ipswich, UK) and transferred to a cold room (5°C) for 48 h in darkness to initiate germination. Pots were transferred into a controlled environment chamber where the plants were grown under controlled temperature (22°C), photoperiod (8:16 h light:dark), and humidity (50%) and maintained with sufficient water.

2.2.2. *Vicia faba*

Seeds of *V. faba* (Johnsons Company, Newmarket, UK) were germinated in pots containing compost (Everris Limited, Ipswich, UK) and placed in a growth cabinet (Sanyo PG660, Loughborough, UK). The plants were grown under controlled temperature (20°C), two different light intensities (~130 and ~480 µmol m^{-2} s^{-1}), photoperiod (12:12 h light:dark), and humidity (57%) with adequate water.

2.2.3. *Lemna minor*

L. minor were collected from ponds around Colchester, Essex, United Kingdom. The plants were surface-sterilised by immersion in 0.5% sodium hypochlorite solution (NaOCl) for 3s-5min

(Gubbins *et al.*, 2011; Shi *et al.*, 2011) and then rinsed several times with distilled water (DW). Plants were grown in Hoagland's solution (pH 6.8), which in 50 L contains the following: $NH_4H_2PO_4$, 5.75g; KNO_3; 30.30g, Ca $(NO_3)_2$ x $4H_2O$; 47.20g; $MgSO_4$ x $7H_2O$; 24.6g; EDTA monosodium salt, 8g; and 50 ml of the trace element stock solution. The latter solution was prepared in 1 L DW and contains the following: H_3BO_3; 2.86g, $MnCl_2$ x $4H_2O$, 1.8g; $ZnSO_4$ x $7H_2O$, 0.22g; CuSO x $5H_2O$, 0.08g; $NaMoO_4$ x $2H_2O$, 0.029g. Plants were grown in a glasshouse with a controlled temperature (25.6°C and 16°C, during a 13:11 h light:dark cycle) and humidity (35-40%). Photosynthetic photon flux density (PPFD) changed with solar radiation, but was supplemented to 300 µmol m^{-2} s^{-1} when solar radiation dropped below 550 µmol m^{-2} s^{-1}. The plants were kept under these conditions for two weeks and the Hoagland's solution was changed every seven days before the start of experiments.

2.3. Procedures of exposing plants to treatments

2.3.1. Seed germination and seedling growth assessments

Before exposure, seeds of *A. thaliana* were surface sterilized with 5% NaOCl for 10 min (USEPA, 1996) then rinsed several times with DW. A filter paper was placed in a 90 mm sterilized Petri dish and 30 seeds were dispersed into the dish, leaving ~ 0.25 cm between each seed. A total of 2.5 mL of either cAg NPs or $AgNO_3$ solution was added. The same volume of DW was added as a control. Petri dishes were covered and sealed with Parafilm (Bemis, USA) then transferred into a glasshouse for seven days under controlled temperature (25.6°C) and humidity (35-40%). Each treatment was performed in six replicates and at four concentrations (5, 12, 50, and 100 mg/L).

After germination was assessed (see section 2.4.1), the germinated seeds were transferred into pots containing compost (Everris Limited, Ipswich, UK) and grown for three weeks in a

controlled environment [temperature (22°C), photoperiod (8:16 h light:dark cycle), and humidity (50%)] and maintained under sufficient water condition.

2.3.2. Soil exposure application

After four weeks from germination, solutions of either cAg NPs or $AgNO_3$ were added to the surface of soil in which *V. faba* was germinated, with a target concentration of 6, 25 or 50 mg/kg soil. The same volume of DW was added to a control group.

2.3.3. Foliar exposure application

Foliar exposure was conducted by injection and deposition. For the injection application, a pair of intact *V. faba* leaves was used. Solutions of either cAg NPs, $AgNO_3$, or mPEG at concentrations of 12, 50 and 100 mg/L were injected into the petioles in one of the leaves, whilst the other leaf on the same plant was injected with DW as a control. To inject the plants, needles (Becton, Dickinson & CO. Ltd., Drogheda, Ireland) sized 25 G $^5/_8$ (0.5 mm x 16 mm) connected to a 2.5 mL syringe (BD Plastipak, Madrid, Spain) were used.

For the deposition application, two different methods were used, a pipette method and a spray pump method. For the pipette method, 200 µL of cAg NPs solutions at concentrations of 12, 50, and 100 mg/L were deposited on *V. faba* leaves as droplets covering almost all of the surface of one leaf, whilst the other leaf was used as control and treated with DW. For *A. thaliana*, the cAg NPs were deposited on the surface of a whole plant, whilst a different plant received DW as a control. Equivalent ionic suspensions of $AgNO_3$ were also applied on *V. faba* and *A. thaliana*.

Prior to the application of treatments by a spray pump, pot surfaces were covered with plastic to prevent the soil from receiving any solution. As different spray pumps may release

different size of droplets (Quadros and Marr, 2011) and thus to avoid or at least to minimize the possible variation in the size of the treatments deposited on the surface of leaves, the same spray pump was used to spray the solutions. The spray pump was washed with Milli-Q water six times and air-dried between solutions to prevent contamination. Under a fume hood, two *V. faba* leaves at the same level were treated, whereby one leaf was sprayed with 2 ml of DW as a control and the other sprayed with either cAg NPs or $AgNO_3$ at 100 mg/L. Leaves not receiving treatment were covered during exposure.

In *A. thaliana*, the whole surface of the plants was sprayed with either cAg NPs or $AgNO_3$ at 12, 50, and 100 mg/L and separate plants were sprayed with DW as a control. Pot surfaces were covered with plastic to prevent soil from receiving any treatment. The exposed plants were placed in a growth cabinet allowing the particles to be taken up by plants and the aerosolized solutions to dry before conducting measurements.

Both injection and deposition experiments were conducted in replicates.

2.3.4. Aquatic exposure application

Twenty four individual plants of *L. minor* with the same number of fronds were placed in a 24-well microplate to which either 2 mL of DW, cAg NPs, $AgNO_3$, or mPEG were added via Hoagland's solution. The final concentrations applied were 12, 50, and 100 mg/L. Each treatment was performed in four replicates. The microplates were covered with perforated lids and the plants were kept in the glasshouse for 96 h under controlled conditions as mentioned in 2.2.3.

2.4. Experimental measurements

2.4.1. Seed germination and seedling assessments

In order to evaluate the toxicity of cAg NPs and their corresponding ionic form, seed germination was assessed at day 7 by measuring plant roots with a ruler, whereby those with 1 mm length or more were classified as germinated. Germination percentage (GP) was calculated by:

$$\% \text{ GP} = \text{number of germinated seeds/total number of seeds X } 100 \qquad (1)$$

Further investigations were conducted to examine whether exposure to cAg NPs and $AgNO_3$ had an effect on the photosynthetic performance of *A. thaliana* seedlings. Thus, fluorescence and gas exchange parameters were assessed. Measurements of these parameters are detailed in sections 2.4.2 and 2.4.3.

2.4.2. Chlorophyll fluorescence measurements

The chlorophyll fluorescence technique involved capturing images in both dark and light adapted states. For dark adapted measurements of the maximum quantum efficiency of PSII photochemistry (F_v/F_m), plants were kept in darkness for 20 min before being exposed to a weak beam of a PPFD (< 1 µmol m^{-2} s^{-1}) and the minimal level of fluorescence (F_o) recorded. Plants were then exposed to a short pulse (typically 800 ms) of high PPFD (i.e. 6000 µmol m^{-2} s^{-1}) in order to measure the maximal level of fluorescence (F_m) (Lawson *et al.*, 2002; Barbagallo *et al.*, 2003; Baker, 2008). The difference between F_o and F_m represents the variable fluorescence (F_v). Thus, F_v/F_m parameter is calculated as $F_v/F_m = (F_m-F_o)/F_m$ and indicates the maximum quantum efficiency

of photosystem II (PSII) photochemistry (typically with values of 0.83 for healthy plants). In light adapted leaves, the terms of *F*' denotes steady state fluorescence in the light and maximum fluorescence measured following a suturing pulse denoted F_m' (Lawson *et al*., 2002). Minimum fluorescence in the light (F_o') was determined using the following equation (Oxborough and Baker, 1997): $F_o' = (F_o/(F_v/F_m + F_o/F_m'))$. The term F_q' represents the difference between F_m' and *F*'. Consequently the F_q'/F_m' is the operating efficiency of PSII photochemistry and is calculated as $F_q'/F_m' = (F_m'-F')/F_m'$ (Baker, 2008). Figure 2.1 shows the protocol of this technique.

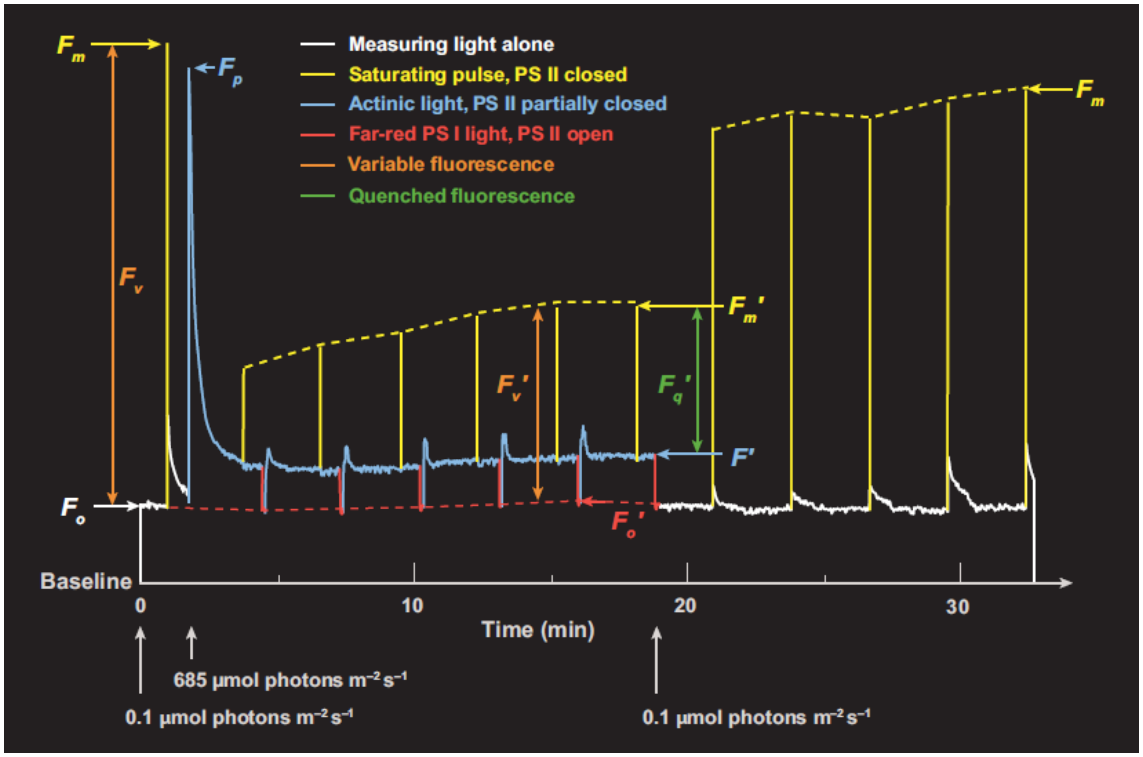

Fig. 2.1. Protocol of fluorescence technique. The fluorescence parameters without a prime are generated from leaves in dark-adapted state. The parameters with a prime are generated from leaves in light-adapted state. From (Baker, 2008).

In order to test to what extend cAg NPs are toxic to the photosynthetic efficiency in *A. thaliana*, *V. faba*, and *L. minor*, the fluorescence parameters F_v/F_m and F_q'/F_m' were measured using a FluorImager Chlorophyll Fluorescence Imaging System (Technologica Ltd., Colchester, UK). F_v/F_m measurements were taken after plants were kept in darkness for 20 min, allowing the primary electron acceptor (Q_A) to be became fully oxidized. Measurements of light-adapted leaves F_q'/F_m', were taken at two different actinic PPFDs (100 and 500 µmol m^{-2} s^{-1}). Readings were recorded at each PPFD level when F_q'/F_m' had stabilized (ca 15-20 min).

2.4.3. Measurements of gas exchange

In order to investigate whether or not cAg NPs affect the photosynthetic rate of plants, gas exchange parameters including stomatal conductance (g_s), assimilation rate (A), and a light response curve (A/PPFD) were determined using a CIRAS portable system (PP Systems, Amesbury, Massachusetts, USA).

Following assessment the seed germination of *A. thaliana* the seedlings were transferred into pots containing soil, and placed in a growth cabinet under controlled temperature (22°C), light (8:16 h light:dark photoperiod) and humidity (50%). When the leaves reached a suitable size (i.e. after week 4) the impact of cAg NPs on plant gas exchange parameters was assessed. To assess A/PPFD, attached leaves of the treated and the control plants were placed in a CIRAS cuvette at 400 μmol^{-1} CO_2 and 1000 µmol m^{-2} s^{-1} PPFD. The machine was supplied with CO_2 using CO_2 cartridges (LI-COR, Lincol, Nebraska, USA). When plants attained a steady state, ~ 15 min, a reading was recorded. PPFD was then decreased to the following values 949, 801, 577, 436, 263, 153, 107, 44, 23, and 0 µmol m^{-2} s^{-1} and a reading recorded at each PPFD level when assimilation rate had stabilized (3-4 min).

To measure the impact of cAg NPs on *A* and g_s, leaves of *A. thaliana* and *V. faba* were placed in a cuvette and CO_2 concentration and leaf temperature were maintained at 400 μmol^{-1} and (22°C), respectively. Measurements were performed inside the growth cabinet using light source of the growth cabinet (at ~130 $\mu mol\ m^{-2}\ s^{-1}$ PPFD) and readings were recorded when parameters were stable (after ~3 min).

2.4.4. Reactive oxygen species detection

Ampliflu Red (or Amplex Red, 10-Acety1-3, 7-dihydroxyphenoxazine; Invitrogen Paysley, UK) is a colourless probe that reacts with hydrogen peroxide (H_2O_2). This reaction leads to the oxidation of Amplex Red (AR) and forms the fluorescent pigment resorufin (7-hydroxy-3H-phenoxazin-3-one) that has maximum excitation and emission peaks at 570 and 590 nm, respectively (Zhou *et al.*, 1997). The probe was prepared in 50 µL aliquots of 10 mM in dimethylsulfoxide and stored at -80°C. Aliquots were diluted in 50 mM sodium phosphate buffer pH 7.5 to obtain a final concentration of 2 mM AR. Sodium phosphate buffer was prepared by dissolving 0.13g of monosodium phosphate monohydrate ($NaH_2PO_4.H_2O$) and 1.09g of disodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$) in 100 mL water. Amplex Red solution was prepared freshly on the day of the experiment. Leaves of *V. faba* that had been injected with either cAgNPs or with DW were cut from plants at the petiole point using a clean-sharp blade. Cutting was performed twice under water in order to ensure no air bubble had occurred and the leaves were able to take up the probe via the transpiration stream (Driever *et al.*, 2009). The cut leaves were transferred quickly into an Eppendorf tube containing AR solution. As AR is light sensitive, light was reduced to 50 $\mu mol\ m^{-2}\ s^{-1}$ during this process, using a "002 Rose Pink" filter (Lee filter, Andover, Hants, UK) to reduce the possibility of ROS production by light. The production of ROS in AR-fed leaves was

imaged using a Peltier-cooled charge coupled device (CCD) camera (Wright Instrument Ltd., Middlesex, UK) controlled by FluorImager V1.01 software (Technologica Ltd., Colchester, UK). A light-emitting diode (LED) was used to provide a constant and event excitation light of 100 µmol m^{-2} s^{-1}. An optical pass filter with a maximum wavelength of 590 nm (Edmund Optics Inc., York, UK) was placed in front of the CCD camera in order to detect the AR reaction.

2.4.5. Analysing of Ag content

In order to measure the content of Ag in plants, injected and deposited leaves of *V. faba* were cut from the petiole using a clean-sharp blade, weighed and dried in an oven (Gallenkamp, UK) at 60°C until the weight was stable. All dried leaves were ground to powder using a mortar and pestle. ~ 50 mg of the powdered samples was transferred into poly tetra fluoro ethylene (PTFE) tubes into which 3 mL nitric acid (HNO_3) was added. Samples were digested for 24 h at 110°C using a block heater (Stuart, SBH200D, Bibby scientific, UK). Samples were cooled for 10 min and 1 mL concentrated hydrogen peroxide acid (H_2O_2) added and samples digested for a further 24 h at 110°C. Samples were cooled for 10 min then transferred into a 15 mL centrifuge tube and made up to 12 mL using Milli-Q water following a triple wash of PTFE. Samples were centrifuged at 5000 rpm for 5 min (Megafuge 40R, Thermo scientific, Germany) and the top 5 mL transferred into a new tube and made up to 15 mL with Milli-Q water. Digested samples were further diluted 100 times before being analysed. Blanks were also prepared following the same steps, but without adding samples. To measure the content of Ag, inductively coupled plasma mass spectrometry (ICP-MS) (PerkinElmer, U.S.A) was used.

2.4.6. Assessing soil microbial community structure

The effect of cAg NPs on soil microbial community structure was assessed. Following the addition of cAg NPs, $AgNO_3$, and DW to the surface of soil in which *V. faba* was germinated, soil samples were collected at two time points: on the day of treatment (i.e. after 3 h) and 96 h post-treatment. After removing the top layer of ~ 0.5 cm, samples were collected to 2.5 cm depth, from different points covering the pot surface area. Samples were stored at –80°C prior to DNA extraction. Genomic DNA was extracted from samples using a PowerSoil® DNA isolation kit (MoBio, Carlsbad, CA) according to the manufacturer's instruction. The bacterial 16S rRNA gene was amplified using the primer pair F341-GC/R534 (Muyzer *et al.*, 1993). PCRs were performed using a Gene Amp® PCR system 9700 Thermocycler (Applied Biosystems). Thermocycling consisted of 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension step of 72°C for 10 min. Agarose gel electrophoresis (1 % w/v agarose in 1x TAE) was run at 120 V for 35 min followed by staining in ethidium bromide (10 mg/mL final concentration) for 30 min.

Denaturing Gradient Gel Electrophoesis (DGGE) was analysed using a D-Code System (Bio-Rad, UK). Fragments (10 µL) from each sample were electrophoresed on 8% (w/v) polyacrylamide (acrylamide:baisacrylamide, 37:1) gels with 40% to 60% denaturant (where the 100% denaturant contains: 7 M urea and 40% formamide in 1xTAE) at 100 V for 16 h in 1xTAE running buffer at 60°C. Gels were then silver stained according to the method of Nicol *et al.* (2005).

DNA extraction was performed in triplicate for each treatment. To elucidate the impact of the ENPs treatments on the microbial community structure, DGGE band presence/absence data was

recorded visually. Changes in band patterns (i.e. increase or decrease) were considered as indicator of the impact of ENPs treatment on the bacterial community.

2.5. Statistical analysis

Student's *t*-test and a One-Way ANOVA were used to statistically analysed data (SPSS Version 19, IBM, Armonk, New York). To examine if the data was normally distributed, a Shapiro-Wilk test was used. To test for differences between results, a post-hoc analysis was carried out following ANOVA. Statistical significance was accepted at a level of $P < 0.05$. Results are presented as mean ± standard error.

Chapter 3. Influence of cAg NPs on seed germination and growth of *Arabidopsis thaliana*

3.1. Introduction

Seed germination is an important process which indicates the beginning of plant development (Cervantes, 2006). It is a physiological process and begins with the absorption of water which is required for the growth and development of the seed embryo. Development occurs as expansion and elongation in the embryo result in the breaking of covering layers and the emergence of radicals, indicating the seed germination process has been completed (Hermann *et al.,* 2007). Germination is also controlled by plant hormones such as ethylene, which play an important role (Miransari and Smith, 2014). During the process of water absorption, ENPs may also be absorbed and enter seeds through coat pores. Entry of Ag NPs into seeds has been reported previously and has been shown to be primarily dependent on ENPs size and concentration (Thuesombat *et al.*, 2014). Once ENPs interact with seeds, inhibitory effects on plant growth is possible. Little is known about this or the consequent effects.

Seed germination is one of the most commonly used endpoints in nanotoxicological studies. In such studies, the concept of seed germination has been widely defined. For example, seed germination has been considered to have occurred when root length is 5 mm long or more (Wang *et al.*, 2012a), equal to or larger than 0.5 mm (López-Moreno *et al.*, 2010), or a minimum of 1 mm (Lin and Xing, 2007). The various effects of ENPs on seed germination have been widely reported. For example, in the presence of Zn NPs, Si NPs, ZnO NPs, Cu NPs, CeO_2 NPs, and Ag NPs inhibitory effects on seed germination in different plants were observed (Lin and Xing, 2007; Stampoulis *et al.*, 2009; El-Temsah and Joner, 2012; López-Moreno *et al.,* 2010; Thuesombat *et al.*,

2014). In contrast, other studies have demonstrated no inhibition, and sometimes enhanced germination in the presence of MWCNTs, ZnO NPs, TiO_2 NPs, and Ag NPs (Barrena *et al.*, 2009; Khodakovskaya *et al.*, 2009; Larue *et al.*, 2012; Wang *et al.*, 2012a; Huang *et al.*, 2014). Currently, it is uncertain whether ENPs have an inhibitory effect on seed germination. Moreover, it is debatable whether seed germination is a sensitive indicator of ENPs toxicity. Thus, further investigation is required in order to assess to what extent ENPs are a source of toxicity on seed germination.

Photosynthesis is a vital process by which plants convert energy from sunlight into chemical energy in order to fuel growth and activity. Under unfavourable conditions, disruptions may occur, leading to a decrease in plant activity and possibly in their productivity. Thereby, photosynthetic performance can be used as a valuable indicator of the physiological state of plants (Baker, 2008). Chlorophyll fluorescence and gas exchange have been used widely to study the effects of different abiotic factors on plants physiology (Barradas *et al.*, 1996; Lawson *et al.*, 2002; Monneveux *et al.*, 2006; Gu *et al.*, 2007; Li *et al.*, 2015).

Chlorophyll fluorescence is a non-invasive tool that is increasing in use (Rohacek *et al.*, 2008) to detect perturbations of leaf metabolism (Barbagallo *et al.*, 2003). Many advantages, for example the ability to monitor a large number of plants simultaneously, are associated with this technique (Baker, 2008). Additionally, it provides valuable information when assessing plant stress as it reflects high sensitivity of PSII to environmental factors (Murchie and Lawson, 2013).

Infra-red gas exchange analysis enables simultaneous measurements of A and g_s to be determined (Parsons *et al.*, 1998). Application of this technique provides valuable information which can be used to monitor how plants respond to environmental conditions (Mitchell, 1992).

In general, environmental stresses, such as ENPs, could induce a decrease in photosynthetic efficiency and gas exchange parameters (Baker, 2008). Although, fluorescence and gas exchange tools have many advantages, they have received little attention so far in nanotoxicological studies.

Entry of ENPs through seeds represents the first route by which ENPs may enter plants discussed in this project. Particularly, in this chapter, the impact of cAg NPs on seed germination and the photosynthetic performance of *A. thaliana* was assessed. Arabidopsis is a model plant used commonly in biological studies. Although it is not a crop plant, *A. thaliana* may be used as a relevant indicator of toxicity within the brassica family which includes important crop species (Rico *et al.*, 2011). Recently, *A. thaliana* has been used to study ENPs toxicity (Kurepa *et al.*, 2010; Lee *et al.*, 2010; Slomberg and Schoenefisch, 2012).

3.2. Assessment of cAg NPs toxicity on seed germination

Figure 3.1 shows the effects of different concentrations of cAg NPs and $AgNO_3$ on seed germination in *A. thaliana*. After 7 days of exposure, the impact of cAg NPs varied with the different concentrations used, compared to the control. Seed germination was not significantly affected by the low concentrations of 5 and 12 mg/L cAg NPs. At 50 and 100 mg/L, however, the cAg NPs caused a significant decrease in *A. thaliana* germination ($P = 0.002$ and $P < 0.001$, respectively). $AgNO_3$ at 5, 12, and 50 mg/L showed further significant decreases compared to controls ($P < 0.001$-$P = 0.001$). Moreover, at all concentrations of cAg NPs tested herein, *A. thaliana* seeds emerged radicals which were not observed with the highest concentration of $AgNO_3$ (100 mg/L), which completely inhibited germination.

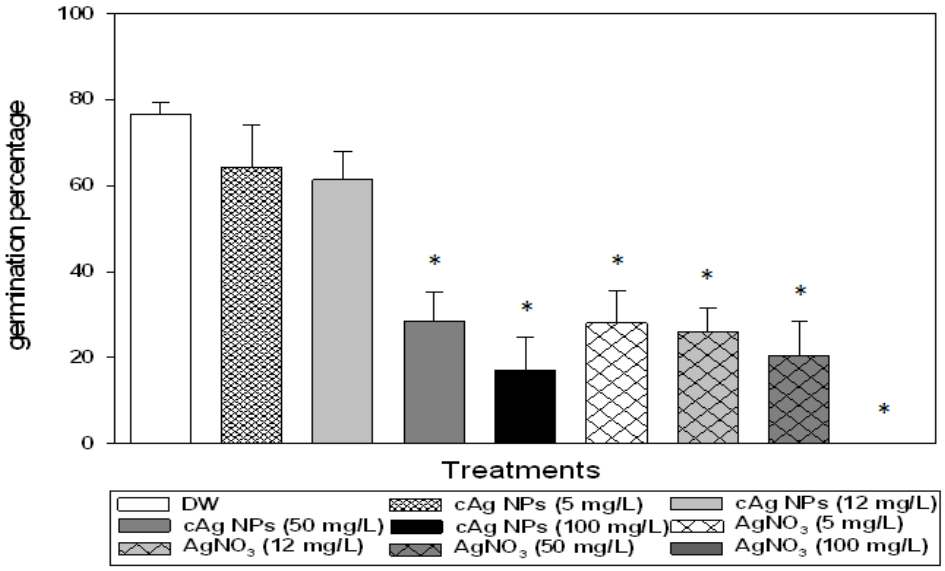

Fig. 3.1. Effect of cAg NPs and $AgNO_3$ at different concentrations (5, 12, 50, and 100 mg/L) on seed germination of *A. thaliana*. Values are given as mean ± SE (n=6). Significant differences are marked with '*' ($P < 0.05$, student's *t*-test).

3.3. Influence of cAg NPs on F_v/F_m and F_q'/F_m'

Plants of the cAg NPs-treated germinating seeds were further investigated after week four, to see whether or not cAg NPs affected fluorescence parameters F_v/F_m and F_q'/F_m' (Fig. 3.2 A-C). The cAg NPs at 12 mg/L had no effect on F_v/F_m. The operating efficiency of PSII in *A. thaliana* at PPFDs of 100 and 500 μmol m^{-2} s^{-1} was also not influenced by cAg NPs. $AgNO_3$ at 5 mg/L also had no effect neither on F_v/F_m nor on F_q'/F_m'.

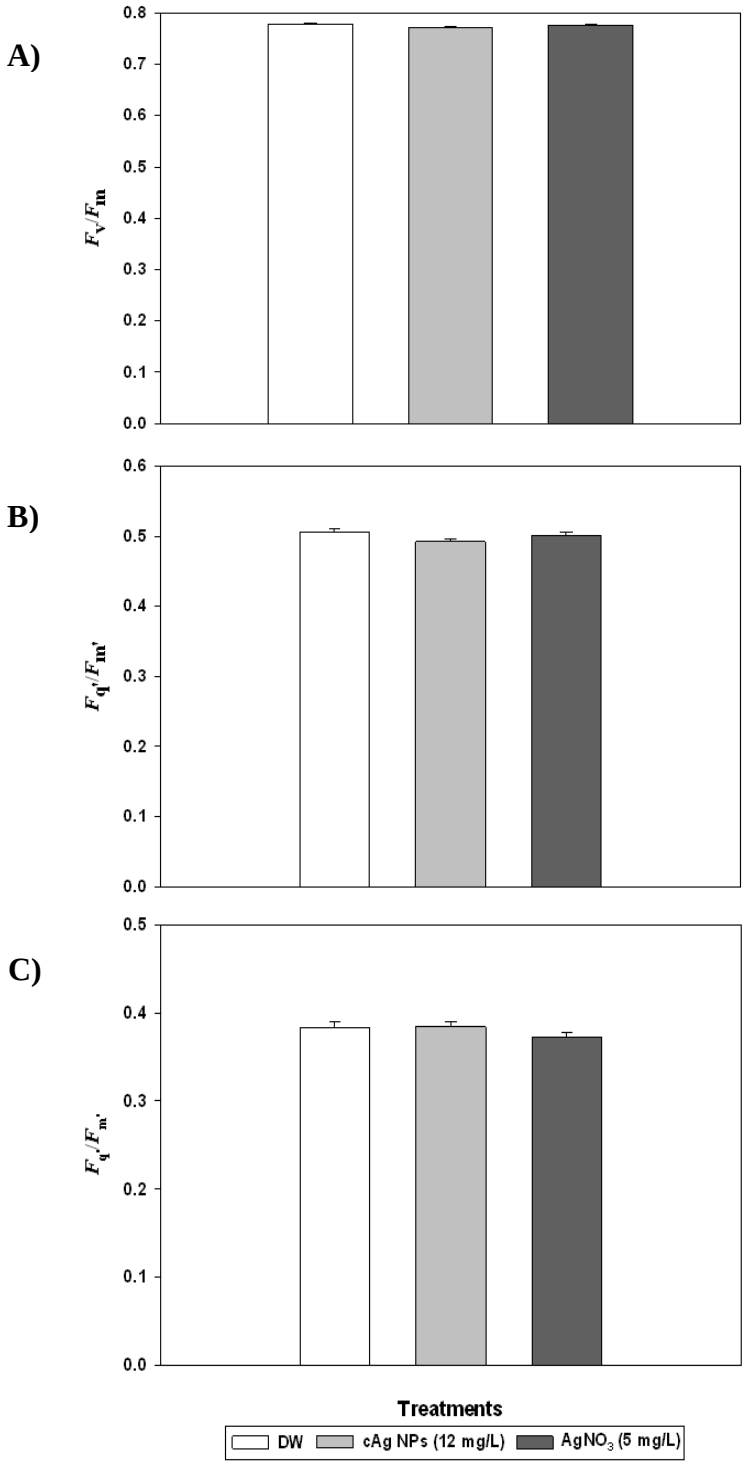

Fig. 3.2. Response of the chlorophyll fluorescence parameters F_v/F_m (A), and F_q'/F_m' at a PPFD of 100 (B) and 500 (C) µmol m^{-2} s^{-1}, to cAg NPs and $AgNO_3$ at 12 and 5 mg/L, respectively. Values are given as mean ± SE (n=5). ($P \geq 0.05$, student's *t*-test).

3.4. Impact of cAg NPs on the response of *A* to PPFD

The impact of cAg NPs on the response of *A* to PPFD was assessed (Fig. 3.3). Compared to the control, all treated plants showed a similar behaviour in which the light saturated rate of photosynthesis was not reached until PPFD was greater than 400 µmol m^{-2} s^{-1}. Thus, neither cAg NPs nor $AgNO_3$ showed any significant effect on *A*/PPFD.

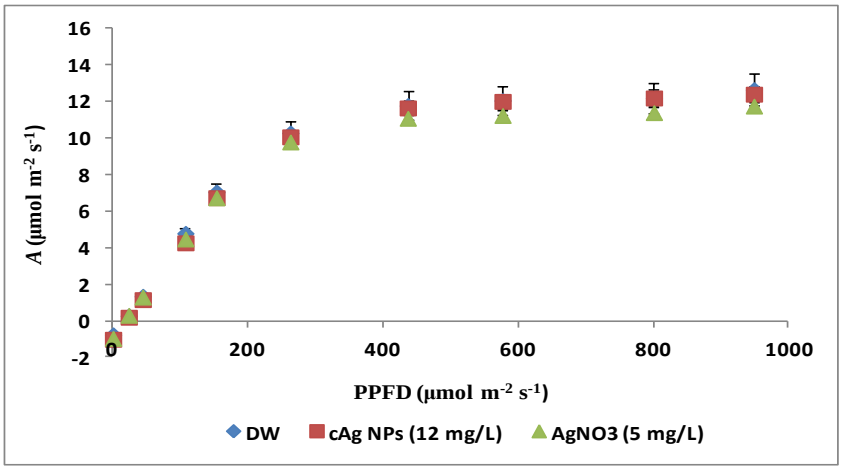

Fig. 3.3. Impact of cAg NPs (at 12 mg/L) and $AgNO_3$ (at 5 mg/L) on the response of CO_2 assimilation rate to PPFD of *A. thaliana* under different light levels ranging between 0 and 1000 µmol m^{-2} s^{-1}. Values are given as mean ± SE (n=10). ($P \geq 0.05$, student's *t*-test).

3.5. Discussion

In this study, cAg NPs at concentrations of 5 and 12 mg/L showed little impact on the germination of *A. thaliana* seeds. However, when the concentration of cAg NPs was increased to 50 and 100 mg/L, significant inhibition was observed, indicating concentration-dependent toxicity. In agreement with these results, others have also reported inhibition to seed germination when exposed to Ag NPs. For example, Thuesombat *et al.* (2014) demonstrated an inhibitory effect of Ag NPs at 0.1-1000 mg/L to rice seeds. In agreement with the present study, these authors found the effect to be concentration-dependent. In addition, El Temash and Joner, (2012) reported seed germination of ryegrass and barley to be inhibited in the presence of 10-100 mg/L Ag NPs. Different ENPs have also been shown to exhibit toxic effects on seed germination in a range of plants. For example, Lee *et al.* (2010) observed a significant decrease in the seed germination of *A. thaliana* exposed to ZnO NPs at 400, 2000, and 4000 mg/L. Exposure to CeO_2 NPs at 500, 1000, 2000 mg/L resulted in a significant decrease in seed germination of alfalfa, corn, tomato, and cucumber (López-Moreno *et al.*, 2010). In contrast, Larue *et al.* (2012) found no effects on seed germination when wheat was exposed to TiO_2 NPs at 100 mg/L. In addition, other studies showed CuO NPs (at 10 and 100 mg/L) and ZnO NPs (at concentrations up to 1000 mg/L) had no effect on seed germination in corn or pea plants (Wang *et al.*, 2012b; Huang *et al.*, 2014). A study by Lin and Xing (2007) demonstrated selectively of seed coats to pass ENPs and report significant effects of Zn NPs and ZnO NPs, whilst no effects were caused by MWCNTs, Al_2O_3 NPs, and Al NPs. Moreover, the impact of ENPs on seed germination is not always negative, and positive effects have also been observed in previous studies. In one study, Ag NPs (at concentrations up to 40 mg/L) capped with gum arabic significantly enhanced germination of *Eupatorium fistulosum* (Yin *et al.*, 2012). Also, MWCNTs (at 10 to 40 μg/mL) significantly enhanced germination of tomato seeds (Khodakovskaya *et al.*, 2009).

Evidently different ENPs can have differential effects on seeds, and factors such as plant species and experimental conditions play a critical role in controlling ENPs toxicity. Indeed, it is known that different plant species have varied responses to environmental variables (Cervantes, 2006).

Since Ag NPs can release Ag ions (Loza *et al.*, 2014), the toxicity of the dissolved silver was assessed in the present study. Results demonstrate that Ag ions affected *A. thaliana* germination significantly at all concentrations tested (5, 12, and 50), and caused complete inhibition at 100 mg/L. Such comparable investigations between ENPs and ions are missing in some previous studies (El Temash and Joner, 2012; López-Moreno *et al.*, 2010; Thuesombat *et al.*, 2014).

It is proposed that the effects demonstrated here on seed germination could indicate entry of cAg NPs through seed coats, as pollutants could not affect plants if they cannot pass via such coats (Lin and Xing, 2007). Nevertheless, ENPs (for example, TiO_2 NPs) have been reported to affect plants without entering the plant itself, by accumulating on root surfaces and blocking water pathways, which consequently affected plant growth (Asli and Neumann, 2009). Dissolution of cAg NPs and the consequent release of Ag ions which might be occurred before and/or following the entry of cAg NPs through seed coats can explain the toxicity observed in the present study. However, it is unclear whether this toxicity is related to the dissolution of cAg NPs inside plant cells and/or in the surrounding media. Moreover, it remains unknown how ENPs affect seed germination as it has been differentially reported to be related either to the ENPs themselves or to their released ions (Barrena *et al.*, 2009; Lee *et al.*, 2010; Yin *et al.*, 2011; Thuesombat *et al.*, 2014).

Ethylene is a plant hormone of crucial importance in seed germination, in particular on cell elongation (Cervantes, 2006). The role of ethylene in promoting seed germination in different species is well documented. For example, Stewart and Freebairn (1969) showed the importance of

ethylene and its role in stimulating the seed germination of lettuce. In addition, exposure to ethylene resulted in stimulation of seed germination of douglas fir (Borno and Taylor, 1975). In a recent study, Lin *et al.* (2013) reported a promotional role of ethylene in the germination of *A. thaliana* seeds. Although the exact mechanism of how ethylene promotes seed germination is not well understood (Miransari and Smith, 2014), such promotion could be associated with the role of ethylene in decreasing levels of abscisic acid (ABA), a hormone that has an inhibitory role on germination (Cervantes, 2006). In the presence of ethylene inhibitors, delayed or no germination occurs. Silver is well known as an ethylene inhibitor and several studies have determined the inhibitory effect of silver on plant development. For example, the effect of Ag ions ($AgNO_3$) on mung bean was investigated and results showed inhibitory effects on seed germination, highlighting the role of Ag ions as ethylene inhibitor (Chaudhuri and Kar, 2008). Likewise, the antagonistic action of Ag ions on ethylene resulted in inhibition to germination and seedling growth in barley (Locke *et al.*, 2000). Turhan (2004) also demonstrated the antagonistic action of Ag ions and reported an inhibitory effect of ethylene on potato plants. Thus, the toxic effect of cAg NPs observed in the present study is thought to be due to the released ions which in turn affected ethylene function and ultimately seed germination.

Chlorophyll fluorescence and gas exchange are useful and informative tools that have been used widely to study the effects of different factors on plants (Lawson *et al.*, 2002; Gu *et al.*, 2007; Li *et al.*, 2015). However, both methods have been little considered to date in the area of nanotoxicity. Thus, this study is one of the first to elucidate the effects of cAg NPs on the photosynthetic performance of *A. thaliana* using such techniques.

Plants of the cAg NPs-treated germinating seeds were further investigated at week five, to see whether or not cAg NPs affected fluorescence parameters F_v/F_m and F_q'/F_m'. Results showed no toxic effects of cAg NPs at 12 mg/L on either parameter. Results also showed no effect of cAg NPs at 12 mg/L on the gas exchange parameter *A*/PPFD. Indeed, such results were unsurprising, as these investigations were conducted four weeks after the observed effects on seeds germination, at a stage in which the plants were well developed. The lack of a toxic effect could also be attributed to the ability of plants to mitigate the toxicants' impacts by, for example, upregulating specific proteins that act as protective mechanisms when plants are under stress (Zhao *et al.*, 2015). Additionally, it could be that the cAg NPs were taken up by the plants through the seeds, but remained trapped in roots without any effect on the activity of the plants. Accumulation of ENPs in plants without any observed toxic effects has previously been reported for different plant species (Parsons *et al.*, 2010; Larue *et al.*, 2012; Thuesobat *et al.*, 2014). However, detection of cAg NPs in roots was not conducted in this study, and such a hypothesis should be evidenced. In agreement with the results of this study, Wang *et al.* (2008) showed no significant impact on F_v/F_m and F_q'/F_m' when *C. reinhardtii* exposed to TiO_2 NPs at 0-100 mg/L. In addition, Zhao *et al.* (2012) demonstrated no effects on gas exchange parameters of corn exposed to CeO_2 NPs at 400 and 800 mg/kg soil.

In conclusion, the results from this study showed that cAg NPs at the concentrations investigated have the potential to affect plants, causing dose-dependent inhibition to seed germination of *A. thaliana*. Although seed germination assessment has many advantages, for example, simplicity and low cost (El Temash and Joner, 2012), it could be an insensitive endpoint in nanotoxicological investigations (Lin and Xing, 2007; Stampoulis *et al.*, 2009). Therefore, additional techniques such as photosynthetic performance should be considered in nanotoxicological

studies. As this study showed an inhibitory effect on seed germination, further studies are required to confirm whether cAg NPs may impact on ethylene function.

Chapter 4. Impact of soil-applied cAg NPs on the associated soil microbes and on *Vicia faba* Physiology

4.1. Introduction

ENPs incorporated in nanotechnological products, such as agricultural fertilizers and pesticides, have already found their way into soils (Gottschalk *et al.*, 2009). The application of agricultural protection products and sludge deposition (Boxall *et al.*, 2007) are clear examples for the direct and indirect, respectively, entry of ENPs into soils. Such contamination of the soil environment is likely to adversely affect soil microbes and plants, potentially resulting in plant growth inhibition and unknown impacts on microbial-mediated processes such as biogeochemical cycles (Brar *et al.*, 2010; Du *et al.*, 2011; Beddow *et al.*, 2014a; Huang *et al.*, 2014; Judy *et al.*, 2015). It was recently reported that a disruption in the interaction between *Rhizobia* and legume plants could occur in the presence of ENPs (Huang *et al.*, 2014). As a result, concerns have been raised about the potential biological impacts of ENPs on plants and associated soil microbes (Anjum *et al.*, 2015).

Soil microbes are of great importance in the environment as they play multiple critical roles including element cycling, pollutant degradation, and stimulation of plant growth (Gajjar *et al.*, 2009). It has been reported that microbial communities are sensitive to ENPs (Calder *et al.*, 2012; Priester *et al.*, 2012; Colman *et al.*, 2013; Beddow *et al.*, 2014a,b; Chunjaturas *et al.*, 2014; Fan *et al.*, 2014; Judy *et al*., 2015). Studying the effect of ENPs on soil microbes is not only important to evaluate the potential impacts of ENPs on microbes, it is also useful to obtain valuable information about the influence of ENPs on soil health and plant growth (He *et al.*, 2011; Fan *et al.*, 2014; Huang *et al.*, 2014). For example, it was reported that the application of ENPs resulted in changes to

soil health by affecting the activity of soil enzymes such as protease, catalase, and peroxidase (Du *et al.*, 2011). Moreover, in the presence of ENPs, plants may mistakenly treat beneficial bacteria as pathogenic species (Huang *et al.*, 2014). Therefore, maintaining both the health of soil and the microbial communities present is necessary in order to provide a healthy environment for quality vegetative production.

Ag NPs are an effective antimicrobial agent and are applied widely in nanotechnological products against pathogenic bacteria (Gajjar *et al.*, 2009). However, they may also pose a risk to beneficial microorganisms when released into the environment. The mechanisms of Ag NP toxicity to microorganisms have been reported to include: damage to cell walls and membranes, altering cell morphology, inhibiting enzymes, and interfering with DNA, consequently leading to cell death (Prabhu and Poulose, 2012; Beddow *et al.*, 2014a; Huang *et al.*, 2014; Fan *et al.*, 2014).

ENPs that ultimately end up in soil can interact directly with plant roots (Colman *et al.*, 2013), and this represents the second route by which ENPs may enter plants. Indeed, ENPs have been reported to enter plants through the walls of root cells via apoplastic pathways (Asli and Neumann, 2009; Du *et al.*, 2011). In addition, ENPs can pass into plants by forming new pores (i.e. by causing physical damage) in epidermal and cortical cells (Lin and Xing, 2008). Following entry, ENPs can pass through the root epidermis, exodermis, cortex and endodermis, or directly from the lateral root into the stele (Peng *et al.*, 2015). Once inside plants, ENPs can translocate into above-ground parts through the xylem, symplastic transport, or through damaged cells or holes (Nowack and Bucheli, 2007) and finally they may accumulate in different parts of the plant. A number of toxic effects on plants caused by ENPs have previously been reported. For example, Thuesombat *et al.* (2014) investigated the effect of Ag NPs at concentrations (up to 1000 mg/L) on rice and

observed a decrease in seed germination and seedling growth. The authors also detected Ag NPs in root and leaf tissues. Wang *et al.* (2012b) also investigated the effect of CuO NPs at concentrations up to 100 mg/L on corn and reported a decrease in seedling growth. Thus, it is possible that plant health may be detrimentally impacted as a result of ENPs release into soils.

Notably, most previous studies investigating the effect of ENPs on plants have focused on aqueous exposure systems which cannot be used to indicate the impact on plants germinated in soils (Calder *et al.*, 2012; Priester *et al.*, 2012). As a result of this, little is known about the toxicity of ENPs, particularly Ag NPs, on plant-soil systems (Fan *et al.*, 2014; Huang *et al.*, 2014). This study, particularly this chapter, investigated the effect of cAg NPs on *V. faba* and associated soil microbial community due to the beneficial role of this system in the environment, for example in nitrogen fixation.

4.2. Analysis of cAg NPs effect on soil bacterial community structure

The impact of cAg NPs at concentrations of 6, 25, and 50 mg/kg on soil microbial structure was investigated after 3 and 96 h of exposure (Figs. 4.1 and 4.2). Compared to the control, the DGGE profiles of treated samples all indicated no significant changes to microbial community structure at all cAg NPs concentrations investigated, during 3 and 96 h. Similarly, after 3 and 96 h there were no significant changes to microbial community structure in soils treated with $AgNO_3$ at equivalent concentrations.

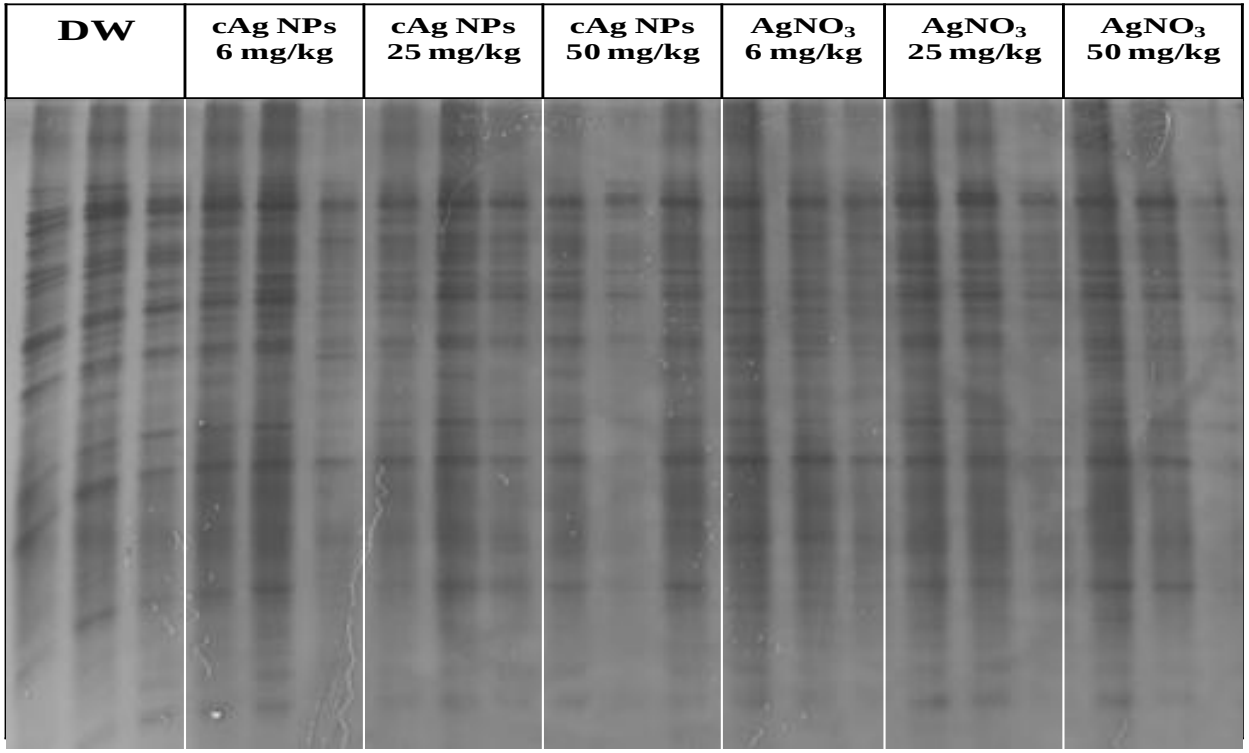

Fig. 4.1. DGGE profiles of 16S rRNA genes of bacterial communities from soils treated with DW and different concentrations of cAg NPs and $AgNO_3$ at 6, 25, and 50 mg/kg soil after 3 h. Triplicate soil community profiles (lanes) for each treatment are presented.

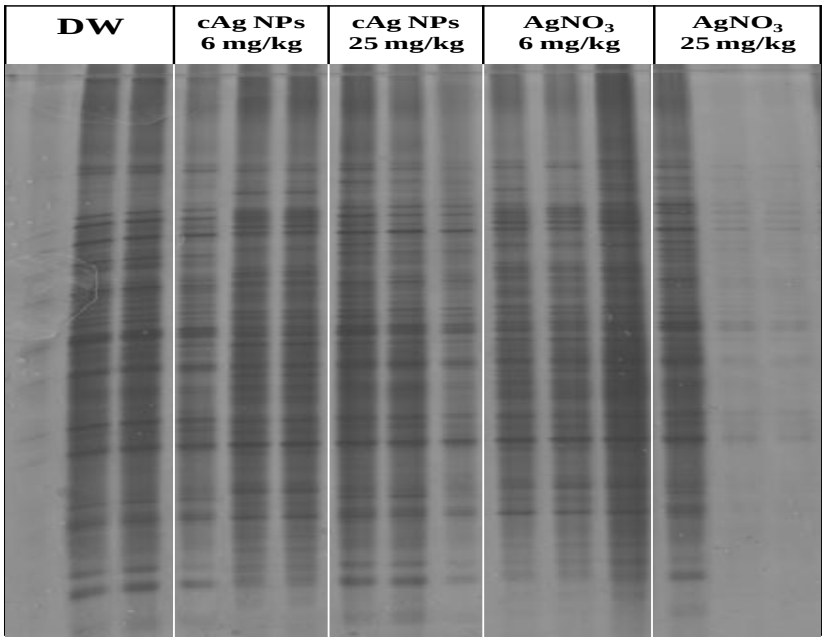

Fig. 4.2. DGGE profiles of 16S rRNA genes of bacterial communities from soils treated with DW and different concentrations of cAg NPs and $AgNO_3$ at 6 and 25 mg/kg soil after 96 h. Triplicate soil community profiles (lanes) for each treatment are presented.

4.3. cAg NPs influence on F_v/F_m

The effect of cAg NPs applied on the surface of soil at 6, 25, and 50 mg/kg on the F_v/F_m of *V. faba* is presented in Fig. 4.3. During 96 h there was no significant effect of cAg NPs on F_v/F_m at any concentration applied, compared to control plants. Similarly, F_v/F_m was unaffected by $AgNO_3$ at all concentrations applied, compared to control plants.

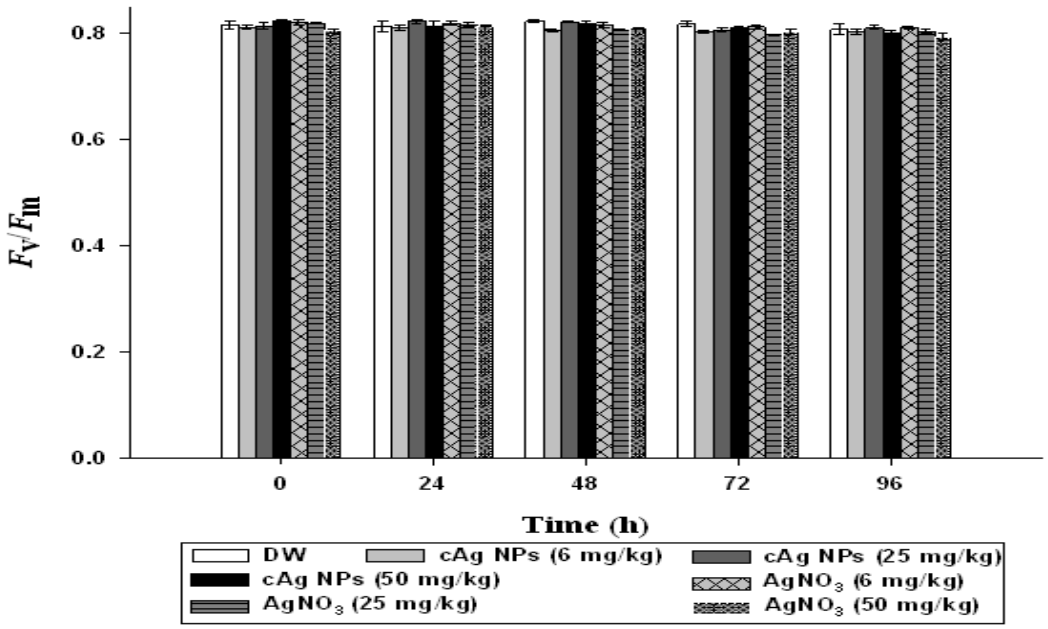

Fig. 4.3. Response of the chlorophyll fluorescence parameter F_v/F_m to cAg NPs and $AgNO_3$ at 6, 25, and 50 mg/kg soil. The values given are mean ± SE (n=3). Comparisons between treatments were performed using one way ANOVA ($P \geq 0.05$).

Alongside with the non-toxic effect of cAg NPs on F_v/F_m, the visual appearance of control and cAg NP exposed plant leaves showed no differences throughout the experiment (Fig. 4.4).

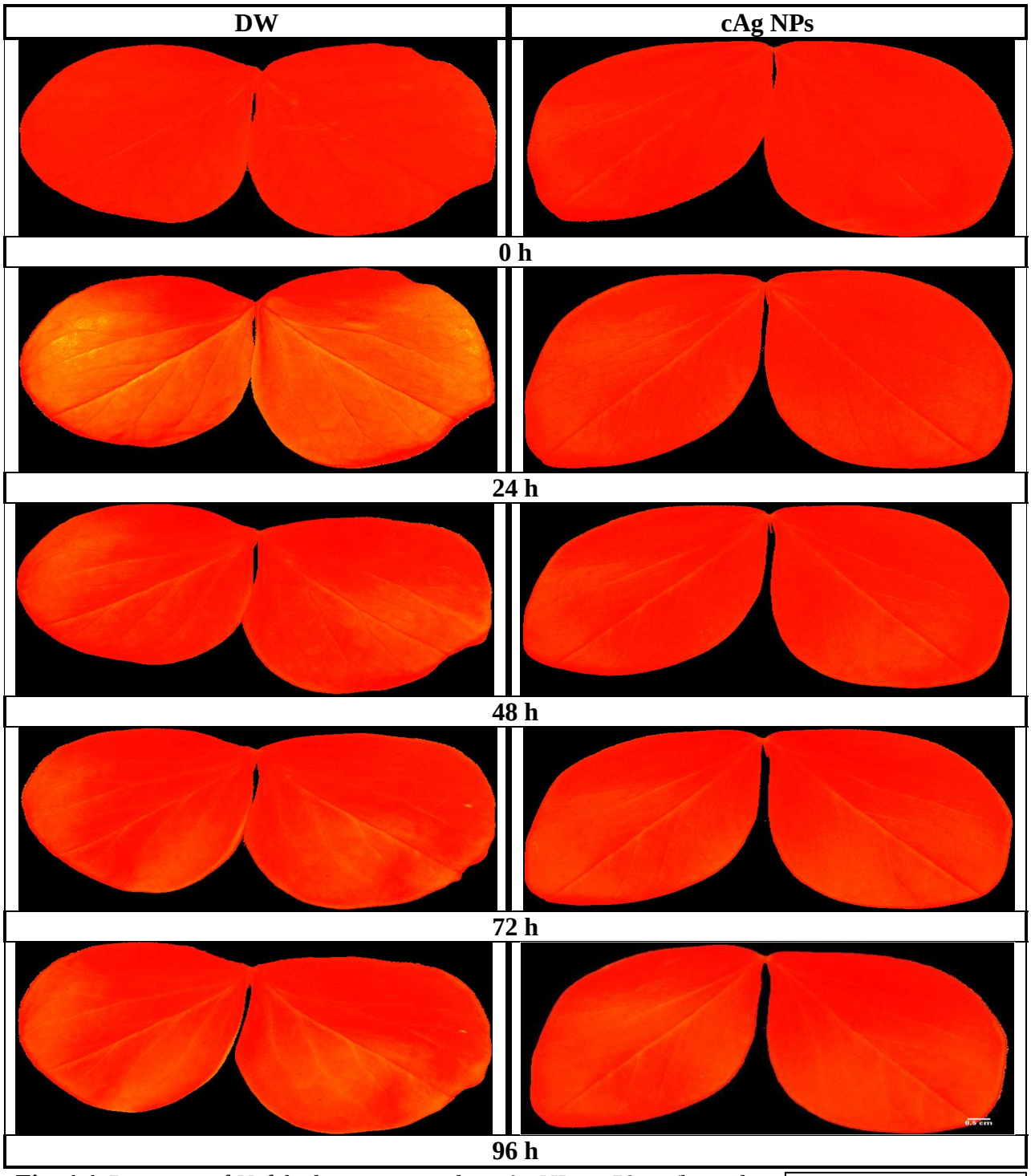

Fig. 4.4. Response of *V. faba* leaves exposed to cAg NPs at 50 mg/kg soil over 96 h. Images represent changes in false colour representing ranges of F_v/F_m values were taken by the FluorImager. Paired leaves on the left represent control plants and paired leaves on the right represent treated plants.

4.4. Impact of cAg NPs on F_q'/F_m'

Figures 4.5 and 4.6 show the effects of cAg NPs at 6, 25, and 50 mg/kg soil on *V. faba* F_q'/F_m' at a PPFD of 100 and 500 µmol m^{-2} s^{-1}, for treated and control plants. At a PPFD of 100 µmol m^{-2} s^{-1}, the F_q'/F_m' of *V. faba* was not significantly affected by cAg NPs at any of the concentrations applied during 96 h, compared with the control. Exposure to $AgNO_3$ also had no significant impact on F_q'/F_m'. Additionally, when the PPFD was increased to 500 µmol m^{-2} s^{-1}, none of the Ag treatments showed any significant toxic effects and plant responses were comparative to controls during the 96 h investigation period.

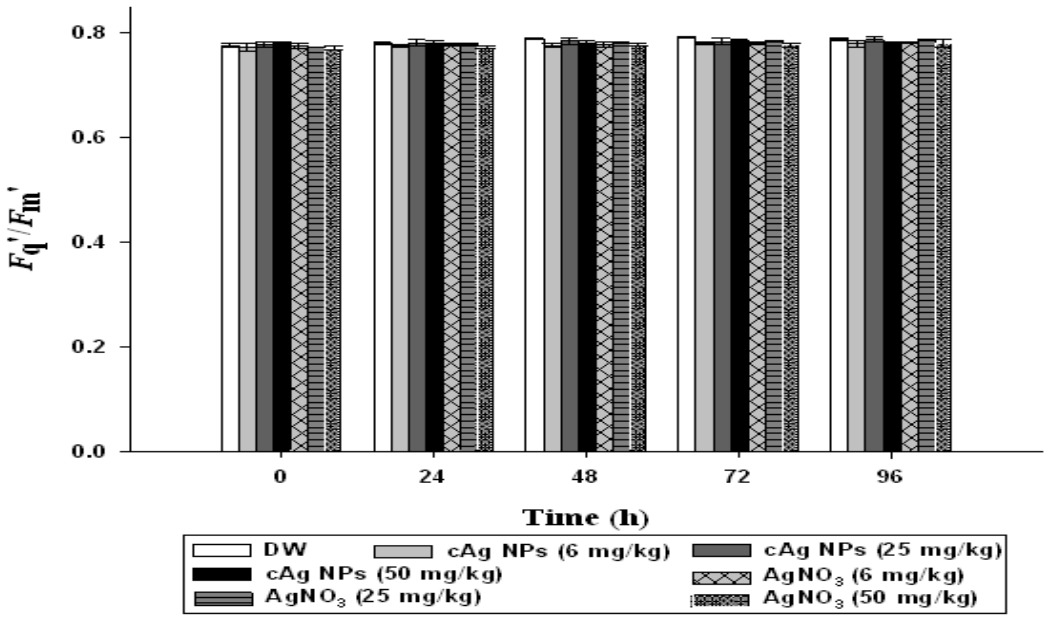

Fig. 4.5. Response of the chlorophyll fluorescence parameter F_q'/F_m' at a PPFD of 100 µmol m^{-2} s^{-1}, to cAg NPs and $AgNO_3$ at 6, 25, and 50 mg/kg soil. The values given are mean ± SE (n=3). Comparisons between treatments were performed using one way ANOVA ($P \geq 0.05$).

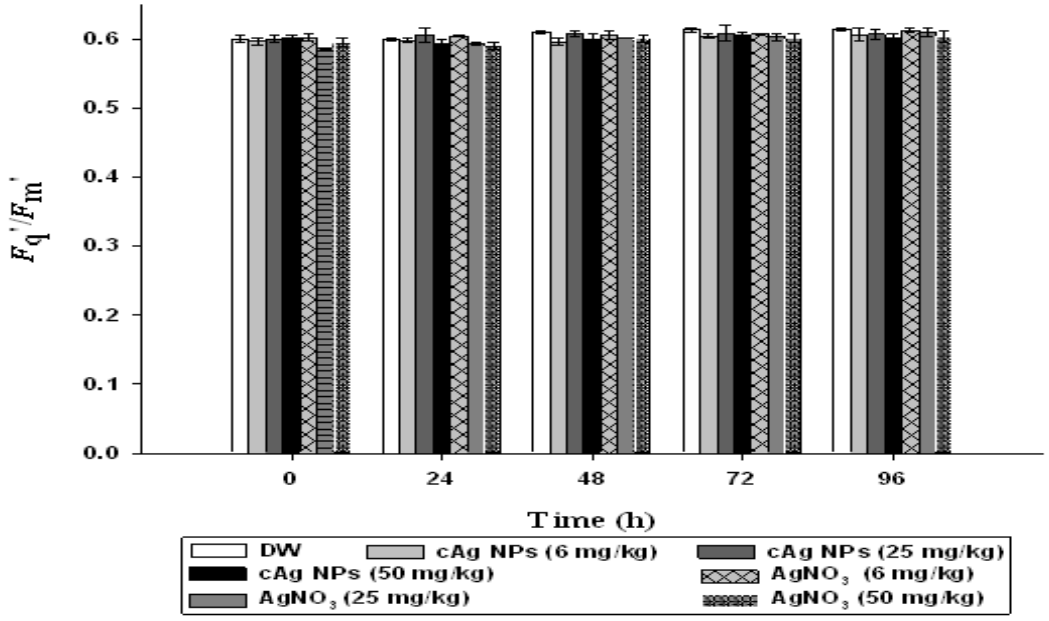

Fig. 4.6. Response of the chlorophyll fluorescence parameter F_q'/F_m' at a PPFD of 500 µmol m^{-2} s^{-1}, to cAg NPs and $AgNO_3$ at 6, 25, and 50 mg/kg soil. The values given are mean ± SE (n=3). Comparisons between treatments were performed using one way ANOVA ($P \geq 0.05$).

4.5. Effect of cAg NPs on *A*

The effect of cAg NPs at 6, 25, and 50 mg/kg soil on *A* is presented in Fig. 4.7. During 96 h, neither cAg NPs nor $AgNO_3$ had a significant toxic effect on *A*.

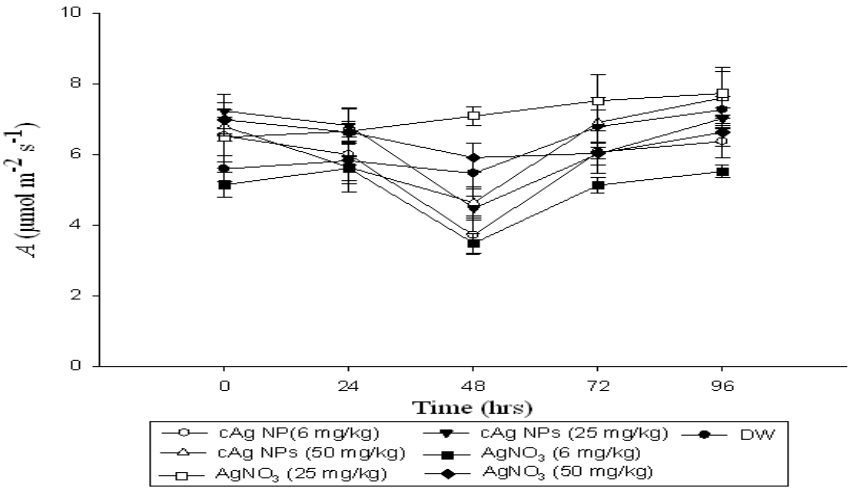

Fig. 4.7. Response of *A*, to cAg NPs and $AgNO_3$ at 6, 25, and 50 mg/kg soil, during 96 h. The values given are mean ± SE (n=4). Comparison between treatments was performed using one way ANOVA ($P \geq 0.05$).

4.6. Discussion

The stability of soil bacterial communities and their activity is of great importance to environmental health. Significant changes to a bacterial community such as loss of a species, can affect the overall function and activity of the community (Bour *et al.*, 2015) and thus influence soil quality and health (Shah *et al.*, 2014).

The results of the present study demonstrate that different concentrations (up to 50 mg/kg) of cAg NPs applied to the surface of soil had no significant effect on soil bacterial community structure, as demonstrated by a lack of significant changes in bacterial DGGE band profiles. Similarly, $AgNO_3$ at the same concentrations had no significant effect on soil bacterial community structure. In agreement with these results, a number of previous studies have shown no effect of Ag NPs on soil microbial communities. For example, Shah *et al.* (2014) revealed that under field conditions, Ag NPs at 550 mg/kg showed no toxicity towards *Bradyrhizobium* and *Rhizobium*. In addition, Calder *et al.* (2012) demonstrated no effect on the beneficial soil bacterium (*Pseudomonas chlororaphis*) exposed to Ag NPs at 1 and 3 mg/L in a loam soil. One possible explanation for a lack of toxicity to soil microbial communities caused by cAg NPs in the present study is the presence of humic acids in the soil. Humic acids may play a role in reducing the toxicity of cAg NPs through increased aggregation of Ag NPs and/or interaction of Ag ions with chloride ions, forming less-toxic Ag complexes (Liu and Hurt, 2010; Calder *et al.*, 2012). Well-dispersed Ag NPs have been suggested to be more effective antimicrobial agents, indicating the role of released ions in toxicity (Lok *et al.*, 2007). Indeed, aggregation of Ag NPs reduces their specific surface area and can lead to a reduction in ion release as well as a reduction in interactions with organisms, resulting overall in reduced toxicity (Beddow *et al.*, 2014a). It is also possible that

interactions between cAg NPs and soil compounds occurred which can mitigate the toxicity of cAg NPs. For example, reactions between sulphur and Ag NPs have previously been shown to decrease the toxicity of Ag NPs to nitrifying organisms (Chio *et al.*, 2009). Likewise, Beddow *et al.* (2014b) related the unperturbed activity of hydrocarbon-degrading microbes in the presence of Ag to a reaction with sulphur.

In addition, it is likely that genes conferring Ag-resistance exist in environmental bacteria (Silver, 2003) and it is not excluded that such strains were present in the soil used in this study which may consequently explain the missing toxicity of cAg NPs (Beddow *et al.*, 2014b). In the latter study, the authors suggested that certain hydrocarbon-degrading microbes may be resistant to silver. Although silver resistance is an important factor to consider in light of the present findings, soil Ag-content was not analysed in the present study so it is not known whether the *in situ* microbial communities were pre-exposed to Ag. Indeed, microbial mechanisms for mitigating the toxicity of Ag NPs are not fully understood. Further investigation is required in order to understand how Ag NPs migrate and interact within soil components and how this affects their toxicity towards microbes and consequently on plant growth.

In contrast to the results of this study, when Beddow *et al.* (2014b) applied the same cAg NPs (up to 50 mg/L) to estuarine sediments, a significant shift in the bacterial community structure occurred. Such differential results could be attributed to the type of medium used. For example, Ag NPs (at 1-3 mg/L) were not toxic towards the bacteria *Pseudomonas chlororaphis* in loamy soil, whilst in sand, loss in bacterial culturability was observed (Calder *et al.*, 2012). Likewise, when comparing six different soil types, silty-clay was the only soil where microbial activity was affected by TiO_2 NPs at 1 and 500 mg/L (Simonin *et al.*, 2015). Chunjaturas *et al.* (2014) also

demonstrated that Ag NPs affected bacterial community structure in one of two soil types investigated, concluding that the toxicity of Ag NPs (up to 500 mg/g) on soil microbial structure is dependent on soil type.

Studying the concentration and the behaviour of ENPs, such as aggregation state, dissolution rate, and interaction with trace elements, in soils and the resulting influence on toxicity are key factors that should be considered in nanotoxicological studies. Factors related to soil properties such as pH, water content, permeability and matrix structure are also important. Consideration of these factors would indeed help to improve current understanding about the behaviour and toxicity of ENPs.

Although it was shown that seed germination is significantly inhibited by cAg NPs (Chapter 3 of this thesis), no significant impacts on photosynthetic performance of *V. faba* were observed when cAg NPs were applied into the soil at concentrations up to 50 mg/kg. This could be explained by the way in which the cAg NPs interacted with the plants. Whilst in the germination experiment the cAg NPs were in direct contact with the seeds, in the soil experiment it is possible that little direct contact occurred between the cAg NPs and plant roots due to interactions between the cAg NPs and soil components (such as organic substances), resulting in reduced bioavailability of the cAg NPs for plants. The results of this study are consistent with the report of Lee *et al.* (2012) who demonstrated little to no effect of Ag NPs at concentrations up to 2000 mg/kg on mung bean and sorghum germinated in soil, compared to those germinated in agar. This was related to the low bioavailability of Ag NPs in soil as aggregation and sorption onto soil particles occurred. A similar hypothesis was reported to explain minor inhibition to corn exposed to different ENPs in soil, compared to those hydroponically germinated (Asli and Neumann, 2009).

Another factor that can reduce the uptake of cAg NPs by plants is water uptake capacity. Plants with a high water uptake capacity will tend to uptake more ENPs (Zhao *et al.*, 2015). For example, Schwade *et al.* (2013) reported a greater water uptake of pumpkin compared to wheat, which consequently increased the translocation of ENPs from the soil into plants. The physiological structure of the plants (i.e. larger pores in pumpkin compared to wheat) could, however, help to explain the difference in ENPs uptake. Although, the application of cAg NPs in the present study was performed after four weeks of plant growth, by which time the root system was well established, it seems that low concentration of cAg NPs was taken up as plants may have low water uptake capacity. Thus, no effect on photosynthetic performance was observed.

Importantly, the interaction of ENPs with soil components does not completely prevent adherence of ENPs to root surfaces or the uptake of ENPs or dissolved ions by plants (Peng *et al.*, 2015). It seems, however, that cAg NPs and/or dissolved Ag ions were taken up by plants with no significant impact on photosynthetic performance. This hypothesis is supported by previous studies including one by Colman *et al.* (2013), whereby Ag accumulated inside plant tissues yet, there was no evidence of toxicity to photosynthesis in sedge, rush, forb, or grass. Likewise, Zhao *et al.* (2015) detected Ce in different corn tissues, whilst no adverse effects on photosynthesis occurred when exposed to CeO_2 NPs at 400 and 800 mg/kg soil. Wang *et al.* (2013a) also observed no effect on the growth of cowpea by ZnO NPs at 500 mg/kg, although Zn was taken up and accumulated in root tissues. Collectively, these data could suggest that the toxicity of ENPs on plants is not necessarily linked with uptake.

An alternative explanation for the lack of toxicity in the present study is that the cAg NP concentrations investigated were too low to have a toxic effect on *V. faba*. Such a hypothesis is

supported by the results of previous studies. For example, Du *et al.* (2011) and Zhao *et al.* (2015) only saw toxic effects on plants at high concentrations of ENPs (up to 1000 mg/kg of TiO_2 NPs and up to 800 mg/kg of ZnO NPs) compared to the concentrations applied in this study.

Considering the lack of impact of cAg NPs observed on soil microbes, the lack of toxicity of cAg NPs towards plants physiology is not surprising. In agreement with this finding, correlations between plants and microbes in their responses to ENPs exposure have previously been reported. In a recent study, Ag NPs and other ENPs at different concentrations (up to 2400 mg/kg) caused a significant shift in soil microbial community composition alongside a reduction in the growth of *Medicago truncatula* (Judy *et al.*, 2015). In addition, Fan *et al.* (2014) reported a disruption in the interaction between plants and their associated microbes, which caused a delay in root nodule development of peas and the subsequent onset of nitrogen fixation, caused by TiO_2 NPs at 250 to 750 mg/L concentrations. Huang *et al.* (2014) also observed similar effects on peas in the presence of up to 750 mg/L ZnO NPs. It is likely that correlated toxicity of ENPs towards microbes and plants result from many factors such as dissolved ions, the attachment of ENPs to roots, and the generation of ROS (Huang *et al.*, 2014).

In conclusion, these results demonstrated that under the conditions used in this study, cAg NPs had no toxic effect on soil microbial community structure or the photosynthetic performance of *V. faba*. These results do not imply that cAg NPs are safe, as they may become toxic following long-term exposure or at higher concentrations than those tested, which could occur as a result of continued release of Ag NPs into the environment. Further studies focusing on different soils, plants, and Ag NP concentrations are required. Particularly, the investigation of high concentrations of Ag NPs under short and long exposure times is of importance. Alongside this, it

is important to focus on the impact of Ag NPs on agricultural soils as they closely linked with food production and consequently with human health.

Chapter 5. Foliar-injection of cAg NPs: impact on the physiology of *Vicia faba*

5.1. Introduction

ENPs can enter the air whether directly or indirectly through different scenarios. The manufacture, use, and incineration of nanotechnological products all represent potential sources of aerial pollution of ENPs (Keller *et al.*, 2013; Larue *et al.*, 2014). Such air-suspended ENPs will eventually return to the ground either by dry or wet deposition (Kurwadkar *et al.*, 2014) and some particles will inevitably reach plant surfaces where they may enter leaves through stomatal or cuticular pathways (Eichert *et al.*, 2008; Hong *et al.*, 2014). Entry through leaves represents the third route by which ENPs may enter plants. Following entry into the leaf, ENPs may be translocated into different parts of the plant, including the roots and fruits, via the vascular system (Wang *et al.*, 2012b; Wang *et al.*, 2013a; Hong *et al.*, 2014).

Although foliar uptake is well known to be a route of the entry of pollutants into plants (Uzu *et al.*, 2010), limited studies to date have considered foliar uptake of ENPs. Moreover, the effects of foliar uptake of ENPs on plants are currently unknown (Laure *et al.*, 2014). Previous studies have investigated the toxicity of ENPs on photosynthetic organisms such as algae (Matorin *et al.*, 2010; Saison *et al.*, 2010; Chen *et al.*, 2012), however, few studies on crop plants are been reported. Furthermore, the mechanisms of ENPs toxicity to plants through foliar uptake are unclear. Thus, the aim of the work in this chapter was to investigate the impact and mechanisms of toxicity of cAg NPs on the photosynthetic performance of *V. faba* following foliar application.

Photosynthesis is an essential process by which plants transfer sunlight energy into chemical energy. The process occurs primarily in chloroplasts, which provide plants with the

energy required for growth. Any disruptions in the metabolic processes associated directly or indirectly to photosynthesis could cause inhibition in plant photosynthesis (Barbagallo *et al.*, 2003). It has been reported previously that ENPs can interact with the interior and exterior of chloroplasts and bind to PSII (Lei *et al.*, 2007a; Giraldo *et al.*, 2014; Ma *et al.*, 2015). PSII has previously been reported to be extremely sensitive to abiotic factors and therefore any disruption in the function and structure of PSII could lead to oxidative stress (Foyer and Shigeoka, 2011). For example, PSII has been shown to be sensitive to metal ions (which may be released from ENPs) (Giardi *et al.*, 2001). Once PSII has been inhibited, several negative circumstances could be occurred, affecting plant growth and productivity and O_2 production (Santos *et al.*, 2013).

The formation of ROS in plants is a common process which increases rapidly under environmental stresses induced by both biotic and abiotic factors (Apel and Hirt, 2004). A rapid increase in ROS can cause oxidative stress which may lead to multiple disruptions to cell walls, cell membranes, proteins, and organelles, ultimately resulting in cell death and plant growth inhibition (Bhattacharjee, 2005; Chen *et al.*, 2012; Wang *et al.*, 2015). The production of ROS following exposure to ENPs has been well documented, and is thought to be a major contributor to ENPs toxicity in plants. For example, H_2O_2 has been shown to increase by several orders of magnitude in the presence of Ag NPs (Jiang *et al.*, 2014) and has been reported to be a strong inhibitor of photosynthetic performance (Foyer and Shigeoka, 2011). Inhibition in the photosynthetic performance was previously, for example, related to alterations in electron transport processes caused by ROS in the presence of ENPs (Oukarroum *et al.*, 2015).

5.2. Toxicity of cAg NPs on F_v/F_m

After injecting leaves with cAg NPs at a range of concentrations (12, 50, and 100 mg/L), F_v/F_m was monitored to assess the impact on plant photosynthetic processes. As shown in Fig. 5.1, at the lowest concentration (12 mg/L), cAg NPs did not cause any significant changes to F_v/F_m over 96 h. In contrast, at higher concentrations of 50 and 100 mg/L, cAg NPs caused a gradual and significant decrease in F_v/F_m over 216 h (Fig. 5.2). Specifically, after 24 h, F_v/F_m showed a significant decline from 0.81 to 0.74 with injection 50 mg/L cAg NPs ($P = 0.012$) and from 0.82 to 0.76 with injection 100 mg/L cAg NPs ($P < 0.001$). A further significant decrease was also observed after 48 h with cAg NPs at both 50 ($P = 0.003$) and 100 ($P = 0.028$) mg/L, decreasing F_v/F_m to 0.73 and 0.72, respectively. Interestingly 72 h after application, plants entered a recovery phase and F_v/F_m increased in plant exposed to both 50 and 100 mg/L cAg NPs to 0.75 and 0.74 respectively. Despite this, F_v/F_m remained significantly lower compared to control ($P = 0.002$ and $P = 0.017$). After 144 h, F_v/F_m had increased to 0.77 and 0.76 in plants exposed to 50 and 100 mg/L cAg NPs, but still remained significantly lower than the control until 216 h especially at 100 mg/L ($P < 0.001$).

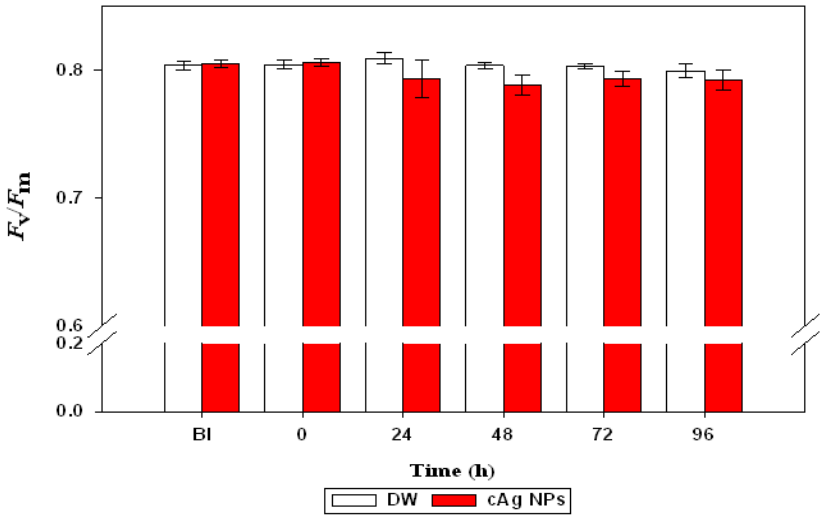

Fig. 5.1. Effect of cAg NPs at 12 mg/L on the chlorophyll fluorescence parameter, F_v/F_m. Leaves were dark adapted for 20 min prior to measurements. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performe using student's *t*-test ($P \geq 0.05$).

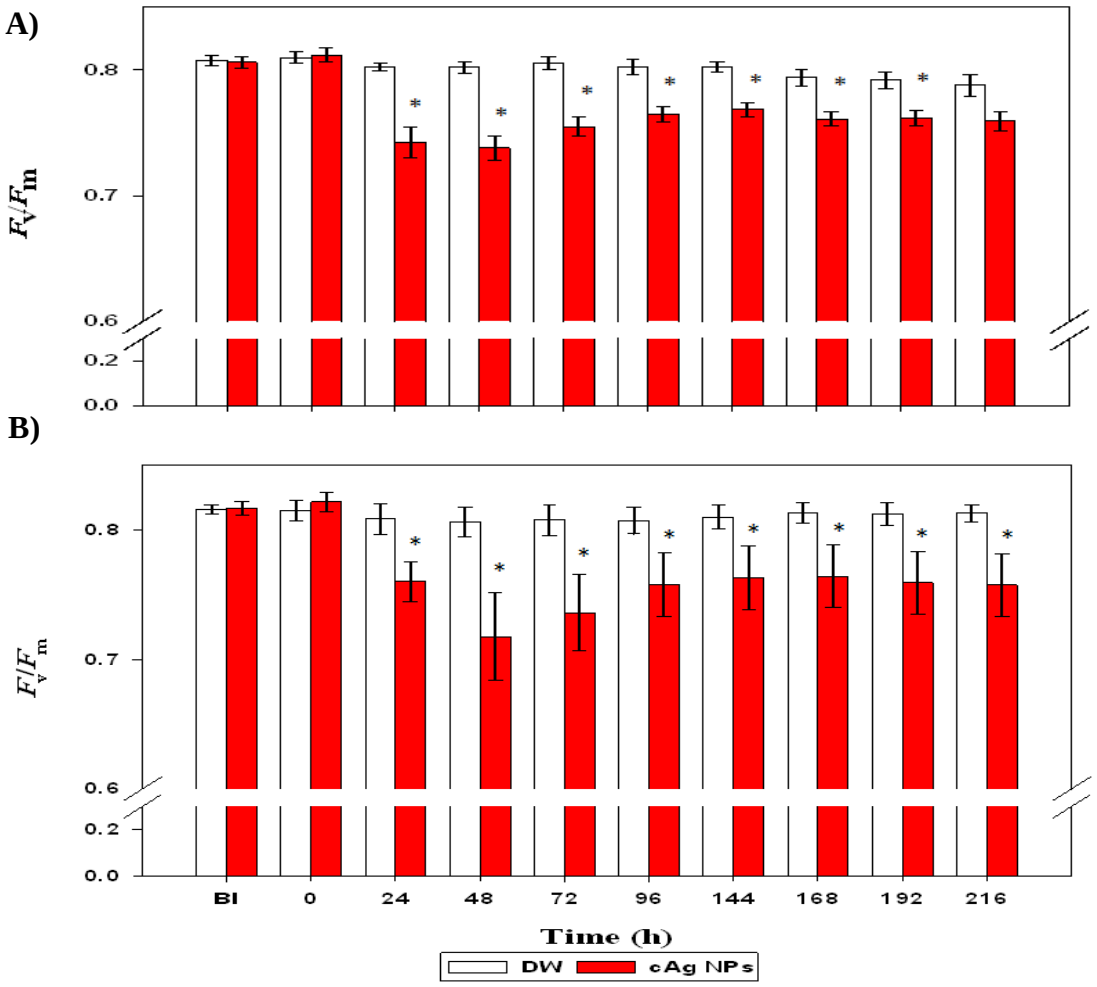

Fig. 5.2. Changes in the chlorophyll fluorescence parameter F_v/F_m induced by cAg NPs at 50 (A) and 100 (B) mg/L. Leaves were dark adapted for 20 min prior to measurements. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$) and significant differences are marked with '*'.

Exposure of *V. faba* to $AgNO_3$ at concentrations of 12, 50, and 100 mg/L was also investigated, to differentiate the toxicity of cAg NPs, and results showed significant changes to F_v/F_m, compared to controls. Specifically, whilst no effect of $AgNO_3$ was observed at 12 mg/L (Fig. 5.3), 50 and 100 mg/L $AgNO_3$ caused an immediate and significant decrease in F_v/F_m (Fig. 5.4). Specifically, between 0 and 24 h F_v/F_m significantly decreased from 0.80 to <0.72 and from

0.80 to 0.67 with 50 and 100 mg/L $AgNO_3$, respectively (P = 0.006-0.011). Although F_v/F_m started to increase after 48 h with 50 mg/L $AgNO_3$ (to 0.73), it took until 72 h to increase with 100 mg/L $AgNO_3$ (to 0.73). From 72 h throughout the experiment, F_v/F_m remained lower than the control. Importantly, in comparison to the cAg NPs, the inhibitory impact of $AgNO_3$ on F_v/F_m was immediate.

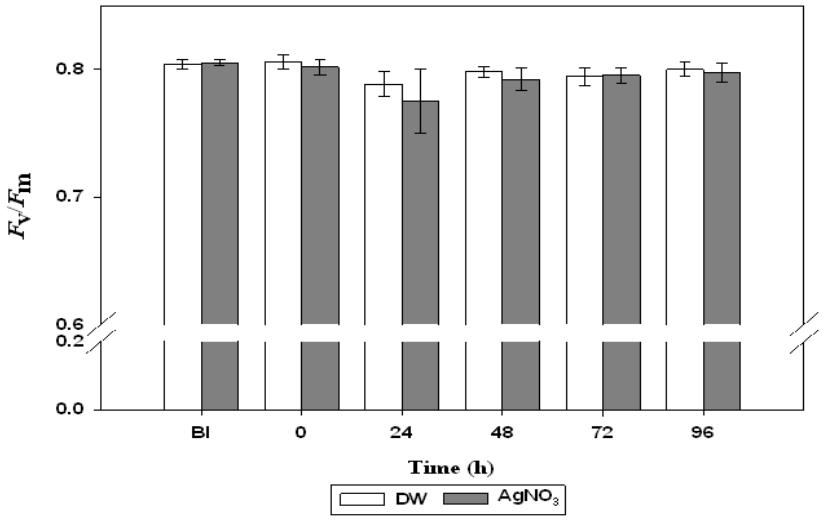

Fig. 5.3. Response of the chlorophyll fluorescence parameter F_v/F_m to $AgNO_3$ exposure at 12 mg/L. Dark adaptation for 20 min was performed prior to measurements. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's t-test ($P \geq 0.05$).

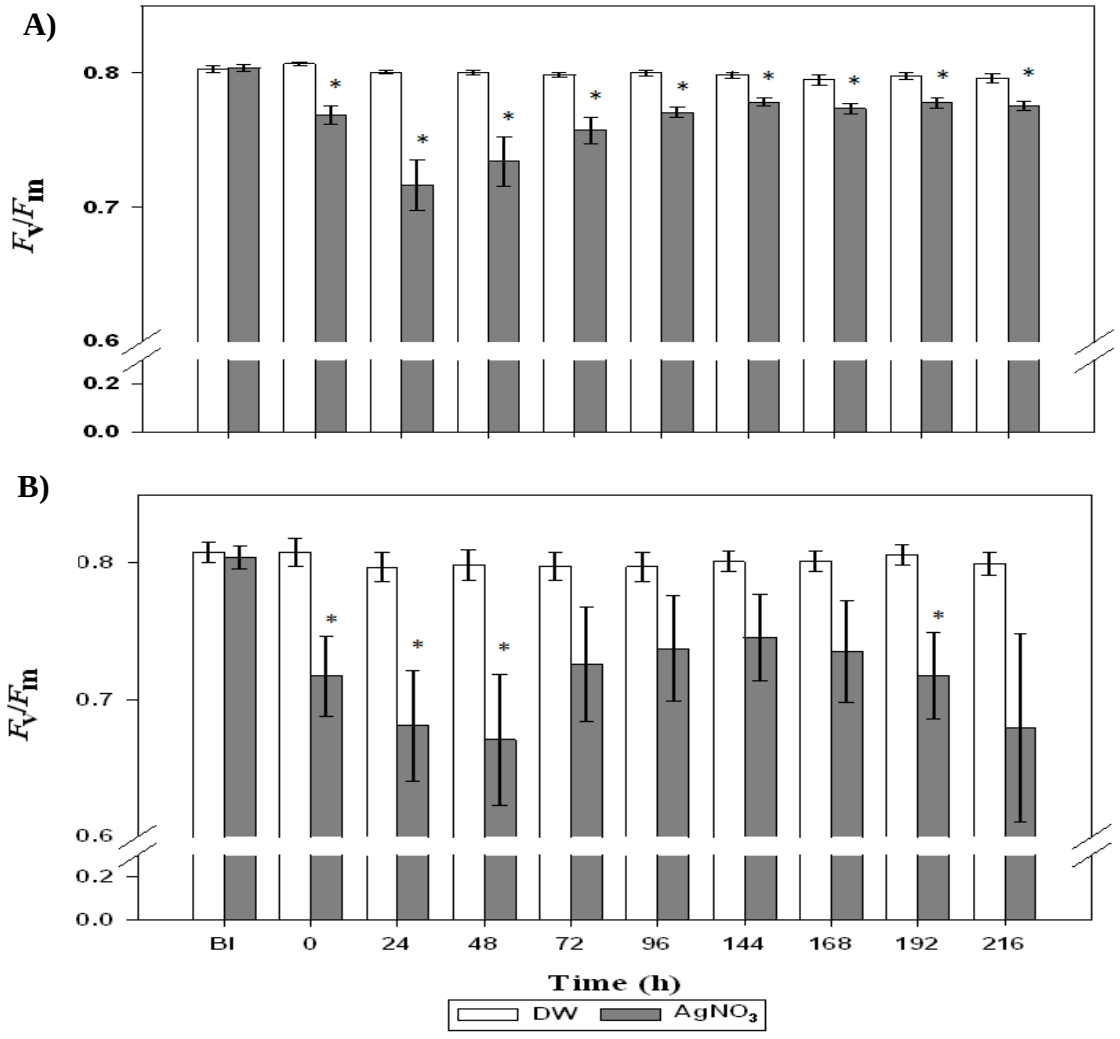

Fig. 5.4. Changes in the chlorophyll fluorescence parameter F_v/F_m induced by $AgNO_3$ exposure at 50 (A) and 100 (B) mg/L. Dark adaptation for 20 min was performed prior to measurements. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$) and significant differences are marked with '*'.

5.3. Impact of cAg NPs on F_q'/F_m'

The response of F_q'/F_m' following application of cAg NPs at 12, 50, and 100 mg/L was investigated. Fig. 5.5 shows that at lower concentration (12 mg/L), neither cAg NPs nor $AgNO_3$ had any significant impact on F_q'/F_m' (at 500 µmol m^{-2} s^{-1} PPFD) over a 96 h period. However, 24

h following exposure to 50 mg/L cAg NPs a significant decrease in F_q'/F_m' was observed and after 144 h F_q'/F_m' declined from 0.64 to 0.57 (100 µmol m^{-2} s^{-1} PPFD) and from 0.50 to 0.39 (500 µmol m^{-2} s^{-1} PPFD) (P = 0.004) (Fig. 5.6). After 216 h, F_q'/F_m' in leaves exposed to cAg NP at both PPFDs decreased further to 0.54 at 100 µmol m^{-2} s^{-1} PPFD and 0.38 at 500 µmol m^{-2} s^{-1} PPFD and was significantly different to controls (P = 0.015 and P = 0.026, respectively).

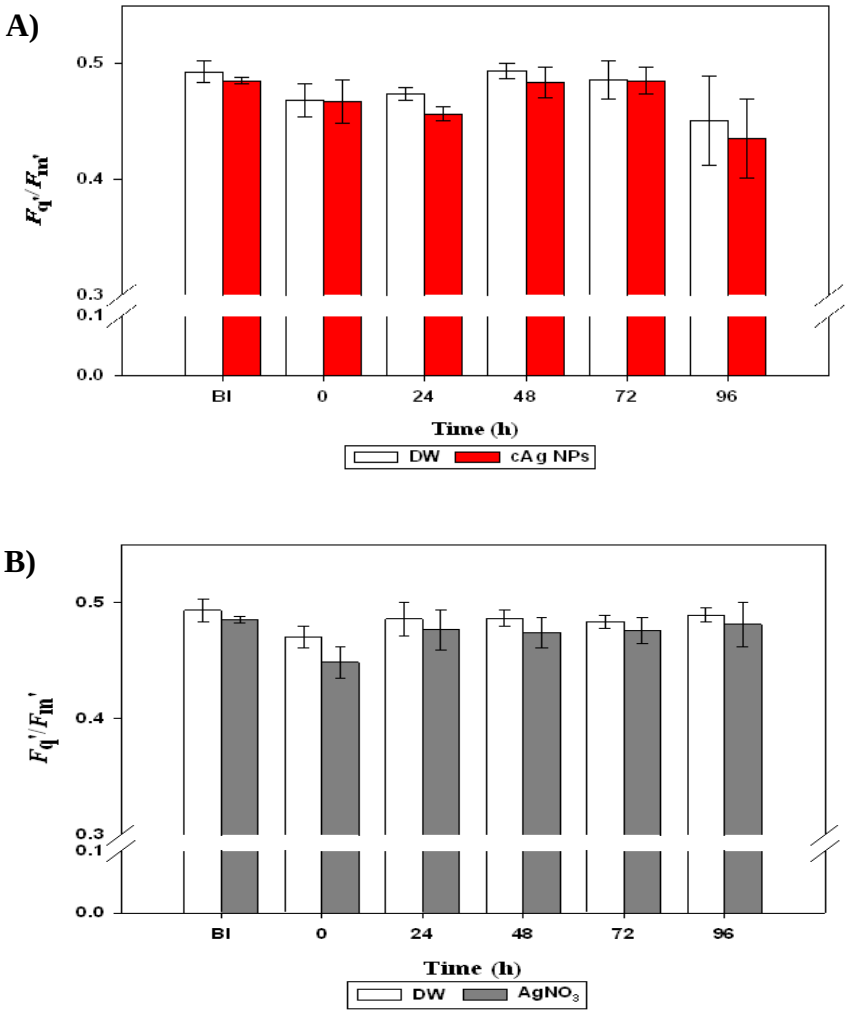

Fig. 5.5. Effect of cAg NPs (A) and $AgNO_3$ (B) at 12 mg/L on the chlorophyll fluorescence parameter F_q'/F_m' at 500 µmol m^{-2} s^{-1} PPFD. BI = before injection. The values are given as mean ± SE (n=5). Comparisons between treatments were performed using student's t-test ($P \geq$ 0.05).

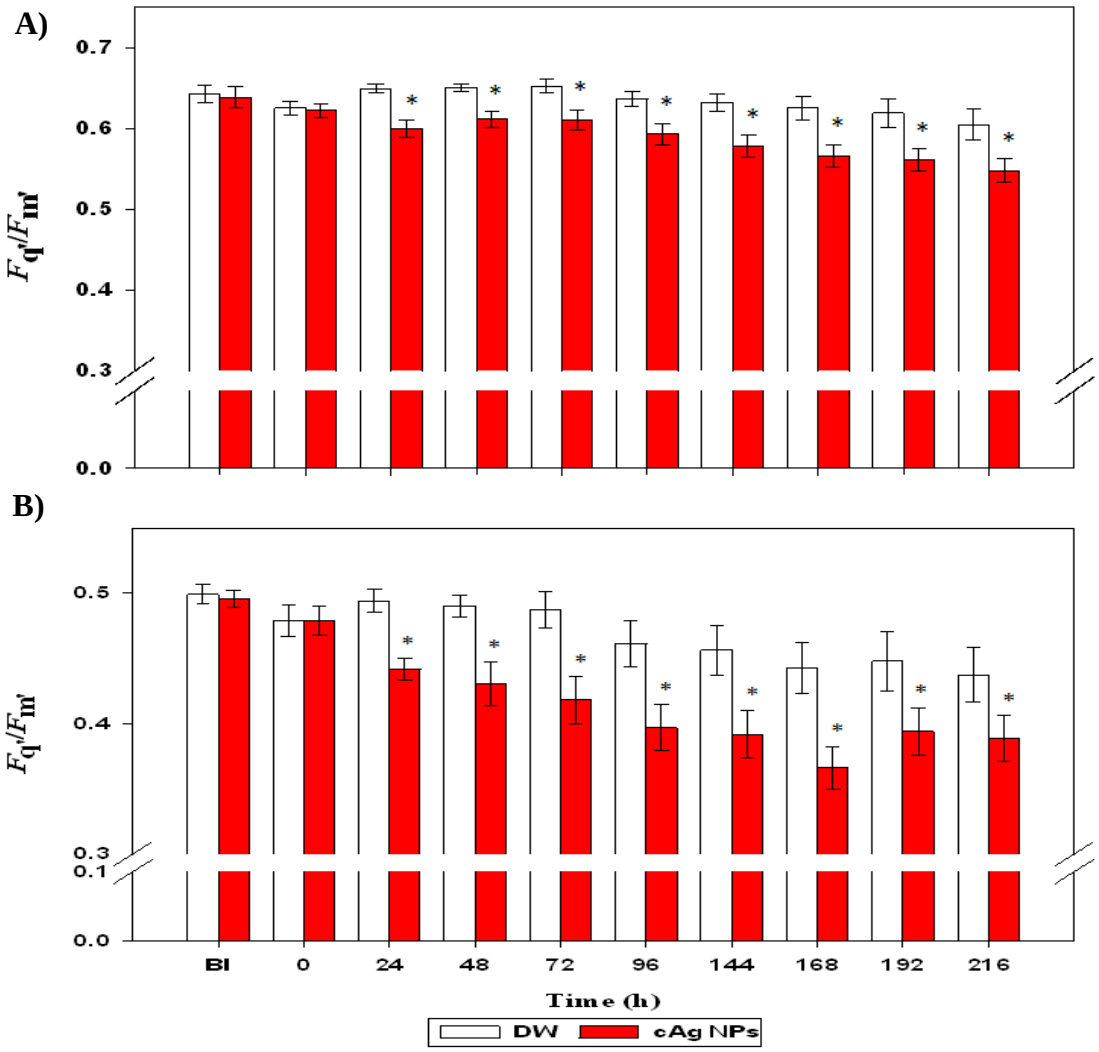

Fig. 5.6. Changes in the chlorophyll fluorescence parameter F_q'/F_m' induced by cAg NPs at 50 mg/L at a PPFD of 100 (A) and 500 (B) µmol m^{-2} s^{-1}. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$) and significant differences are marked with '*'.

Unlike exposure to cAg NPs, $AgNO_3$ caused an immediate and significant decline in F_q'/F_m' at both PPFDs after 0 h (Fig. 5.7). Specifically, $AgNO_3$ at 50 mg/L caused a significant decrease in F_q'/F_m' from 0.65 to 0.51 (100 µmol m^{-2} s^{-1} PPFD) and from 0.51 to 0.34 (500 µmol m^{-2} s^{-1} PPFD) ($P = 0.006$ and $P = 0.003$, respectively). Following this, the $AgNO_3$ treated leaves at

both PPFDs showed recovery over the next 24 h, although F_q'/F_m' remained significantly decreased in comparison to the controls ($P < 0.001$ and $P = 0.005$, respectively), even after 216 h.

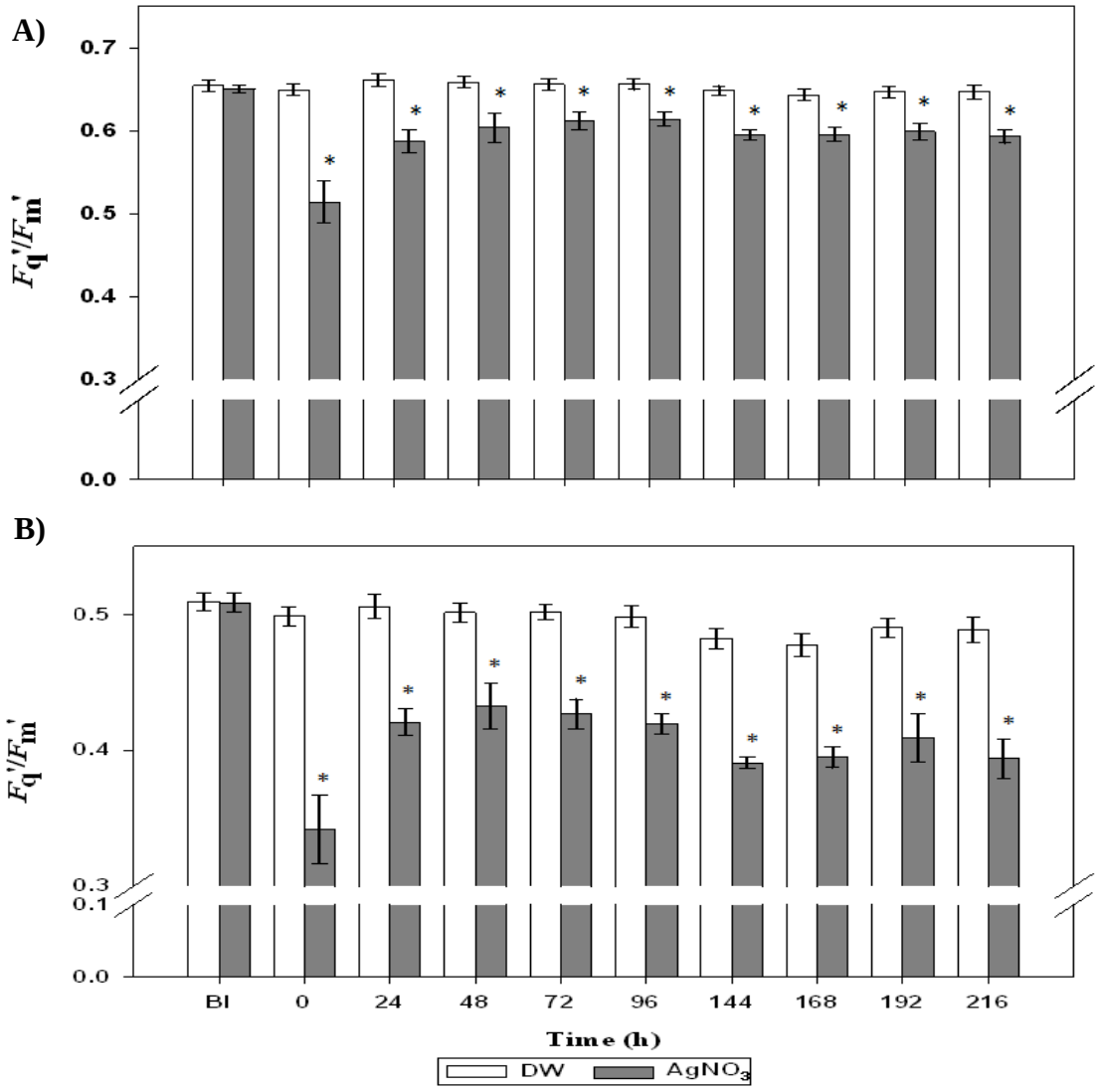

Fig. 5.7. Effect of $AgNO_3$ at 50 mg/L on the chlorophyll fluorescence parameter F_q'/F_m' at a PPFD of 100 (A) and 500 (B) µmol m^{-2} s^{-1}. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$) and significant differences are marked with '*'.

At a high cAg NPs concentration of 100 mg/L, F_q'/F_m' significantly decreased at both PPFDs (Fig. 5.8). At a PPFD of 100 µmol m^{-2} s^{-1}, no significant effects were observed until 48 h after application when F_q'/F_m' dropped significantly from 0.77 to 0.71 at 216 h (P = 0.045). In addition, cAg NPs significantly decreased F_q'/F_m' (at a PPFD of 500 µmol m^{-2} s^{-1}) at 48 h from 0.57 to 0.53 (P = 0.002). However, F_q'/F_m' remained significantly lower in comparison to controls throughout the experiment, and by 216 h F_q'/F_m' was only 0.46 (P < 0.001).

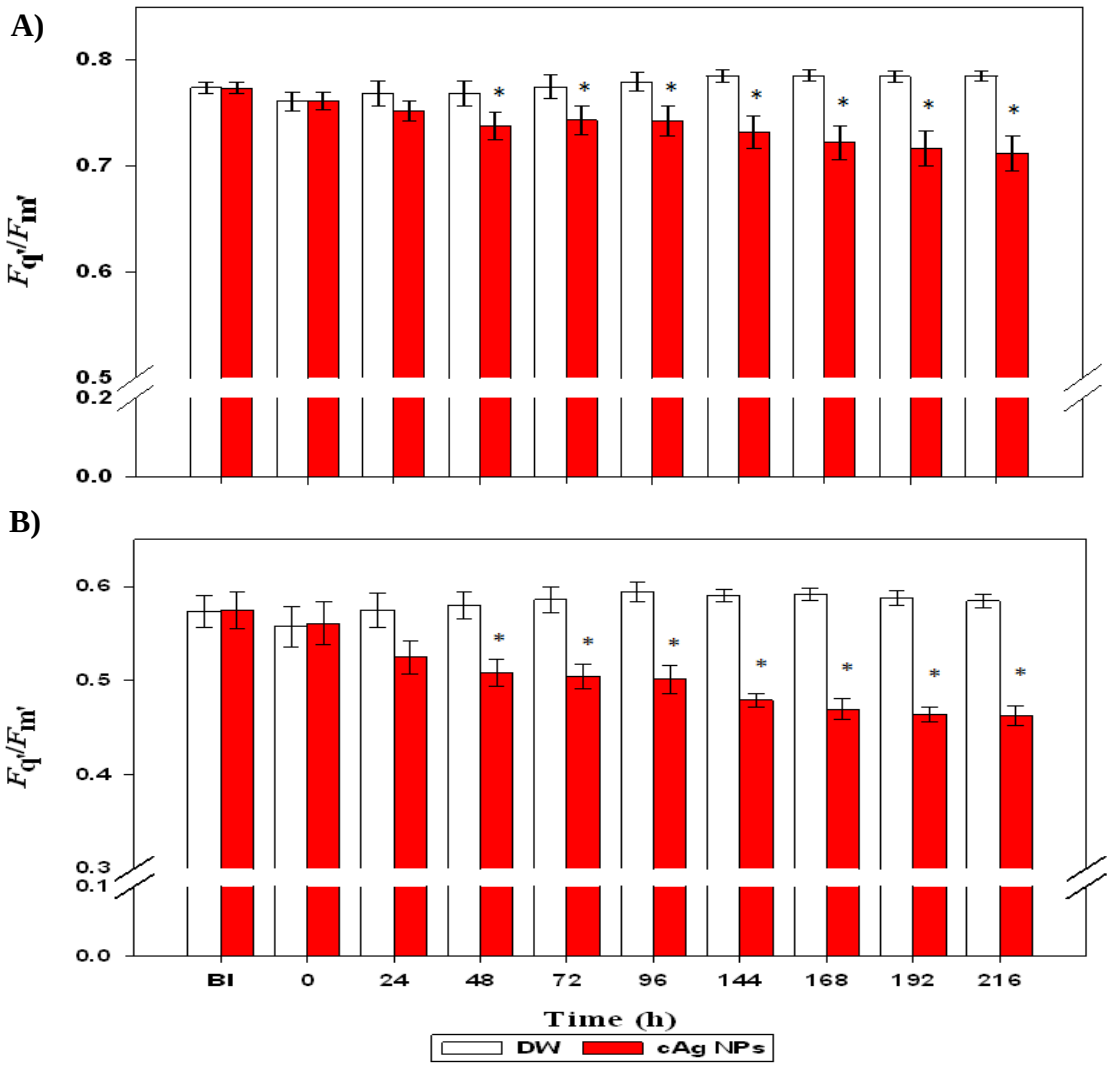

Fig. 5.8. Response of the chlorophyll fluorescence parameter F_q'/F_m' to cAg NP exposure at 100 mg/L at 100 (A) and 500 (B) µmol m^{-2} s^{-1} PPFD. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$) and significant differences are marked with '*'.

In contrast to cAg NPs, $AgNO_3$ had an immediate and significant impact on F_q'/F_m', resulting in a decrease from 0.76 to 0.62 (100 µmol m^{-2} s^{-1} PPFD) and from 0.53 to 0.40 (500 µmol m^{-2} s^{-1} PPFD) ($P = 0.001$) (Fig. 5.9). Following this, the treated leaves showed evidence of recovery within 24 h with an increase from 0.62 to 0.70 (100 µmol m^{-2} s^{-1} PPFD) and from 0.40 to

0.48 (500 µmol m^{-2} s^{-1} PPFD). However, F_q'/F_m' in treated leaves remained significantly reduced in comparison to controls up to 192 h after application (P = 0.019).

Interestingly, whilst leaves treated with $AgNO_3$ at 50 and 100 mg/L showed recovery after 24 h at both PPFDs, those treated with cAg NPs at the same concentrations did not show any recovery and continued to decrease following 24 h exposure and throughout the experiment.

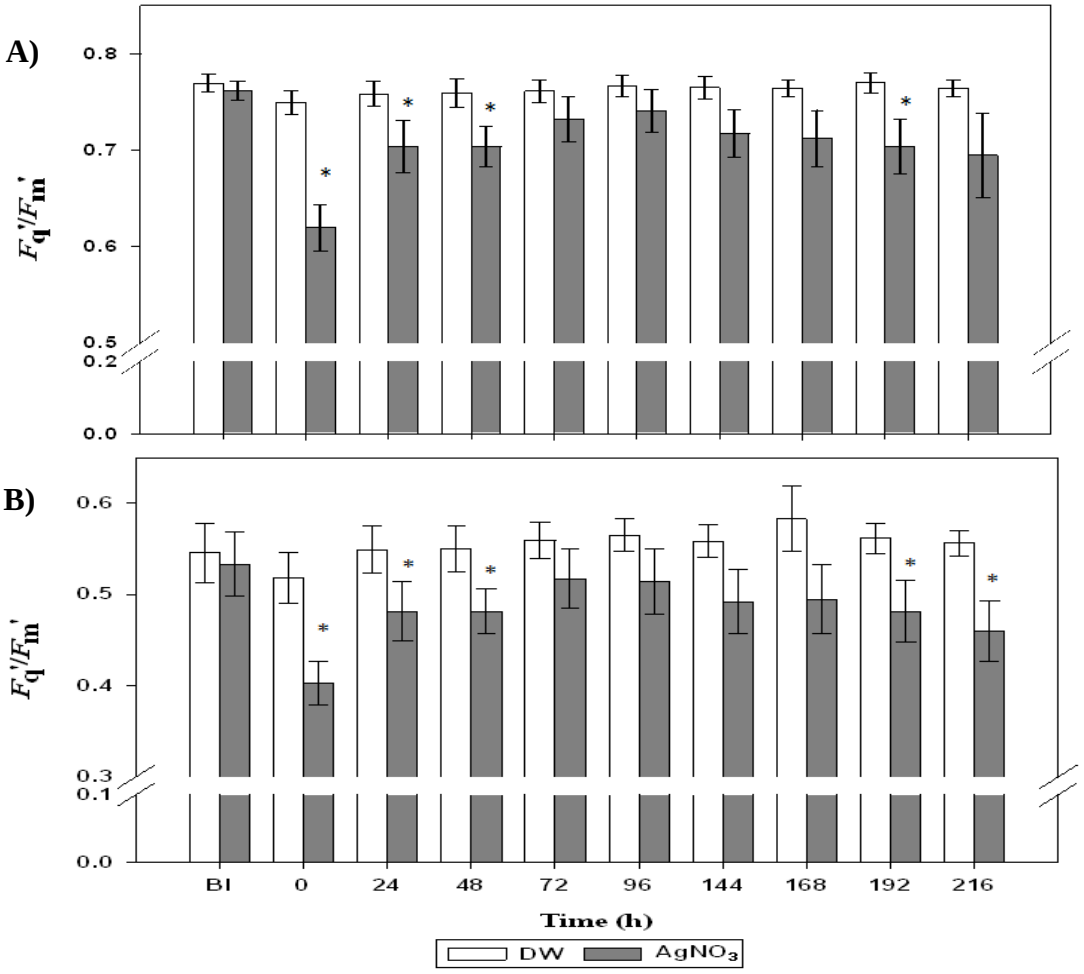

Fig. 5.9. Changes in the chlorophyll fluorescence parameter F_q'/F_m' induced by $AgNO_3$ exposure at 100 mg/L at a PPFD of 100 (A) and 500 (B) µmol m^{-2} s^{-1}. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$) and significant differences are marked with '*'.

5.4. The role of light on the toxicity of cAg NPs

The role of light on the impact of cAg NPs on F_v/F_m was considered. When growth PPFD inside the growth cabinet was increased (before planting) from ~130 µmol m^{-2} s^{-1} to ~480 µmol m^{-2} s^{-1}, F_v/F_m significantly decreased from 0.80 to 0.72 within 24 h in plants exposed to cAg NPs at 100 mg/L (P = 0.025) (Fig. 5.10). Interestingly, no further decrease was observed at 48 h as shown previously at low PPFD as treated leaves began a recovery phase leading to an increase in F_v/F_m above 0.78 by 96 h. However, F_v/F_m remained lower in comparison to controls throughout the remaining time of the experiment.

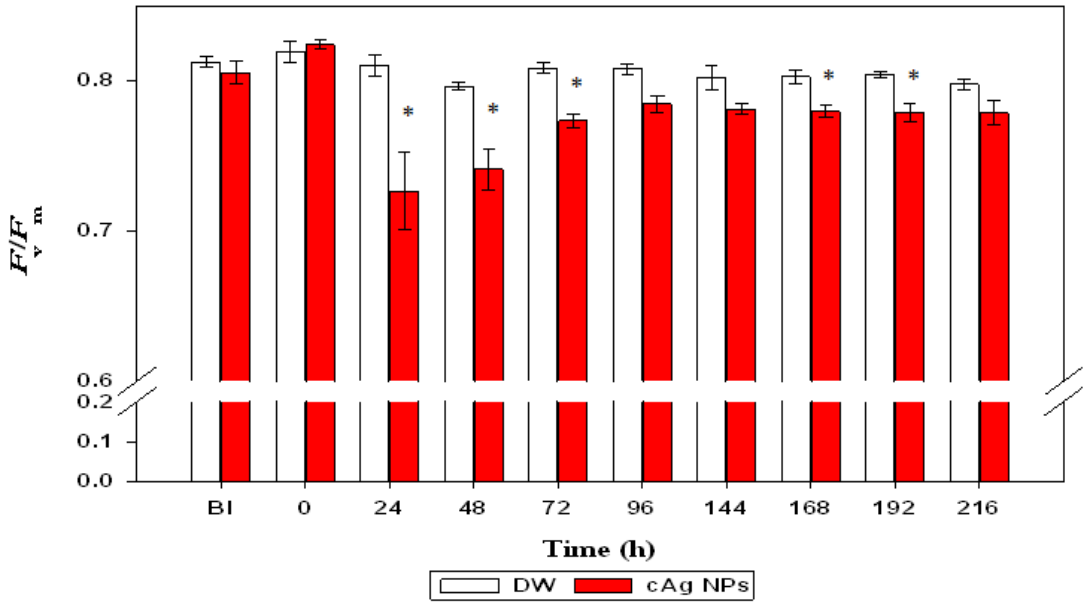

Fig. 5.10. Response of the chlorophyll fluorescence parameter F_v/F_m to cAg NPs at 100 mg/L when PPFD inside the growth cabinet increased to ~480 µmol m^{-2} s^{-1}. Dark adaption for 20 min was performed prior to measurements. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's *t*-test (P < 0.05) and significant differences are marked with '*'.

cAg NPs also affected F_q'/F_m' significantly (Fig. 5.11). Specifically, a significant decrease in F_q'/F_m' began after 48 h as it decreased from 0.78 to 0.75 and from 0.60 to 0.51 at a PPFD of 100 and 500 µmol m^{-2} s^{-1}, respectively (P = 0.030-0.039). However, F_q'/F_m' continued to decrease throughout the experiment, reaching 0.73 (100 µmol m^{-2} s^{-1}) and 0.50 (500 µmol m^{-2} s^{-1}) after 216 h.

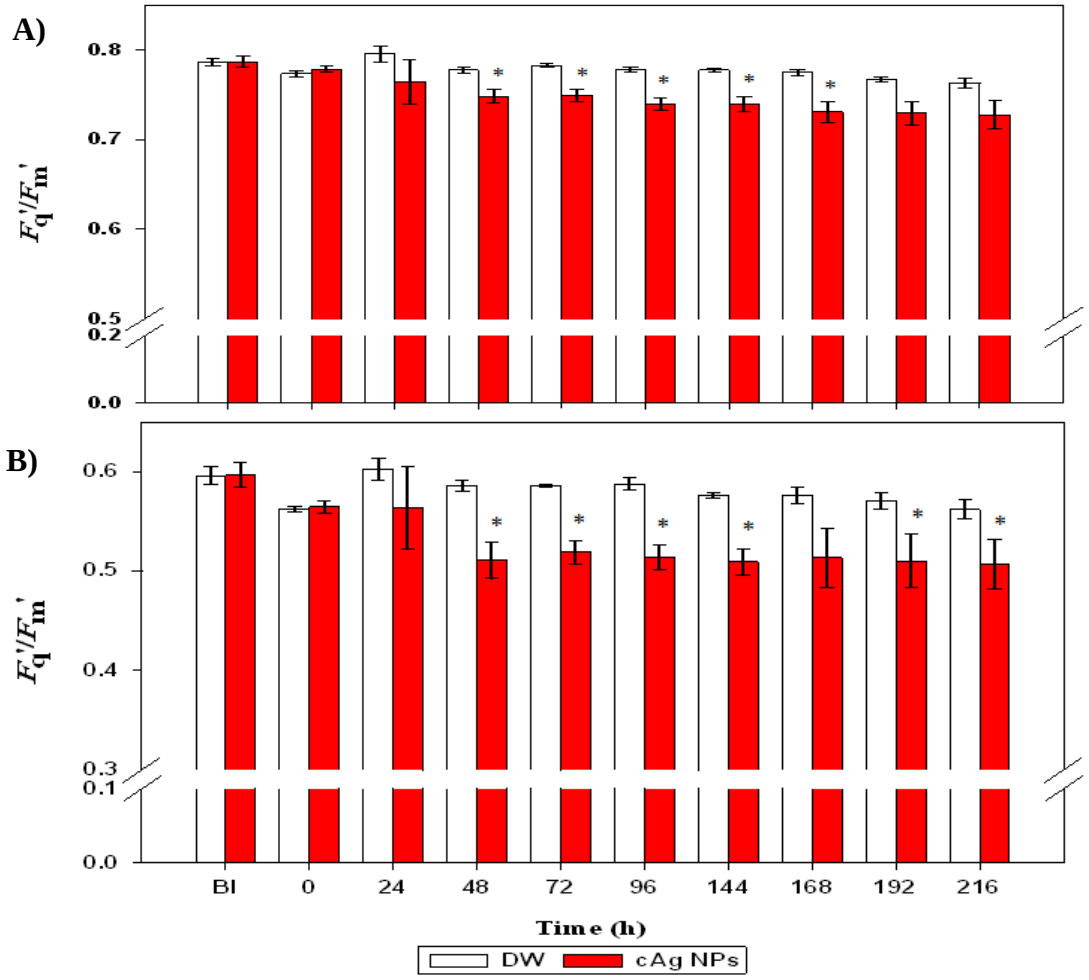

Fig. 5.11. Changes in the chlorophyll fluorescence parameter F_q'/F_m' induced by cAg NPs at 100 mg/L at a PPFD of 100 (A) and 500 (B) µmol m^{-2} s^{-1} when PPFD inside the growth cabinet increased to ~ 480 µmol m^{-2} s^{-1}. BI = before injection. The values given are mean ± SE (n=5). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$) and significant differences are marked with '*'.

5.5. Influence of injecting cAg NPs on the spatial pattern of F_v/F_m and visual appearance of leaves

Following injection of *V. faba* with cAg NPs, plants developed the appearance of brown coloured spots after 24 h and spread over the whole leaf surface. As shown in Figs. 5.12 and 5.13, these spots lead to localized decreases in F_v/F_m, and was more evident in leaves treated with 100 mg/L cAg NPs compared to those treated with 50 mg/L. Additionally, the brown spots were clearly visualized on both the abaxial and adaxial sides of the leaves (Figs. 5.14 and 5.15). Despite the recovery phase experienced by plants in F_v/F_m, the brown spots remained present on the leaves throughout the experiment. Interestingly, such spots were not observed when leaves were injected with $AgNO_3$. Alternatively, black areas just around the point of injection appeared immediately following $AgNO_3$ injection, resulting in quick and deep damage.

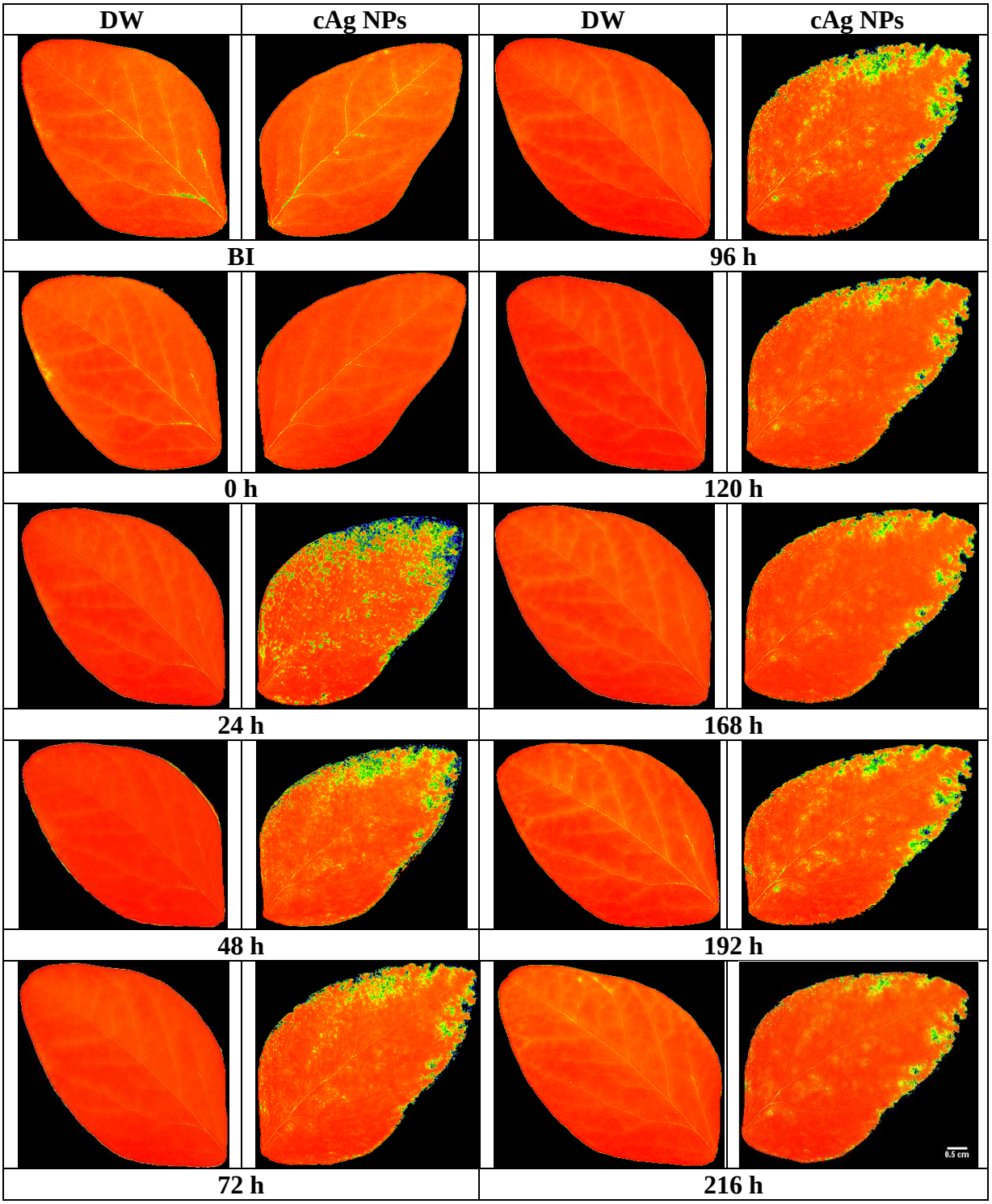

Fig. 5.12. Visual presentation of the impact of the control and cAg NPs at 50 mg/L on *V. faba* leaves represented by changes in false colour images corresponding to F_v/F_m range values as visualized by the imager.

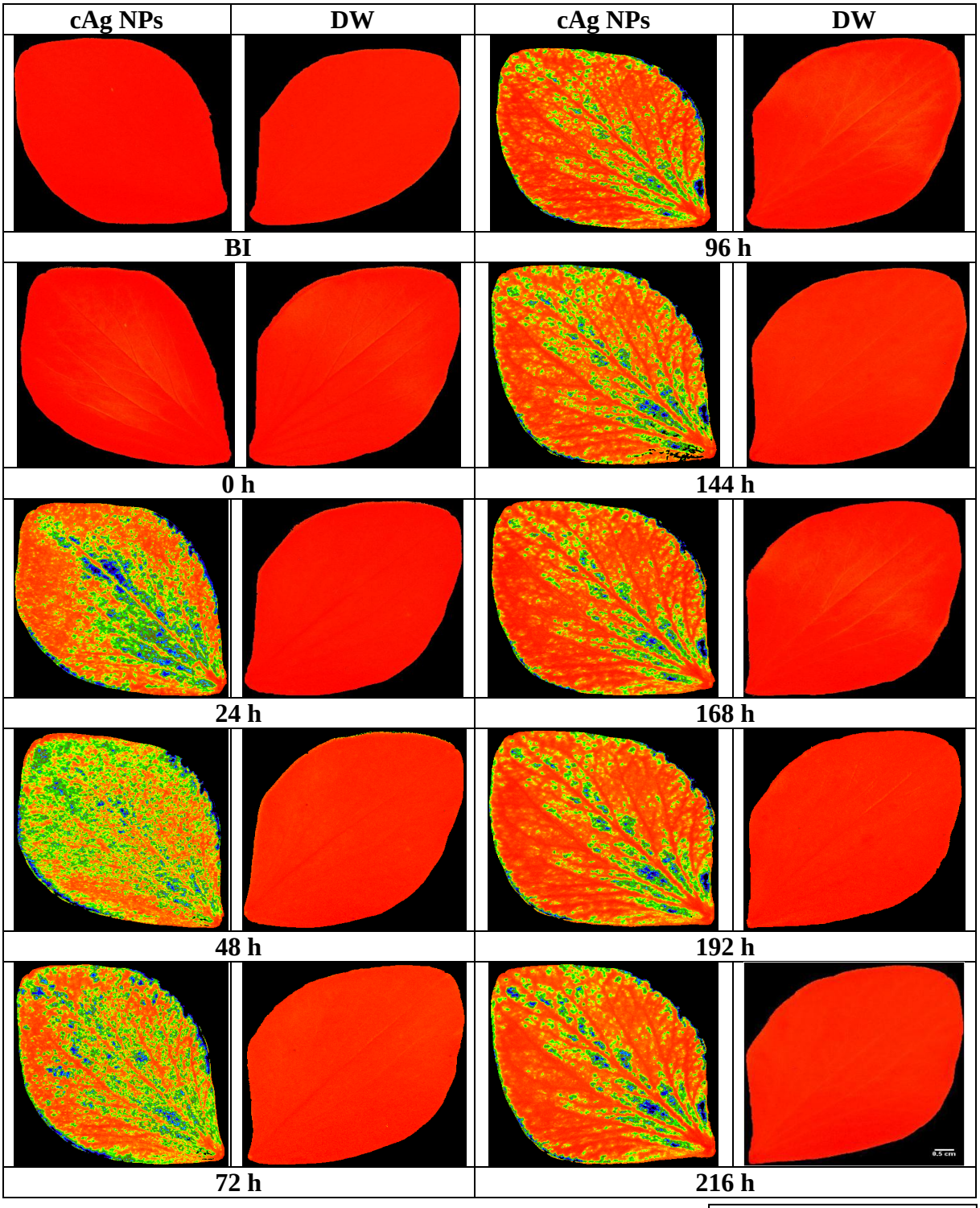

Fig. 5.13. Visual presentation of the impact of the control and cAg NPs at 50 mg/L on *V. faba* leaves represented by changes in false colour images corresponding to F_v/F_m range values as visualized by the imager.

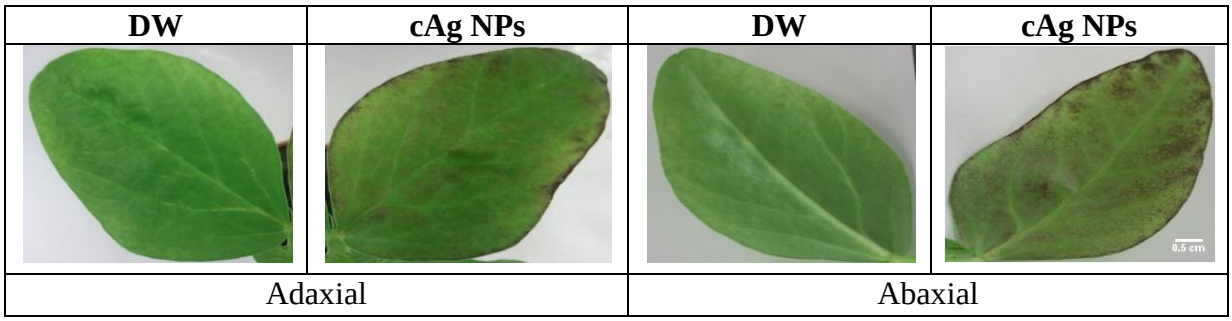

Fig. 5.14. Visual changes in leaf appearance induced by cAg NPs at 50 mg/L as shown by using digital camera (FinePix AV 2000, Fujifilm) after 216 h.

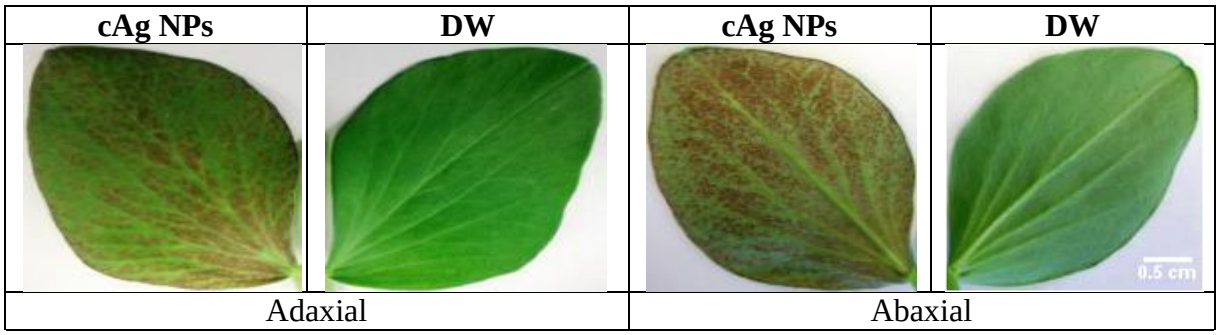

Fig. 5.15. Visual changes in leaf appearance induced by cAg NPs at 100 mg/L as shown by using digital camera (FinePix AV 2000, Fujifilm) after 216 h.

5.6. Assessment of cAg NPs effect on *A*

The impact of cAg NPs at 100 mg/L on *A* is presented in Fig. 5.16. During 48 h experiment, cAg NPs caused a decrease in *A* by more than 50% compared to the controls. To measure the total amount of CO_2 fixed by the treated and control leaves, the integrated areas under the curve (from 20 to 30 h) were calculated. The results showed that leaves treated with cAg NPs at 100 mg/L fixed significantly less CO_2 (247 mmol m^{-2}) compared to control leaves (587 mmol m^{-2}) ($P < 0.001$).

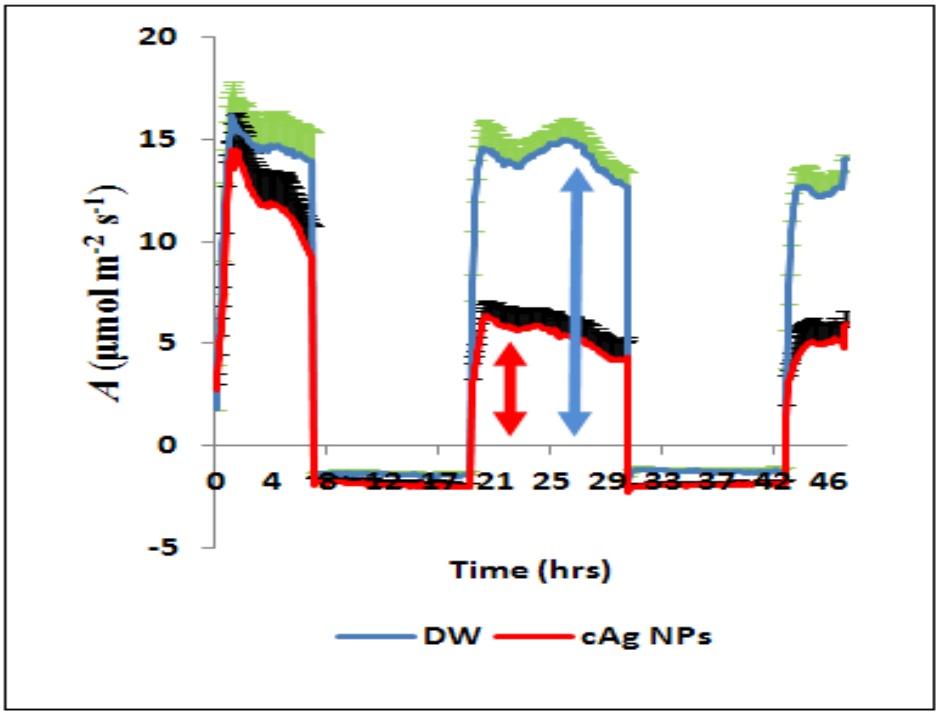

Fig. 5.16. Response of *A* to cAg NPs at 100 mg/L. CIRAS was applied in this experiment. Blue and red arrows indicate the areas measured to calculate the total amount of CO_2 fixed by treated and untreated leaves. The values given are mean ± SE (n=4). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$).

5.7. Effect of cAg NPs on g_s

For 48 h after injection of cAg NPs at 100 mg/L, changes in g_s of exposed leaves were recorded. Stomatal conductance decreased following injection of cAg NPs compared with untreated leaves (Fig. 5.17). Specifically, between 20 and 30 h after injection, g_s in control leaves was approximately double than that of the treated leaves.

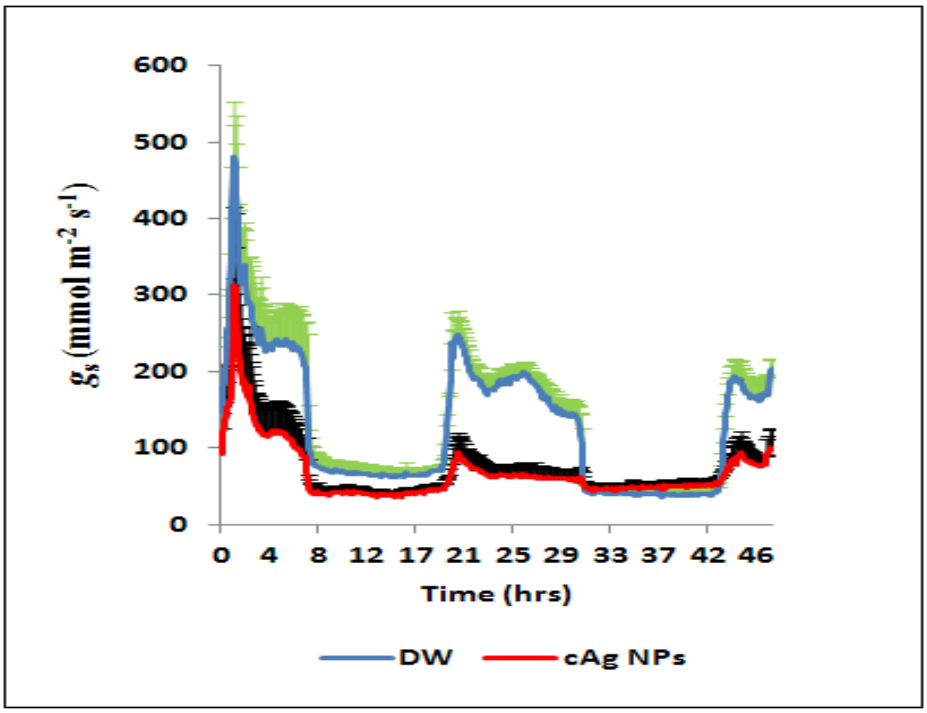

Fig. 5.17. Response of g_s to cAg NPs at 100 mg/L. Following the injection of *V. faba* leaves readings of g_s were recorded over 48 h. CIRAS was applied in this experiment. The values given are mean ± SE (n=4).

5.8. Reactive oxygen species detection

In order to determine whether the generation of ROS (H_2O_2) was involved in the toxicity of cAg NPs, AR was used to stain the treated leaves in order to localize the generated ROS. Images of stained leaves injected with cAg NPs at 12 and 100 mg/L are shown in Figs. 5.18 and 5.19. 24 h after injection, both control and cAg NP-exposed leaves (at 12 mg/L) produced H_2O_2, which was found mainly in the leaf veins. H_2O_2 was continuously detected in both cAg NP-exposed and control leaves up to 96 h. Surprisingly, when the concentration of cAg NPs was increased to 100 mg/L, the AR fluorescence signal of treated leaves was absent or negligible. In contrast, the AR fluorescence signal in control leaves remained present over 216 h.

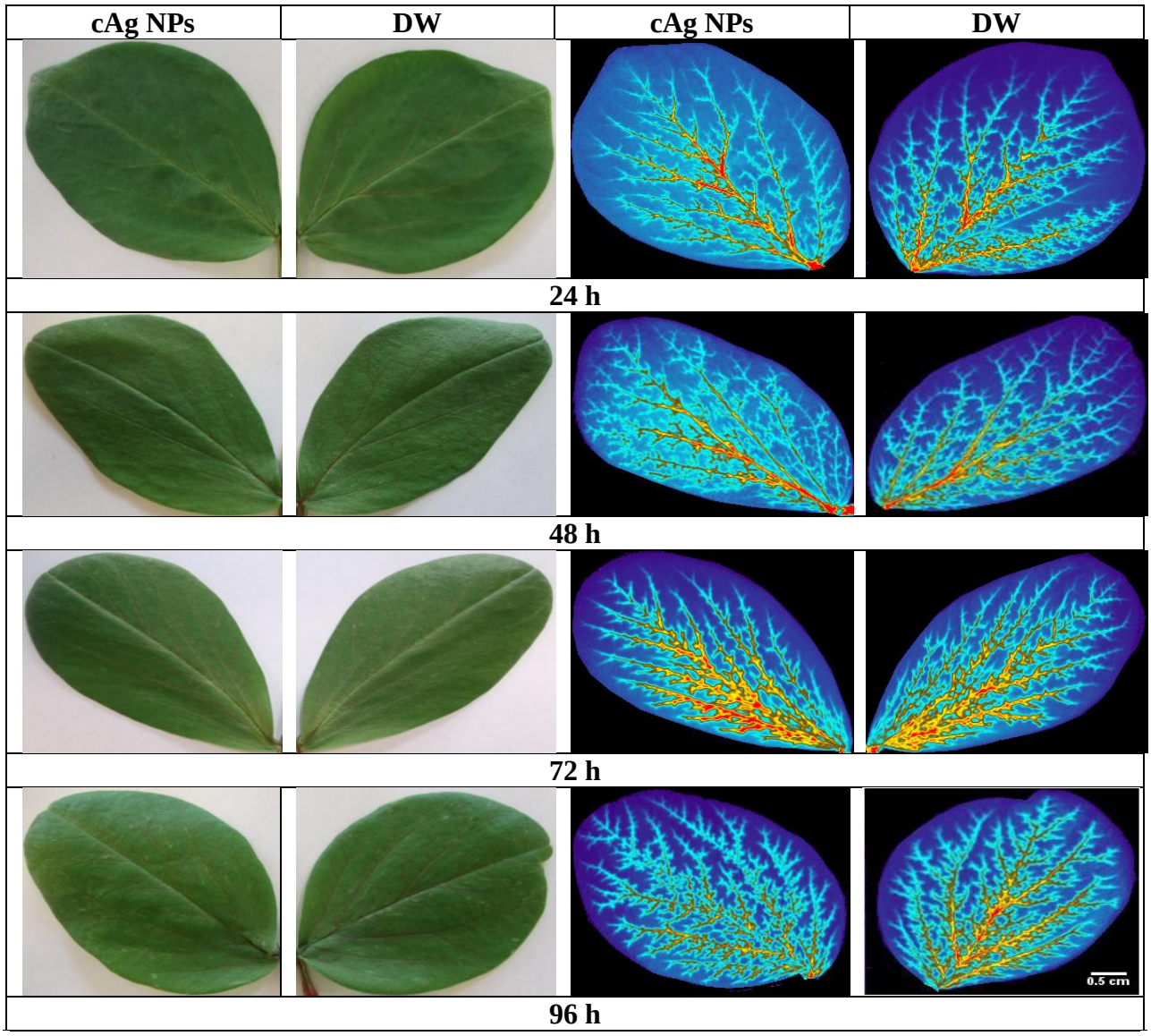

Fig. 5.18. Impact of cAg NPs at 12 mg/L on the generation of H_2O_2. The uptake of AR and its reaction with H_2O_2 is evident in both control and leaves injected with low concentration of cAg NPs at 12 mg/L. Images on the left of the middle line were taken by a digital camera (FinePix AV 2000, Fujifilm) and images on the right of the middle line were taken by the CCD camera.

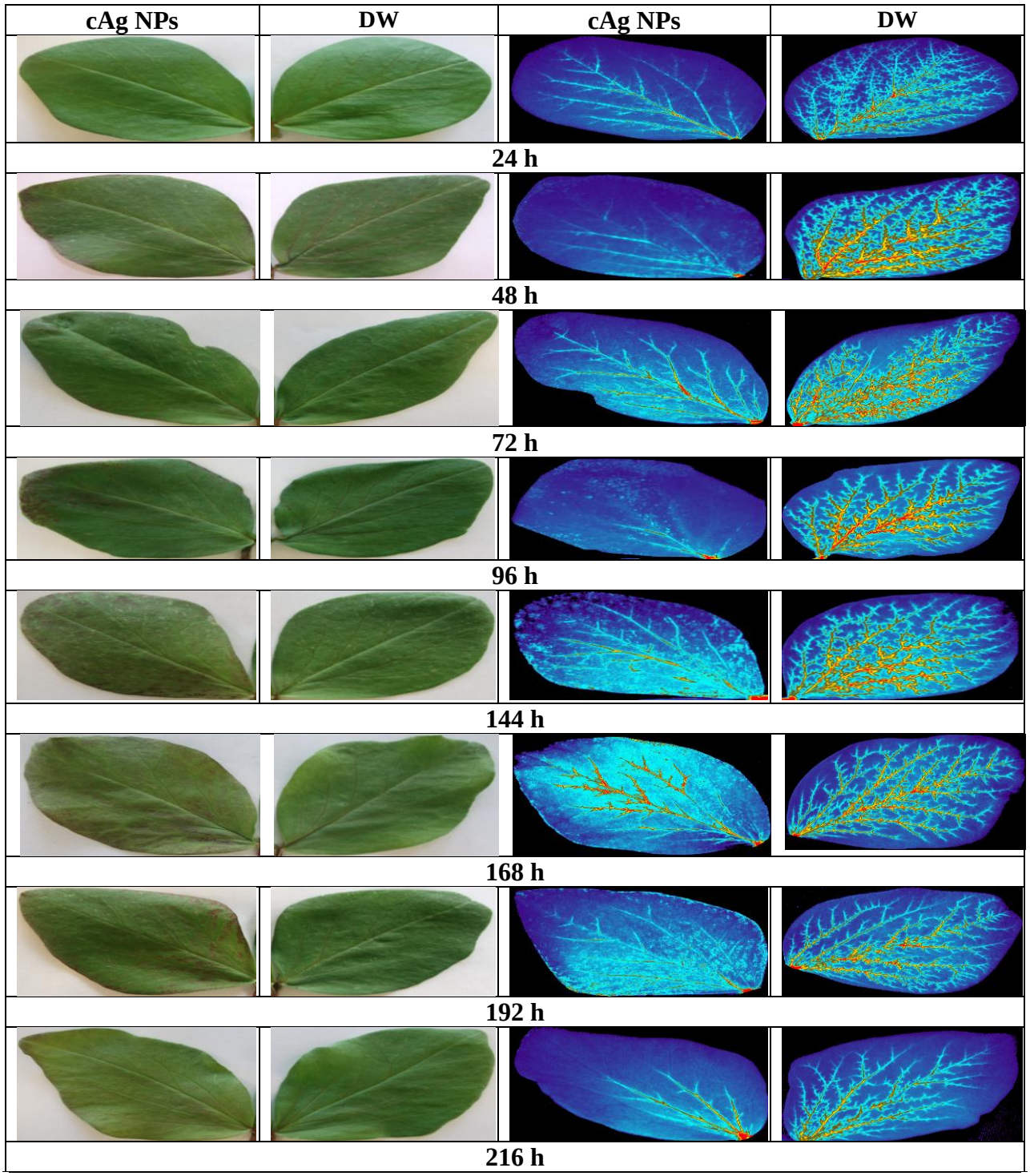

Fig. 5.19. Effect of cAg NPs at 100 mg/L on the generation of H_2O_2. The uptake of AR and its reaction with H_2O_2 is evident in control leaves, however, negligible or no uptake and interaction were occurred in leaves injected with high concentration of cAg NPs at 100 mg/L. Images on the left of the middle line were taken by a digital camera (FinePix AV 2000, Fujifilm) and images on the right of the middle line were taken by the CCD camera.

5.9. Ag content in leaf tissues

The concentration of Ag in the leaves injected with cAg NPs and $AgNO_3$ at 50, and 100 mg/L was measured using ICP-MS (Fig. 5.20). As can be seen after 216 h the concentration of Ag in leaves injected with 50 and 100 mg/L $AgNO_3$ (173 and 914 µg/g, respectively) was greater than those injected with cAg NPs (73 and 76 µg/g, respectively).

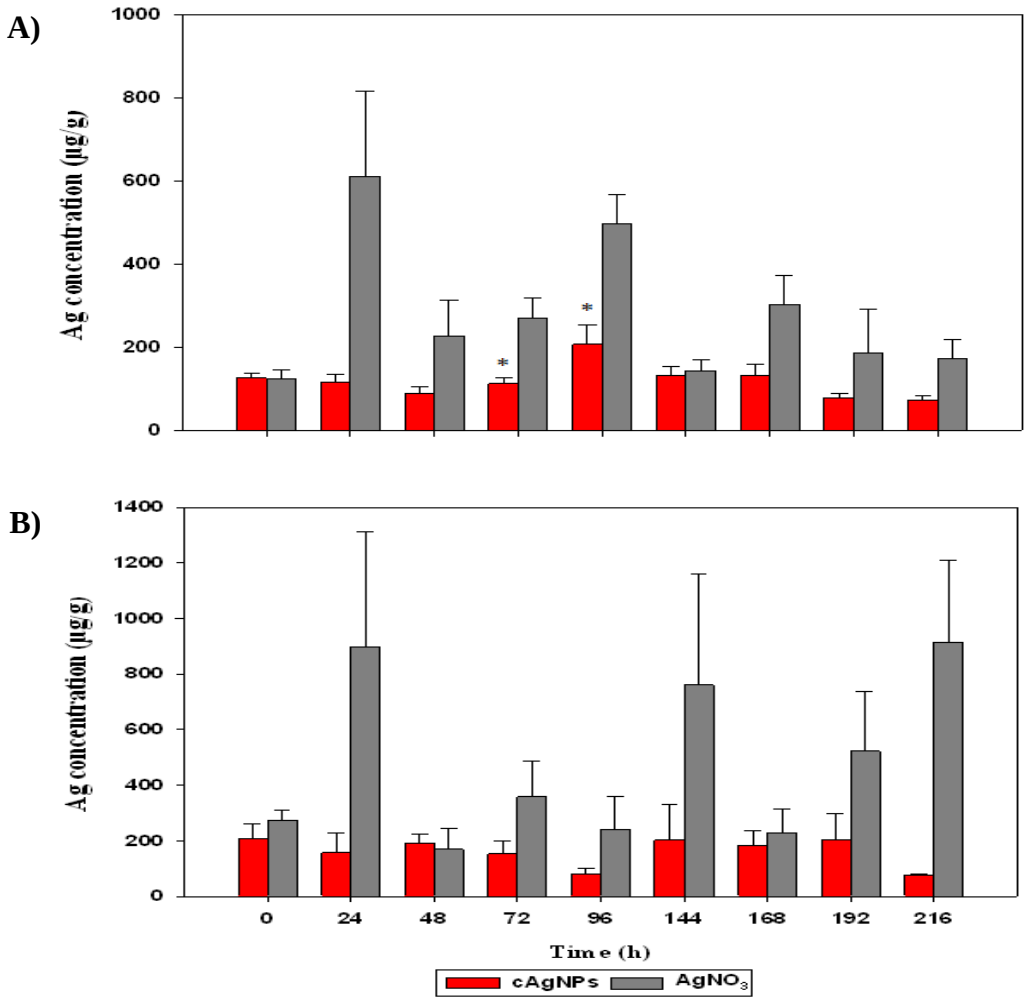

Fig. 5.20. Ag concentration over 216 h in leaves injected with cAg NPs and $AgNO_3$ at 50 (A) and 100 (B) mg/L. The values given are mean ± SE (n=3). Comparisons between treatments were performed using student's *t*-test ($P < 0.05$) and significant differences are marked with '*'.

5.10. Discussion

Photosynthesis is an essential process providing plants with the basic products required for growth and daily activity. Abiotic factors specifically heavy metals have been reported to be extremely toxic to photosynthesis, leading to several disruptions (Han *et al.*, 2008; Parmar *et al.*, 2013). Despite this, little is currently known about the potential effects of Ag NPs on photosynthesis. In the present study, photosynthetic performance was considered to help understand the potential impact of cAg NPs on *V. faba*. Specifically, F_v/F_m, F_q'/F_m', A, and g_s were investigated. Additionally, the detection of H_2O_2 was targeted as ROS generation is thought to contribute towards the toxicity of Ag NPs in plants. Also, the content of Ag inside life tissues was assessed.

The results of the present study demonstrated that cAg NPs caused a significant decrease in F_v/F_m and F_q'/F_m' which was also linked with a decrease in A, and g_s. Specifically, at low concentration applied in this study (12 mg/L), cAg NPs had no significant impact on F_v/F_m compared to controls. However, when the concentration of cAg NPs increased to 50 and 100 mg/L, F_v/F_m significantly decreased after 24 h. At 0 h no effects were observed, suggesting that the toxicity of cAg NPs is not directly from the NPs. Similarly to cAg NPs, $AgNO_3$ at 12 mg/L did not have any significant effects on F_v/F_m, yet at higher concentrations of 50 and 100 mg/L a significant decrease in F_v/F_m occurred. In agreement with the results of this study, Dewez and Oukarroum (2012) demonstrated an inhibitory effect on F_v/F_m caused by up to 10 μmol/L of Ag NPs (50 nm) in the green alga *C. reinhardtii*. In addition, Jiang *et al.* (2012) reported a significant decrease in F_v/F_m when *Spirodela polyrhiza* was exposed for 72 h to both Ag NPs and ionic silver at 5 mg/L, which was time-dependent.

It is well established that the toxicity of Ag NPs involves Ag ions as they can lead to the formation ROS and result in oxidative stress (Jiang *et al.*, 2014). Indeed, it was hypothesized in the present study that Ag ions may reduce F_v/F_m by inactivating RCs (Misra *et al.*, 2001). Furthermore, it appeared that the cAg NPs at higher concentrations reduced the PSII efficiency of *V. faba* by inducing alterations in the photochemical activity of PSII which consequently caused photoinhibitory effects. Photoinhibition has been reported to occur as a result of the inactivation of PSII, leading to irreversible perturbation in the function of D1 RCs protein. This photoinhibition is usually followed by a repair cycle in which *de novo* synthesis will be activated and thus the damaged D1 is reassembled (Tyystjarvi and Aro, 1996; Yokthongwattana and Melis, 2006; Sun *et al.*, 2010). As no effects of cAg NPs on F_v/F_m were observed at 0 h in the present study, the mechanism of cAg NPs toxicity was thought likely to be from the release of Ag ions over time, and the subsequently generated ROS. This hypothesis is supported by the contrasting immediate effect of $AgNO_3$. It was found that once inside plants, Ag NPs can undergo several changes, leading to the release of Ag ions (Larue *et al.*, 2014). Photosynthetic enzymes such as reductases and ferredoxins, in addition to carbohydrates such as glucose and fructose, can lead to the reduction of ENPs into ionic forms (Wang *et al.*, 2012b). The delay in cAg NPs toxicity in the following 48 h could indeed indicate that the release of Ag ions may be time-dependent (Kittler *et al.*, 2010). Other studies have also demonstrated a time-dependent release of metal ions in different media. For example, ZnO NPs require 1 h to 30 days to dissolve (Collins *et al.*, 2012; Lee and An, 2013; Wang *et al.*, 2013a). This finding supports in turn the hypothesis that the dissolution was time-dependent and the dissolved Ag ions are responsible for the gradual observed toxicity of cAg NPs.

Referring the toxicity of cAg NPs to the dissolved ions could also be supported by the results of previous studies. For example, Boucher and Carpentier (1999) reported a strong decrease

in F_v/F_m when spinach was exposed to different metal ions (mercury (Hg^{2+}), lead (Pb^{2+}), and Cu^{2+}) at concentrations ranging from 25 to 1250 mg/L. Likewise, Pietrini *et al.* (2010) exposed different plants to 50 μm cadmium (Cd), a strong inhibitor of photosynthetic activity, and observed a decrease in F_v/F_m. In addition, the effect of different concentrations (up to 500 μM of manganese (Mn) on *Citrus grandis* was investigated and results revealed a decrease in F_v/F_m (Li *et al.*, 2010).

F_q'/F_m' was also considered in this study in order to investigate the impact of cAg NPs on plant photosynthesis. Results revealed that at 12 mg/L, neither cAg NPs nor $AgNO_3$ had any significant effect on F_q'/F_m'. Increased concentrations of cAg NPs and $AgNO_3$ (50 and 100 mg/L), however, caused a significant decrease in F_q'/F_m'. Although F_q'/F_m' decreased more with cAg NPs at 50 mg/L compared to 100 mg/L, this does not necessarily imply that the lower concentration is more toxic. However, the difference could be related to variability in the properties of the light sources fitted in the two different machines used during the study.

At present, the mechanisms by which cAg NPs affect F_q'/F_m' are not entirely clear, although it is possible that dissolved Ag ions may adversely affect F_q'/F_m'. In agreement with this Boucher and Carpentier (1999) reported a strong decrease in F_q'/F_m' when spinach was exposed to different ions including Hg^{2+}, Cu^{2+}, and Pb^{2+}. Likewise, in comparison between four rice plants treated with Cd, Wang *et al.* (2013c) observed a decrease in F_q'/F_m'. Additionally, Vassilev *et al.* (2003) observed a decrease in electron transport within PSII as a result of exposure to Cu ions. The mechanism of this toxicity could be, however, attributed to an inhibitory effect of Ag ions on the secondary electron acceptor (Q_B) (Mohanty *et al.*, 1989). Indeed, the site of toxicity could also include the destination between the primary electron acceptor (Q_A) and Q_B, and compartments beyond Q_B (Mohanty *et al.*, 1989; Parmar *et al.*, 2013).

Also, F_q'/F_m' could be affected by processes beyond PSII, including PSI activity and Calvin cycle reactions which makes the interpretation of cAg NPs toxicity complex. For example, the mechanism of cAg NPs toxicity (induced by ions) could begin by affecting Q_A and Q_B, followed by inhibition of PSI, ultimately preventing electrons from being transported from PSII. It was demonstrated that exposing peas to metal ions resulted in inhibition to PSI and PSII (Chugh and Sawhney, 1999), nevertheless PSII has been shown to be more sensitive than PSI in previous studies (Ramakrishnan and Murthy, 2013).

Another explanation for the decrease in F_q'/F_m' observed herein could be attributed to the binding of cAg NPs to the chloroplast. It has previously been shown that ENPs can enter leaves and interact with the interior and the exterior of chloroplasts (Giraldo *et al.*, 2014; Ma *et al.*, 2015). This may lead to change the transport of electrons from chlorophyll to cAg NPs (Falco *et al.*, 2015). Giraldo *et al.* (2014) reported that ENPs could absorb light which would have been captured by chloroplast, but they cannot transfer this energy to the electron transport chain leading to an inhibition in photosynthetic activity. Gold nanoparticles (Au NPs) have been shown similar affinity to accept electrons from chlorophyll, quenching the emission intensity of chl *a* (Barazzouk *et al.*, 2005). In addition, a study by Falco *et al.* (2011) demonstrated that the quenching of chlorophyll fluorescence in soybean was mainly attributed to the transfer of electrons from chlorophyll to Au NP.

As photosynthesis is mainly dependent on light absorption, it is crucially important to understand whether the toxicity of cAg NPs is influenced by light. For this reason, light intensity inside the growth cabinet was increased and the results clearly showed rapid decreases in F_v/F_m and F_q'/F_m' after the injection of cAg NPs at 100 mg/L. This was followed by faster recovery,

especially in F_v/F_m, within 48 h at the higher PPFD. In contrast, at a lower PPFD, plants required a longer time (72 h) to begin such recovery. Although understanding the role of light on ENPs toxicity is still limited (Lee and An, 2013), light has been suggested to play a critical role in changing ENPs properties (Nowack *et al.*, 2012) and enhancing their toxicity (Ma *et al.*, 2014) by facilitating dissolution and the consequent formation of ROS (Santos *et al.*, 2013; Ma *et al.*, 2014). In agreement with this, Lee and An (2013) observed that at high light intensity (8.20 mW cm^{-2}) the dissolution of ZnO NPs and the consequent release of Zn ions was greater compared to low light intensity (5.68 mW cm^{-2}). This finding, indeed, supports the hypothesis that when light intensity inside the growth cabinet was increased herein, dissolution of cAg NPs was accelerated. This in turn is thought to have increased the production of ROS and thereby accelerate the toxicity as observed by a decrease in F_v/F_m and F_q'/F_m'. In contrast to the results of this study, in the presence of TiO_2 NPs, photosynthesis of spinach was promoted due to improve light capture which was facilitated by an increase in LHCII content (Lei *et al.*, 2007a). These contrasting findings are likely to be related to variation in many factors including ENPs type and properties, plant species, and application methods.

Both A and g_s are among the least investigated end-points in nanotoxicological studies. Results suggest that cAg NPs are a source of toxicity to A and g_s. In agreement with this, Zhao *et al.* (2015) reported that ZnO NPs at 800 mg/kg soil decreased A and g_s in corn by 12% and 15%, respectively. In contrast, CeO_2 NPs at 400 and 800 mg/kg soil showed no toxicity to A and g_s (Zhao *et al.*, 2012; Zhao *et al.*, 2015). Such conflicting results between studies are expected, due to the differences in ENPs and plant species between studies. It is hypothesised that the inhibition of A and g_s in the present study was due to the Ag ions. This explanation is widely accepted in different studies investigating the effects of ions on plants. For example, Li *et al.* (2010)

investigated the effect of Mn^{+} at concentrations up to 500 µM on *C. grandis* and reported inhibitory effects on A and g_s. In addition, exposure to Al^{+} decreased A and g_s in the same plant (Chen *et al.*, 2005). Furthermore, Cd^{+} at different concentrations significantly decreased A and g_s in different plants (Pietrini *et al.*, 2010; Ying *et al.*, 2010).

It has been suggested that the presence of ENPs inside leaves can affect stomata and thereby negatively impact photosynthetic performance (Wang *et al.*, 2013b; Hong *et al.*, 2015). One question that remains to be addressed is whether or not the decreases in fluorescence and gas exchange parameters observed in the present study were related to a direct effect on the stomata or was a result of sequential damages began in PSII. In order to determine this, the impact on intercellular CO_2 concentration (C_i) was also targeted. As results demonstrated interestingly no effect on C_i (Appendix A), the impact of cAg NPs on the photosynthetic performance is thought more likely to have been caused by inhibition to photosynthesis apparatus, rather than stomatal factors. This hypothesis needs further examination, however.

A similar hypothesis about the unaccompanied link between the decreases in the photosynthetic performance and C_i has also been put forward in a number of previous studies. For example, Chen *et al.* (2005) observed a decrease in F_v/F_m, F_q'/F_m', A and g_s (by Al ions) yet no effect on C_i, which indicated the unaccompanied consequence of stomatal limitation. Alternatively, the authors attributed this reduction to a disruption in PSII. In addition, Li *et al.* (2010) observed a similar decrease in the F_v/F_m, A, and g_s of *C. grandis*, treated with Mn ions, whilst C_i was increased. The authors therefore concluded that this was due to damages the whole electron transport chain rather than stomatal factors. Furthermore, a decrease in A in four different rice plants treated with Cd was related to limitations in photosynthesis apparatus, rather than stomatal limitation (Wang *et al.*, 2013c). Nevertheless, other studies have attributed the effect of ENPs on

photosynthetic performance to stomatal limitation. For example, Hong *et al.* (2015) applied aerially CeO_2 NPs and CuO NPs (up to 200 mg/L) on cucumber leaves and observed a decrease in A and g_s which was suggested to be due to the blocking of stomata by the applied ENPs.

Taken together, it seems that as a result of their impact on the RCs and decreasing the rate of electron transport, the presence of cAg NPs ultimately affected the carbon fixation process. It is thus worth highlighting that the toxicity of cAg NPs on the photosynthetic performance of plants is multi-dimensional and may include impacts on the efficiency of RCs, electron transfer, and carbon fixation process.

ENPs toxicity is extensively attributed to their ability to produce ROS and cause oxidative stress (Santos *et al.*, 2013). The generation of ROS in the presence of Ag NPs has previously been reported with animals, bacteria, and algae, yet little information is currently known for plants (Jiang *et al.*, 2014). To confirm whether the toxicity observed in the present study was caused by the formation of ROS, the generation of H_2O_2 was measured. Unexpectedly, whilst H_2O_2 was detected in leaves exposed to cAg NPs at 12 mg/L (which is the lowest concentration used in this study), at 100 mg/L (which is the highest concentration applied in this study) very little or no H_2O_2 was detected. Although the observed toxicity is thought to be mainly attributed to the generated H_2O_2 (mediated by Ag ions), it seems likely that when cAg NPs at high concentration applied more Ag ions were released and thus H_2O_2 were generated at extreme level. Previously, the production of ROS has been shown to be dependent on the concentration of ENPs and released ions (Wang *et al.*, 2015). At this high concentration of 100 mg/L, however, it is possible that the concentration of H_2O_2 was high and exceeded the AR capacity for detection. Although AR has been reported to be sensitive in detecting H_2O_2 (Zhou *et al.*, 1997), its sensitivity seems to be dependent on the concentration of generated H_2O_2 as it was reported that for accurate determination of H_2O_2, AR

should be present at a higher concentration than the generated H_2O_2 (Mohanty *et al.*, 1997). However, 2',7'-dichlorodihdrofluorescein-diacetate (DCFH-DA) used in other studies (Oukarroum *et al.*, 2013; Jiang *et al.*, 2014; Perreault *et al.*, 2014; Wang *et al.*, 2015) could be used as an alternative probe for H_2O_2 detection in future nanotoxicological studies.

Another factor that may have participated in the lack of detection of H_2O_2 with 100 mg/L cAg NPs in the present study is the activation of the plant's antioxidant defence system at its maximum capacity which can remove H_2O_2. Variation in the capacity of antioxidants at different concentrations of metal ions has previously been reported. In their study, Han *et al.* (2008) observed an increase in antioxidant activity of *U. armoricana* with the increase in Cu concentration (up to 100 μg/L). This hypothesis is unlikely, however, to explain the lack of detection of H_2O_2 in the present study as an inhibitory effect of cAg NPs remained throughout the experiment, indicating the presence of H_2O_2.

Plants exposed to ENPs have been shown to be disrupted by ROS (Oukarroum *et al.*, 2013), thus activation of protection systems is required to scavenge ROS and mitigate toxicity. Protection systems against ROS involve enzymatic and non-enzymatic antioxidants such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), malondialdyhide (MDA), and glutathione (GSH). Such antioxidants can reduce ROS induced by the presence of Ag NPs (Hsu-Kim, 2007). Jiang *et al.* (2014) demonstrated that under oxidative stress caused by ROS induced by Ag NPs (up to 10 mg/L), a significant increase in the activity of SOD, CAT, and POD was observed. The same authors also observed a significant increase in the content of non-enzymatic antioxidants GSH and MDA with Ag NPs. The mechanism of how these antioxidants reduce ROS may be explained by Ag ions binding and the formation of less-toxic complexes. For example, Ag ions were found to be bound to GSH when lettuce was exposed to Ag NPs at concentrations up to 100 μg/g (Larue *et al.*,

2014). Following such scavenging, recovery of plants from stress could occur, which may explain the increase in F_v/F_m after 48 h observed herein. In a recent study by Zhao *et al.* (2015), recovery in photosynthesis was also observed when corn was exposed to ZnO NPs at 400 and 800 mg/kg. Indeed, these results are indicative of the participation of enzymatic and/or non-enzymatic antioxidants in the detoxification of ROS. Importantly, the total removal of ROS is often not possible (Foyer and Shigeoka, 2011), which explains the incomplete recovery of F_v/F_m, even after 216 h post-application in the present study.

In addition to the activation of enzymatic and non-enzymatic antioxidants, plants may also have increased the efficiency of unaffected RCs to meet the requirement of electrons to obtain the state of Q_A reduction. Partial inhibition of RCs was previously suggested when Cu and Hg ions inhibited the activity of some RCs whilst others were not affected (Samson and Popovic, 1989).

Capping agents are widely applied in order to increase ENPs stabilization (Perreault *et al.*, 2014). However, such agents could make ENPs toxic substances which have previously been shown to have adverse effects on seed germination and plant growth (Stampoulis *et al.*, 2009). In the present study, when the capping agent (mPEG) alone was injected into plants, no negative effects were observed (Appendix B), indicating that the toxicity was solely related to the cAg NPs. Similarly, Beddow *et al.* (2014a) did not observe any detrimental effects on the growth of *E. coli* and *B. subtilis* exposed to mPEG alone, whilst growth was significantly negatively affected by the cAg NPs. In addition, the capping agent citrate (used to cap Ag NPs) had no effect on the growth of mung beans (Lee *et al.*, 2012).

Bioavailable ENPs have been found in various places inside plant tissues including the cytoplasm, cell wall, intercellular spaces, and biomemberanes (Racuciu and Creanga, 2007; Lee *et al.*, 2008; Foltęte *et al.*, 2011; Mazumdar and Ahmed, 2011; Lee *et al.*, 2012). Such bio-

accumulated ENPs can affect plants (Wang *et al.*, 2012b) and potentially cause a risk to the end consumer (Nowack and Bucheli, 2007; Thuesombat *et al.*, 2014). In the present study, the visual appearance of brown spots which spread on both the abaxial and adaxial surfaces in treated leaves was another toxic effect observed. This is not surprising since such phenomenon has also been observed in several studies. For example, when mung bean and sorghum were exposed to Ag NPs (Lee *et al.*, 2012), when mung bean and wheat were exposed to Cu NPs (Lee *et al.*, 2008), when cucumber was exposed to CeO_2 NPs (Hong *et al.*, 2014), and when rice was exposed to CuO NPs (Peng *et al.*, 2015; Wang *et al.*, 2015). One hypothetical explanation for this phenomenon is that the cAg NPs were dissolved, and the released ions were then aggregated in clusters which appeared as brown spots as previously observed when lettuce were exposed to Ag NPs (Larue *et al.*, 2014). Meanwhile, it is not excluded that some cAg NPs themselves may have aggregated, as it was found when pumpkin plants were exposed to carbon-coated Fe NPs (Corredor *et al.*, 2009). Although further investigation on the nature of cAg NPs inside leaves was not carried out in the present study, it is important to clarify the identity of these dots and their locations. Hence, application of TEM or scanning electron microscopy (SEM), for example, would be valuable in order to monitor the physical characteristics of cAg NPs over the experimental time period. Such information would help to understand how cAg NPs interact with plants and impose their toxicity.

ICP-MS is widely-applied in nanotoxicological studies to measure the concentration of accumulated ENPs and their released ions (Priester *et al.*, 2012; Colman *et al.*, 2013). In the present study, results of ICP-MS revealed that the concentration of Ag in plants increased with increasing cAg NPs dose, indicating a dose-dependent response. Lee *et al.* (2012) also showed a similar dose dependent-response when mung bean and sorghum were exposed to Ag NPs. The results of this study showed also that the concentration of Ag in the cAg NPs-treated leaves was

lower than that in the $AgNO_3$-treated leaves. This agrees with the results of Colman *et al.* (2013), who detected significantly more Ag in the tissue of different plants exposed to $AgNO_3$ compared to Ag NPs.

Unexpectedly the results of the present study demonstrated that the content of Ag in $AgNO_3$-treated leaves varied during the exposure time. Despite this, little variation was observed in cAg NPs-treated leaves. Although, the same steps of digestion and analysis were applied, unknown reasons may have caused these variations.

In conclusion, the cAg NPs demonstrated toxicity towards *V. faba*, and at certain concentrations above 12 mg/L they exceeded the threshold of tolerance. Despite this, $AgNO_3$ were more toxic than cAg NPs. It was determined that cAg NPs concentration, light level, and Ag ions concentration are key factors influencing the toxicity of cAg NPs. Attempts were made to elucidate the mechanisms of cAg NPs toxicity, however, further work is required in this area. The results also indicate that photosynthetic performance is sensitive and thus it is recommended to be considered when studying the impact of ENPs on plants. Importantly, whilst leaves injected with cAg NPs showed significant decreases in photosynthetic parameters, none of these decreases were observed in unexposed control leaves, indicating that the injection process itself did not cause any impact on the plants. Thus, the injection method developed in the present study provides a good model that can be applied for the delivery of ENPs to plants, in order to enhance understanding on the impact of ENPs on photosynthetic performance. This study is one of the first to investigate the effect of ENPs on the photosynthetic performance of crop plants. Further studies are required in this area, due to the importance of such plants in food production.

Chapter 6. The influence of foliar-deposited and- sprayed of cAg NPs on photosynthetic performance in *Vicia faba* and *Arabidopsis thaliana*

6.1. Introduction

Foliar uptake of air pollutants by plants is evidenced from previous studies (Uzu *et al.*, 2010). Of particular concern is the uptake of ENPs as emerging and novel toxicants. Results of previous studies have confirmed the possibility that plants may take up atmospheric ENPs in significant amount (Schreck *et al.*, 2012; Hong *et al.*, 2014; Larue *et al.*, 2014).

Following entry into plants via stomatal or cuticular pathways (Eichert *et al.*, 2008), ENPs can have a positive or negative effect. For example, it has been reported that ENPs can play a role in enhancing energy harvesting for photosynthesis, electron transport, photoreduction activity of PSII, and oxygen evolution (Lei *et al.*, 2007b; Giraldo *et al.*, 2014). In contrast, the presence of ENPs inside leaves has also been suggested to block stomata and negatively affect gas exchange (Wang *et al.*, 2013b). Thus, the present and future impact of ENPs on plants remains uncertain. Moreover, little information is currently known about the role of ENPs on photosynthesis. Therefore, the aim of the work in this chapter was to study the potential impact of cAg NPs on photosynthetic performance in *A. thaliana* and *V. faba*.

6.2. Impact of cAg NPs on F_v/F_m

After depositing (200 µL) and spraying (2 mL) of cAg NPs and $AgNO_3$ at different concentrations (up to 100 mg/L) on *A. thaliana* leaves, F_v/F_m was monitored to assess the impact on plant photosynthetic processes. As presented in Figs. 6.1 and 6.2, concentrations of cAg NPs and $AgNO_3$ at 12, 50, and 100 mg/L deposited on leaves had no significant effect on F_v/F_m

compared to controls during the time of investigation. Similarly, no significant changes to F_v/F_m occurred when plants were sprayed with cAg NPs and $AgNO_3$ of the same concentrations at the end of the experimental time.

Results also demonstrated that none of the investigated concentrations of cAg NPs (12, 50, and 100 mg/L) had any significant impact on F_v/F_m of *V. faba* (Appendix C).

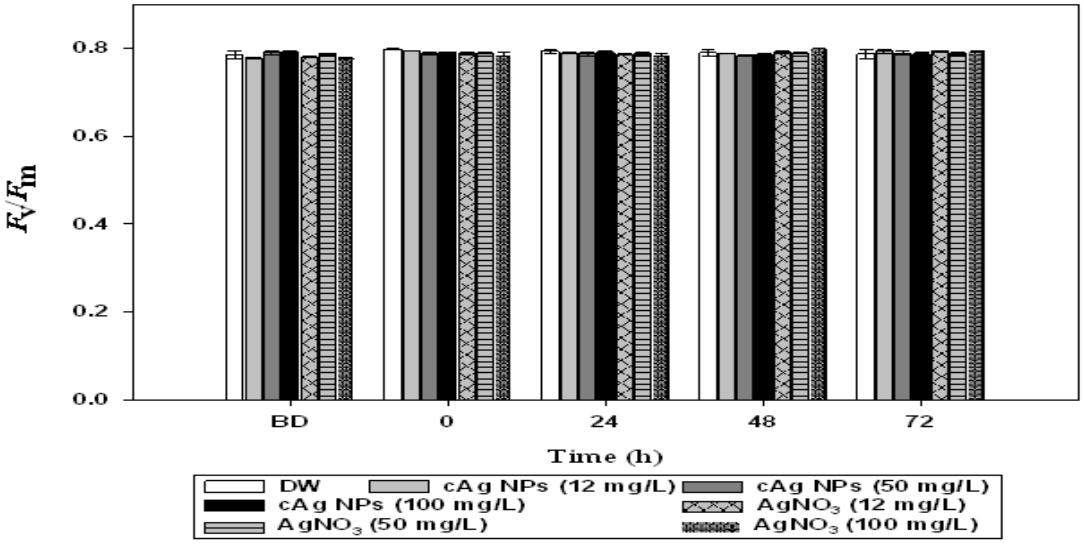

Fig. 6.1. Changes in the chlorophyll fluorescence parameter F_v/F_m induced by the deposition of cAg NPs and $AgNO_3$ at12, 50, and 100 mg/L during 72 h. Leaves of *A. thaliana* were dark adapted for 20 min prior to measurements. BD = before deposition. The values given are mean ± SE (n=3). Comparisons between treatments are performed using one way ANOVA ($P \geq 0.05$).

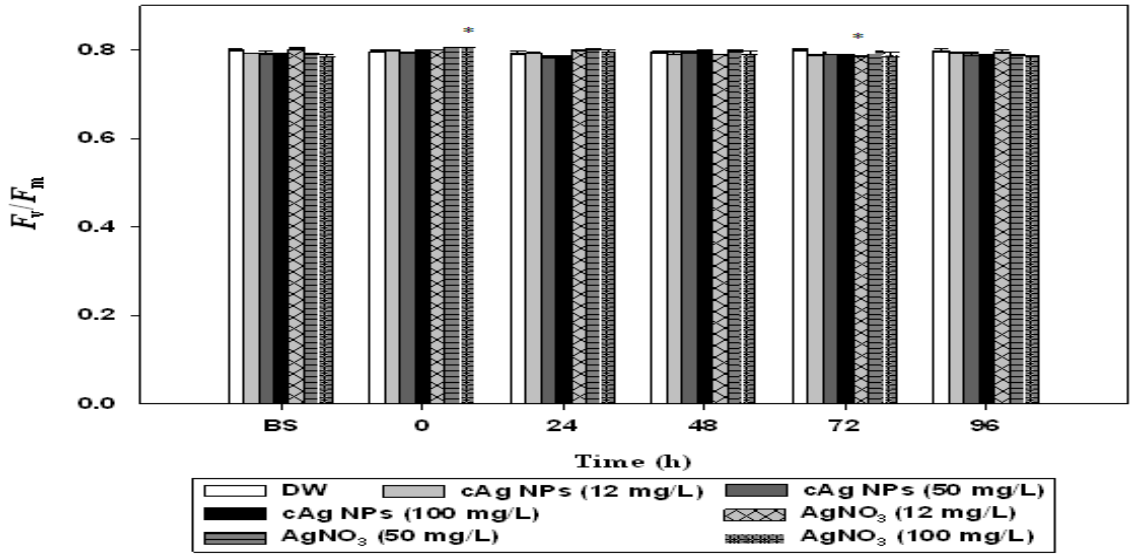

Fig. 6.2. Effect of sprayed cAg NPs and $AgNO_3$ at 12, 50, and 100 mg/L on the chlorophyll fluorescence parameter F_v/F_m during 96 h. Leaves of *A. thaliana* were dark adapted for 20 min prior to measurement. BS = before spraying. The values given are mean ± SE (n=3). Comparisons between treatments were performed using one way ANOVA ($P < 0.05$) and significant differences are marked with '*'.

6.3. Effect of cAg NPs on F_q'/F_m'

The efficiency of F_q'/F_m' at 100 and 500 µmol m^{-2} s^{-1} PPFD, following foliar exposure of cAg NPs and $AgNO_3$ at concentrations of 12, 50, and 100 mg/L was investigated. When cAg NPs were deposited (200 µL) and sprayed (2 mL) on *A. thaliana* leaves there was no significant effect on F_q'/F_m' at 100 and 500 µmol m^{-2} s^{-1} PPFD over the experimental period (Figs. 6.3 and 6.4). $AgNO_3$ at the same concentrations also had no effect on F_q'/F_m' at both PPFDs, by the end of the experimental time.

Deposition and spray of cAg NPs on *V. faba* at the same concentrations also had no effect on F_q'/F_m' at 100 and 500 µmol m^{-2} s^{-1} PPFD during the experimental time period (Appendix C).

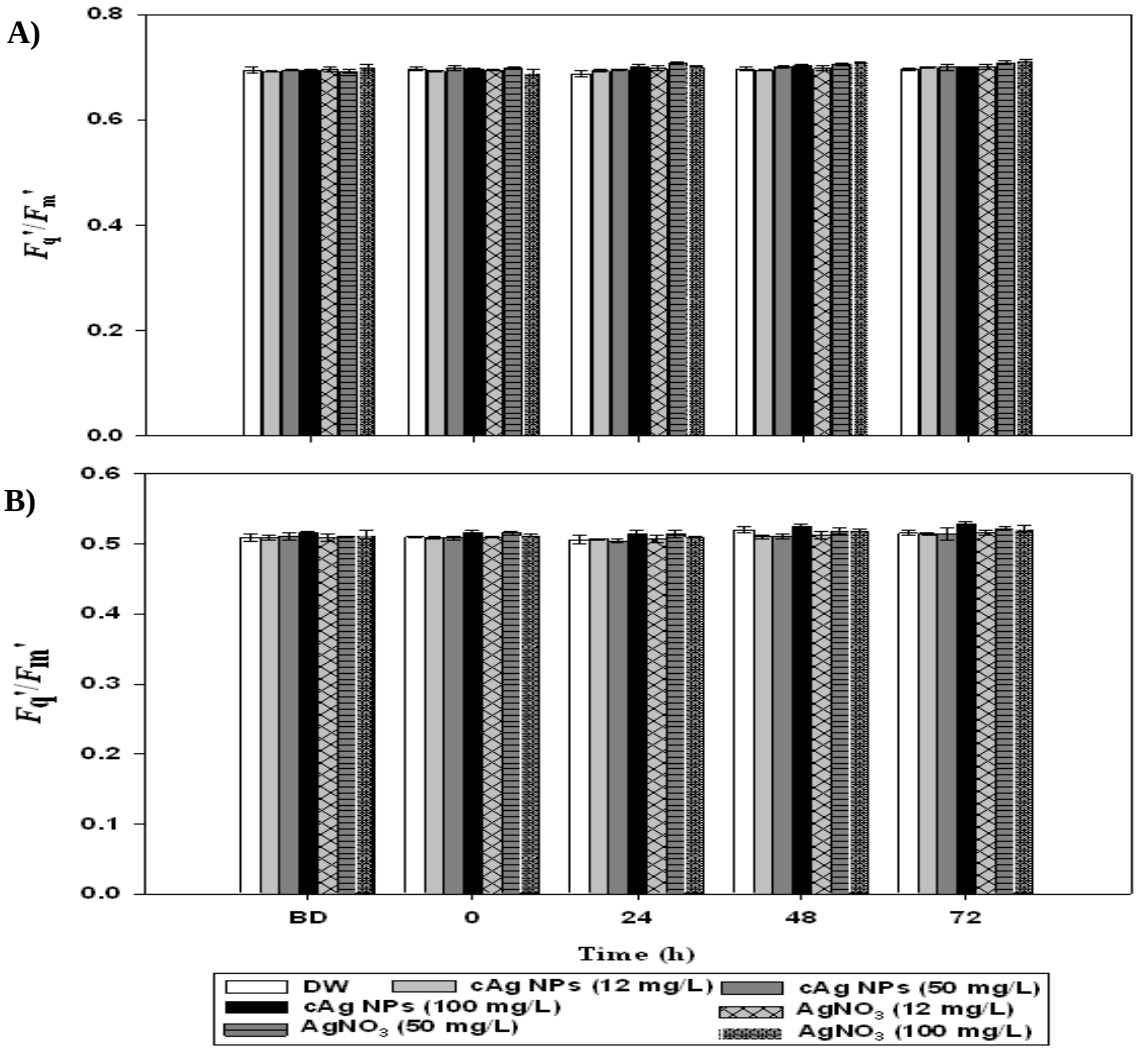

Fig. 6.3. Response of the chlorophyll fluorescence parameter F_q'/F_m' at a PPFD of 100 (A) and 500 (B) µmol m^{-2} s^{-1}, to the deposited cAg NPs and $AgNO_3$ at 12, 50, and 100 mg/L during 72 h. BD = before deposition. The values given are mean ± SE (n=3). Comparisons between treatments were performed using one way ANOVA ($P \geq 0.05$).

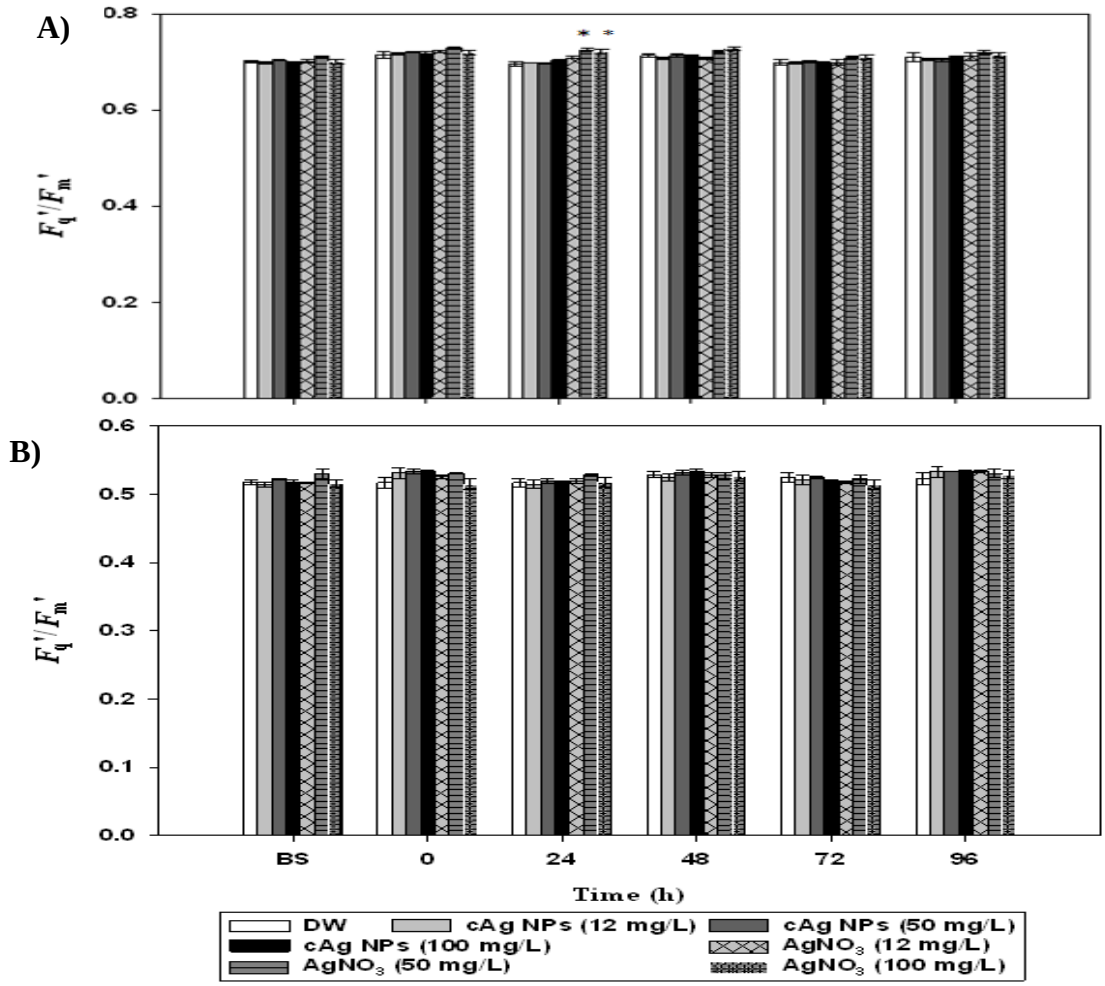

Fig. 6.4. Effect of the sprayed cAg NPs and $AgNO_3$ at 12, 50, and 100 mg/L on the chlorophyll fluorescence parameter F_q'/F_m' at a PPFD of 100 (A) and 500 (B) µmol m^{-2} s^{-1} during 96 h. BS = before spraying. The values given are mean ± SE (n=3). Comparisons between treatments were performed using one way ANOVA ($P < 0.05$) and significant differences were marked with '*'.

6.4. Assessment of cAg NPs effect on *A*

Following the spray of cAg NPs and $AgNO_3$ on plants, changes in *A* were recorded. Fig. 6.5 shows that at the end of the experimental exposure period, neither cAg NPs nor $AgNO_3$ had any significant impact on the *A* of *A. thaliana*.

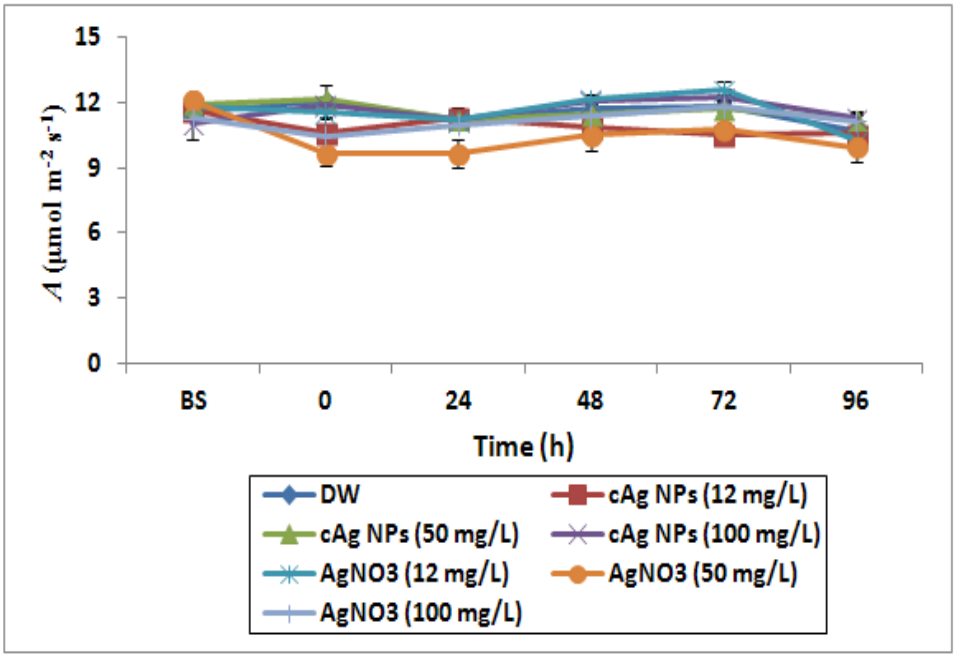

Fig. 6.5. Response of *A*, to cAg NPs and $AgNO_3$ sprayed on *A. thaliana* leaves at 12, 50, and 100 mg/L during 96 h. BS = before spraying. The values given are mean ± SE (n=3). Comparison between treatments was performed using one way ANOVA ($P \geq 0.05$).

6.5. cAg NPs impact on g_s

In order to determine whether or not cAg NPs had any toxic effects on plant physiology, changes in g_s were recorded. Impacts of the deposited and the sprayed cAg NPs and $AgNO_3$ at concentrations of 12, 50, and 100 mg/L on the g_s of *A. thaliana* are presented in Fig. 6.6 and 6.7. Throughout the experiment, no significant effect of cAg NPs and $AgNO_3$ were observed.

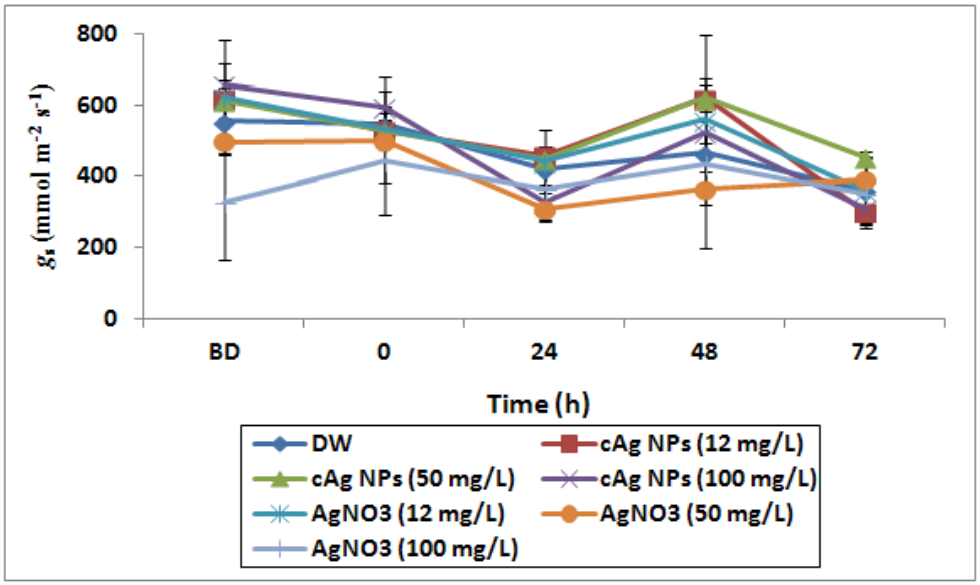

Fig. 6.6. Response of g_s, to cAg NPs and $AgNO_3$ deposited on *A. thaliana* leaves at 12, 50, and 100 mg/L during 72 h. BD = before deposition. The values given are mean ± SE (n=3). Comparison between treatments was performed using one way ANOVA ($P \geq 0.05$).

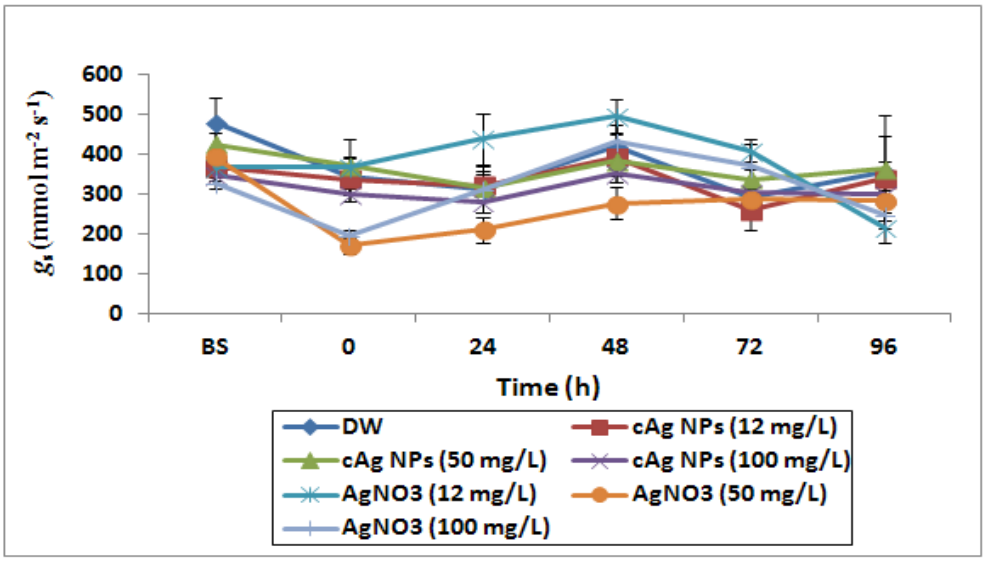

Fig. 6.7. Effect of the sprayed cAg NPs and $AgNO_3$ at 12, 50, and 100 mg/L on g_s during 96 h. BS = before spraying. The values given are mean ± SE (n=3). Comparison between treatments was performed using one way ANOVA ($P \geq 0.05$).

6.6. Ag content in leaf tissues

The concentration of Ag after washing *V. faba* leaves exposed to cAg NPs and $AgNO_3$ at a concentration of 100 mg/L by means of deposition is presented in Fig. 6.8. The concentration of Ag in leaves treated with $AgNO_3$ was greater than for leaves treated with cAg NPs at all time points, with the exception of 24 h post-treatment. Whereas the content of Ag in the cAg NPs-exposed leaves did not exceed 40 µg/g, $AgNO_3$-exposed leaves contained almost triple the concentration of cAg NPs-exposed leaves by the end of the experiment (96 h).

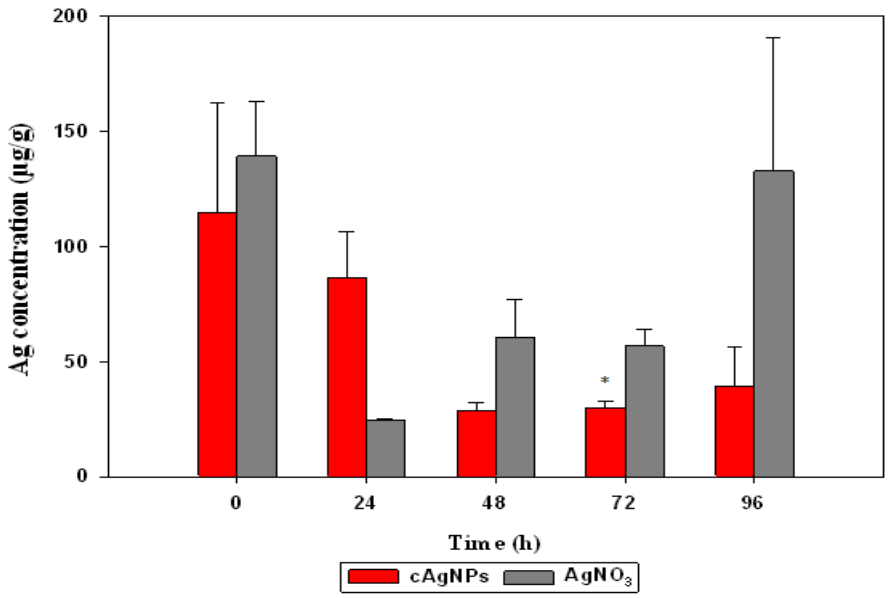

Fig. 6.8. Total amount of bio-accumulated Ag in leaves exposed to cAg NPs and $AgNO_3$, by mean of deposition, at 100 mg/L during 96 h of exposure. Student's *t*-test ($P \geq 0.05$) was performed to compare between treatments. The values given are mean ± SE (n=3).

6.7. Discussion

To date, greater attention has been given to the entry of ENPs into plants through seeds and roots, while little is currently known about foliar uptake and subsequent effects on plant photosynthesis. Foliar uptake could present one of the major ways in which ENPs enter plants and accumulate in food chain, leading to toxic impacts on plants and health risks to humans (Eichert *et al.*, 2008).

In the present study, the photosynthetic performance of different plants (*A. thaliana* and *V. faba*) was monitored following foliar application by spraying and deposition of cAg NPs and $AgNO_3$ at different concentrations. Results revealed that cAg NPs up to 100 mg/L had no impact on the photosynthetic parameters. These results are in agreement with previous studies. For example, Qi *et al.* (2013) reported no disruptions in F_v/F_m and F_q'/F_m' in tomato leaves spraying with TiO_2 NPs at concentrations of 50 and 100 mg/L. Shaw *et al.* (2014) demonstrated that foliar applied CuO NPs (at 0.5, 1, and 1.5 mM) to Syrian barley did not cause any toxicity to F_v/F_m. In addition, Zhao *et al.* (2012) and Zhao *et al.* (2015) investigated the effect of CeO_2 NPs at 400 and 800 mg/kg soil on A and g_s in corn, and showed no effect on these parameters. These findings support the results of the present study in which cAg NPs did not exhibit any toxicity when applied aerially by means of deposition and spraying. Despite this, toxic effects may still occur under different methods of application or when repeating applications.

The lack of toxicity observed in the present study may be explained by the means of cAg NPs application (i.e. a manual spray pump and pipette). These methods produced diverse droplet sizes and it is likely that these droplets dried on the leaf surface, whereupon cAg NPs agglomerated with each other or aggregated with other components available on the leaf surface, leading to big

particles (larger than nanosize) (Quadros and Marr, 2011). Aggregation of cAg NPs is likely to reduce penetration efficiency compared to separated particles. Although the cAg NPs used in the present study were capped, the capping agent may not prevent the cAg NPs from aggregating, as it has previously been shown that in the presence of surfactants, aggregation of ENPs can occur (Brar *et al.*, 2010). As the uptake of Ag NPs depends on particle size, the entry of cAg NPs into leaves is thought to have been minimized, thus no toxicity was observed. In agreement with this, Jiang *et al.* (2014) observed that Ag NPs were more toxic than micron-sized Ag particles. The authors indicated the role of size in facilitating entry, and subsequently toxicity, to plants. Indeed, the diameter of cuticular pores is estimated to be approximately up to 4.8 nm (Eichert and Goldbach, 2008) thus will exclude entry of most ENPs. Although stomatal pores are estimated to be approximately five to ten orders of magnitude larger than cuticular pores (Eichert *et al.*, 2008; Eichert and Goldbach, 2008), they may still be small for the entry of aggregated cAg NPs. Meanwhile, it could be hypothesised that stomata adjust aperture to prevent entry of aggregated cAg NPs.

The role of ENPs size in determining their entry into leaves is supported by the findings of (Eichert *et al.*, 2008) who proved the entry of ENPs at 43 nm into *V. faba* leaves, but not those aggregated to 1.1 μm, which were trapped on the leaf surface and not taken up. Likewise, Wang *et al.* (2013b) observed an improvement in the penetration of small Fe_2O_3 NPs into watermelon leaves over those of a larger size. However, even if some cAg NPs and/or dissolved Ag ions entered leaves through the cuticle and/or stomata, it seems that they were not able to enter chloroplasts, or the concentration was not great enough to cause any toxicity. Penetration of ENPs is not necessarily linked with toxicity, however, as no effect on wheat photosynthesis was observed

after being exposed to TiO_2 NPs (at 100 mg/L), despite finding TiO_2 NPs inside plant tissues (Larue *et al.*, 2012).

In addition to ENPs size, other factors can also control the entry of ENPs into plants, including application method, working environment (light and water), and leaf structure (waxy cuticule and trichomes) (Wang *et al.*, 2013b). Application scheme, in particular the repetition of ENPs exposure, is one of the most crucial factors affecting uptake and toxicity. When comparing the single application of cAg NPs applied in this study to several applications used in other studies (Hong *et al.*, 2014; Hong *et al.*, 2015), it seems that multiple applications of ENPs are responsible for increased ENPs toxicity. In general, multiple applications will lead to excessive ENPs contact with plants, thus increasing the possibility of adverse effects. In one study, Hong *et al.* (2014) repeated the application of CeO_2 NPs (up to 320 mg/L) three times every four h (to increase the chance of the entry), and demonstrated a toxic effect in cucumber. Regardless of whether the spray of ENPs is environmentally relevant or not, the application scheme of ENPs onto plants should be taken into consideration for interpreting the results.

The working environment could also contribute to the extent of entry of cAg NPs into leaves in the present study. For example, humidity is an important factor in the growth environment for plants and can greatly affect the penetration of ionic molecules through leaves (Schreiber, 2005). Thus, under high humidity, the interaction between ENPs and plants is extended as stomatal and cuticular pathways will facilitate the entry of ENPs, potentially resulting in adverse effects. In the present study, however, high humidity was not available inside the growth chambers, thus quick-drying of cAg NPs droplets occurred, leading to a short-term interaction between cAg NPs and plants. In contrast, when plants maintained in water-saturated condition, ENPs were reported to enter the leaves of *V. faba* (Eichert *et al.*, 2008). When the same authors re-wetted the

evaporated residues of ENPs applied, even greater penetration was observed. However, under such condition it seems very likely that stomata tend to be fully opened, facilitating entry of ENPs.

Another factor that also can affect foliar uptake of ENPs is plant morphology. In a study by Schreck *et al.* (2012) foliar uptake by different plants was investigated, results revealed varied metal contents which was attributed to the morphology and physiology of plants. Particularly, parsley appeared to trap more ENPs due to the nature of its rough leaves. In contrast, as silicon covers ryegrass leaves, these leaves exhibit less content of ENPs.

Investigating the state of cAg NPs once dried on the leaf surface would help to clarify whether or not cAg NPs were taken up by the plants. The application of TEM and/or SEM is advisable in order to examine whether ENPs can be taken up by plants and monitor changes in the physical characteristics of those ENPs able to enter leaves.

In contrast to the results of the present study, other studies have found that foliar exposure of plants to ENPs can lead to either positive or negative effects on grain yield, leaf area, pigment amount, seed number, pod number, and photosynthesis (Lei *et al.*, 2007a,b; Moaveni *et al.*, 2011; Morteza *et al.*, 2013). For example, in a recent study, Hong *et al.* (2015) investigated the impact of CeO_2 NPs and CuO NPs at different concentrations (50, 100, and 200 mg/L) on cucumber leaves, and reported a decrease in *A*, particularly at the higher concentrations. In contrast, the same authors demonstrated an increase in g_s at 50 mg/L of CeO_2 NPs. Additionally, Lei *et al.* (2007a and 2007b) found that TiO_2 NPs enhanced photosynthesis and greatly improved growth in spinach. Similarly, Gao *et al.* (2008) demonstrated an increase in *A* when *Ulmus elongate* seedlings were exposed to 0.03% TiO_2 NPs, particularly at low light intensity (800 µmol m^{-2} s^{-1}). It is indeed not surprising to obtain such enhancement as TiO_2 NPs have been reported to have multiple favourable effects on plants, including activating the protective mechanisms against ROS, manufacturing pigments,

capturing light and transforming light to active electrons, stimulating of rubisco activase, enhancing the reduction of $NADP^+$ into NADPH and producing ATP, maintaining the integrity of the chloroplast membrane, and ultimately enhancing photosynthesis (Morteza *et al.*, 2013; Qi *et al.*, 2013; Lei *et al.*, 2007b).

Accumulation of ENPs and their dissolved ions on the surface or inside leaves, when introduced by foliar application, can occur (Schreiber, 2005; Corredor *et al.*, 2009; Wang *et al.*, 2013b). As the results herein demonstrated, Ag was detected in the leaf tissue during the experimental period, showing a greater amount in leaves exposed to $AgNO_3$ compared to those exposed to cAg NPs. Such accumulated Ag does not necessarily indicate uptake of cAg NPs as they could represent those aggregated on the surface of leaves. Although, leaves were washed three times before being digested, absolute removal of cAg NPs and $AgNO_3$ from the leaf surface is difficult, as incomplete removal of ENPs from exposed-plants has been previously reported (Fan *et al.*, 2014; Huang *et al.*, 2014; Peng *et al.*, 2015). Therefore, both penetrated Ag, if any, and surface Ag could be represented by the Ag detected herein. In agreement with the results of this study, Larue *et al.* (2014) detected Ag on the surface and in various regions inside lettuce leaves after being aerially exposed to Ag NPs at 1, 10, and 100 μg/g. In addition, Hong *et al.* (2015) detected Ce in cucumber leaves at concentrations that increased relative to increasing CeO_2 NPs concentration (50, 100, and 200 mg/L).

Dissolution of ENPs both on the surface and inside leaves has been reported previously. For example, Wang *et al.* (2013b) suggested that $Mg(NO_3)_2$ and $MgSO_4$ ENPs (44 nm and 45 nm, respectively) dissolved inside the leaves of watermelon. In contrast, dissolution of other ENPs occurred on the surface of different plants leaves (Schreck *et al.*, 2012). Generally, the dissolution of ENPs can be affected by several factors including the presence of microorganisms on leaves,

plant pH, and the physicochemical properties of the ENPs themselves (Gandois *et al.*, 2010; Larue *et al.*, 2014; Peng *et al.*, 2015).

In conclusion, foliar application of cAg NPs by means of spray and deposition had no effect on the photosynthetic performance of *A. thalinan* or *V. faba* plants. In general, the behaviour and the toxicity of foliar-applied ENPs on the leaf surface can be influenced by several factors including leaf morphology, the amount of wax and exudates present on leaves, the density and morphology of trichomes, any microorganisms present on leaf surface, the physicochemical properties of the ENPs themselves (Larue *et al.*, 2014) and stomatal aperture density (Eichert *et al.*, 2008). Moreover, although spray pumps were previously used to expose plants to ENPs, for successful foliar uptake exposure procedures should also include aerosol-generators, whereby particle size and number concentration can be controlled (Wang *et al.*, 2013b).

Chapter 7. Influence of cAg NPs on the physiological functions of the aquatic plant *Lemna minor*

7.1. Introduction

Fast growth of nanotechnology has led to the use of ENPs in a wide range of products. It has already been shown that ENPs can find their way into aquatic ecosystems in significant amounts during the life cycle of ENPs-containing products (Wang *et al.*, 2008; Geranio *et al.*, 2009). Wastewater flows and antifouling paints on the hulls of boats are clear examples of indirect and direct entry of ENPs into aquatic ecosystems (Saison *et al.*, 2010; Keller *et al.*, 2013). Such contamination of aquatic environments with ENPs raises concern as it is likely to adversely affect aquatic organisms, including plants.

L. minor is an important aquatic plant providing food and shelter for other aquatic organisms (Lewis, 1995; Song *et al.*, 2012). Due to multiple features including its small size, rapid growth, ease of culture, and high capacity to uptake toxicants (Shi *et al.*, 2011; Oukarroum *et al.*, 2015), *Lemna* has been widely used to study the potential impact of ENPs. Previous studies have shown that *Lemna* is extremely sensitive to ENPs, particularly Ag NPs (Gubbins *et al.*, 2011). The toxicity of ENPs to *Lemna* may be induced by the ENPs themselves and/or by their released ions (Oukarroum *et al.*, 2013; Jiang *et al.*, 2014). Moreover, capping agents could also affect the toxicity of ENPs to *Lemna* (Perreault *et al.*, 2014). Thus, there remains uncertainty about whether ENPs are toxic to aquatic plants.

Most published studies considered the impact of ENPs on *Lemna* to date have focused on limited endpoints such as frond numbers, chlorophyll content, and ROS production (Gubbins *et al.*, 2011; Shi *et al.*, 2011; Song *et al.*, 2012; Oukarroum *et al.*, 2013; Santos *et al.*, 2013; Jiang *et al.*,

2014). Little attention has thus far been given to investigating the effect of ENPs on the photosynthetic performance of *Lemna*. The aim of the work in the present chapter therefore was to investigate the impact of cAg NPs on the photosynthetic performance of *L. minor*. In order to specify the mechanism of the toxicity of cAg NPs, the impact of Ag ions and mPEG were also investigated.

7.2. Impact of cAg NPs on F_v/F_m

The effect of cAg NPs and $AgNO_3$ at different concentrations (12, 50, and 100 mg/L) on the photosynthetic performance of *L. minor* is presented in Fig. 7.1. The results showed that after 96 h of exposure, there were no significant impacts of cAg NPs on F_v/F_m at the concentrations applied, compared to the control plants. In contrast, $AgNO_3$ at 50 and 100 mg/L inhibited F_v/F_m within 48 h, whilst had no significant impact at 12 mg/L. This inhibition was also represented by changes in false colour representing ranges of F_v/F_m values (Fig. 7.2).

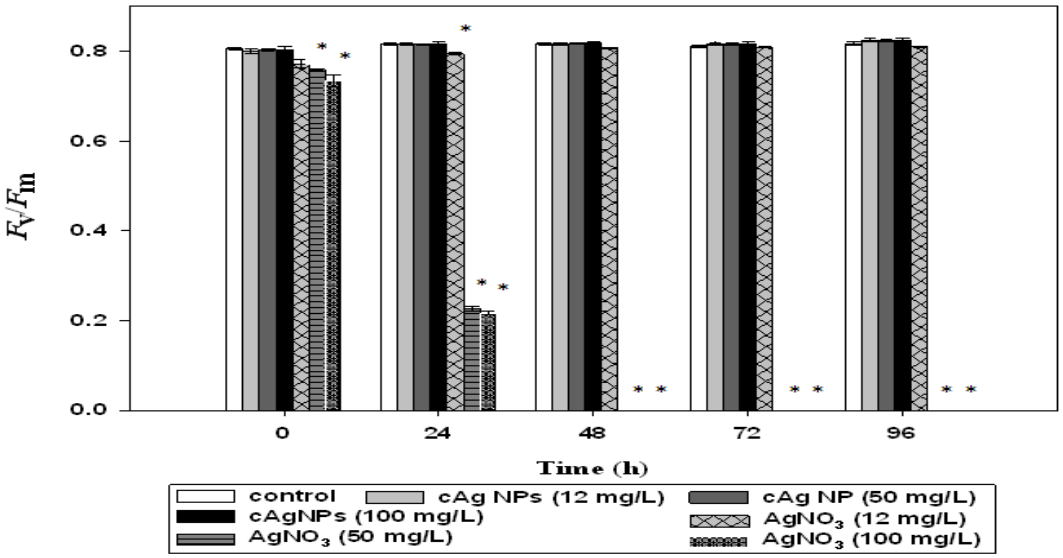

Fig. 7.1. Changes in the chlorophyll fluorescence parameter F_v/F_m of *L. minor* induced by cAg NPs and $AgNO_3$ at 12, 50, and 100 mg/L. The values given are mean ± SE (n=4). Comparisons between treatments were performed using one way ANOVA ($P < 0.05$) and significant differences are marked with '*'.

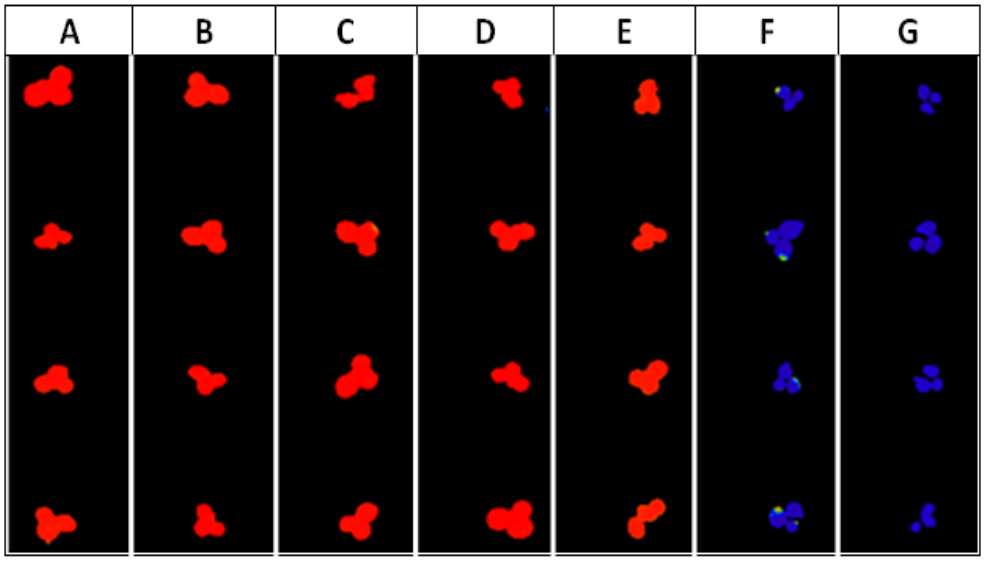

Fig. 7.2. Visual presentation of the impact of control (A) and cAg NPs at 12 (B), 50 (C), and 100 (D) mg/L and $AgNO_3$ at 12 (E), 50 (F), and 100 (G) mg/L on *L. minor* after 24 h represented by changes in false colour representing ranges of F_v/F_m values.

7.3. Influence of cAg NPs on F_q'/F_m'

Figures 7.3 and 7.4 show the impact of different concentrations of cAg NPs and Ag NO_3 on F_q'/F_m' at 100 and 500 PPFDs, for treated and control plants. At both PPFDs, the F_q'/F_m' of *L. minor* was not significantly impacted by cAg NPs after exposure for 96 h. In contrast, exposure to $AgNO_3$ at 50 and 100 mg/L caused inhibition of F_q'/F_m' at both PPFDs within 48 h, whilst at 12 mg/L caused a significant decrease at PPFD of 500 µmol m^{-2} s^{-1} (P = 0.004).

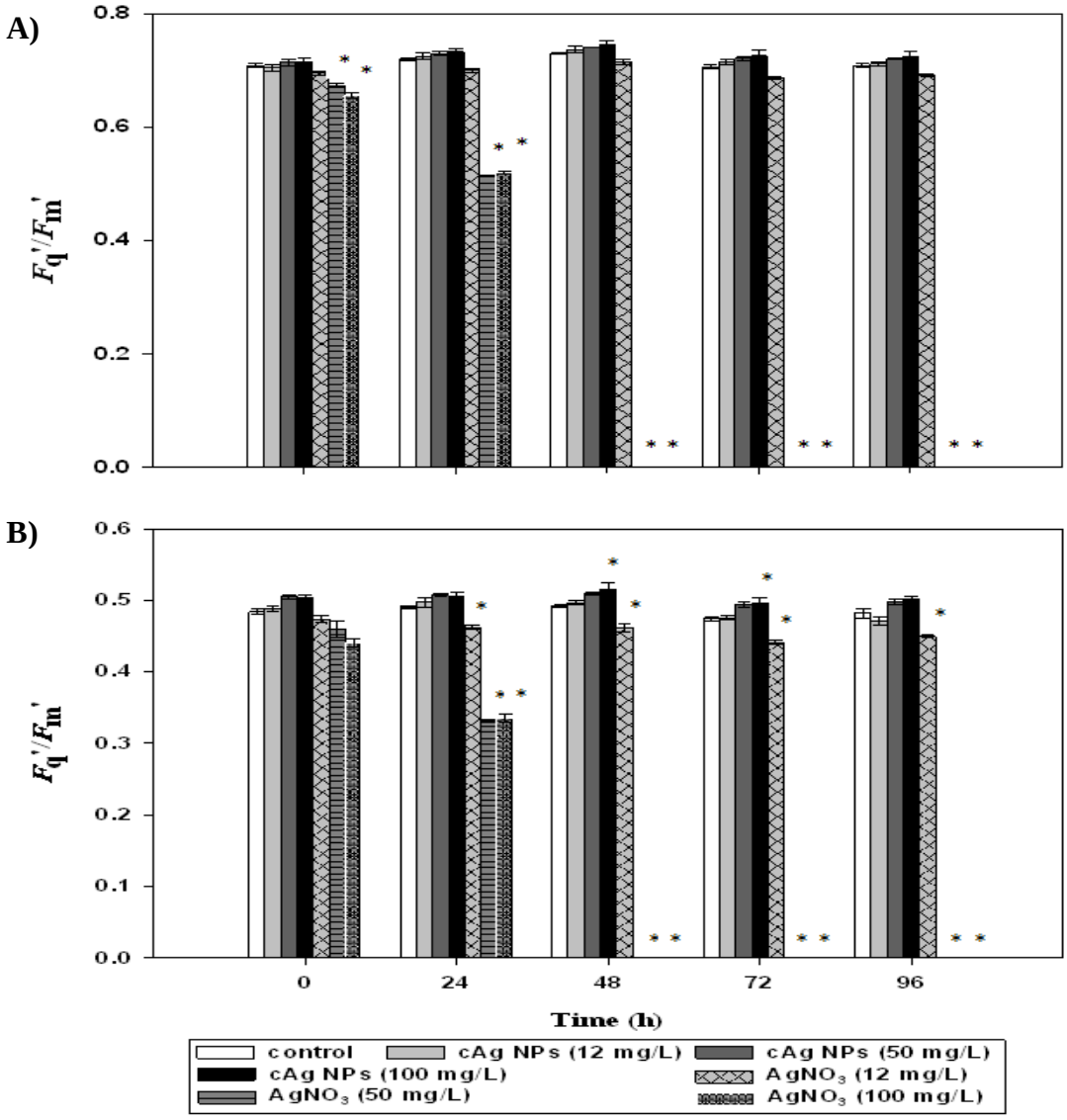

Fig. 7.3. Response of the chlorophyll fluorescence parameter F_q'/F_m' at PPFDs of 100 (A) and 500 (B) µmol m^{-2} s^{-1} when *L. minor* was exposed to cAg NPs and $AgNO_3$ at 12, 50, and 100 mg/L. The values given are mean ± SE (n=4). Comparisons between treatments were performed using one way ANOVA ($P < 0.05$) and significant differences are marked with '*'.

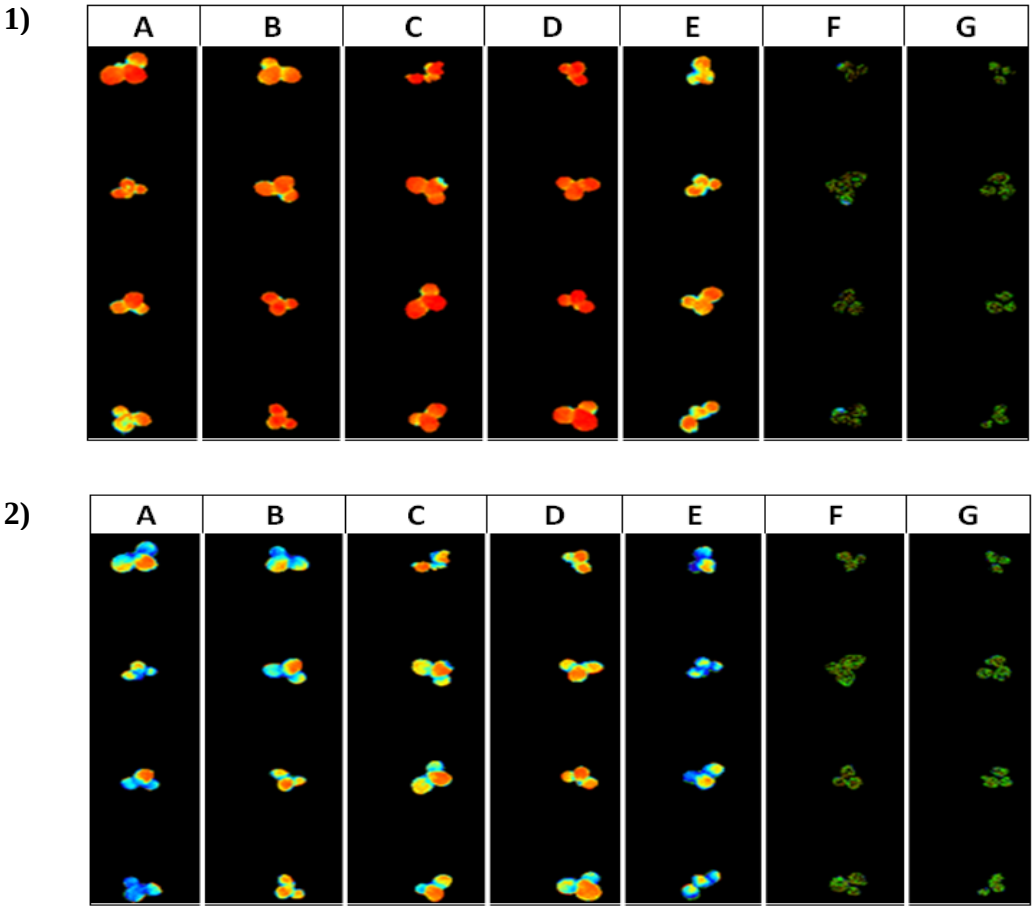

Fig. 7.4. Visual presentation of the impact of control (A) and cAg NPs at 12 (B), 50 (C), and 100 (D) mg/L and $AgNO_3$ at 12 (E), 50 (F), and 100 (G) mg/L on *L. minor* after 24 h represented by changes in the colour of F_q'/F_m' at PPFDs of 100 (1) and 500 (2) µmol m^{-2} s^{-1}.

7.4. Discussion

The release of ENPs into aquatic ecosystems could have serious implications for the equilibrium of such systems (Santos *et al.*, 2013). Some of ENPs will be rapidly taken up by organisms within the water column, others will become incorporated into the sediment (Bour *et al.*, 2015). Thus, ENPs have the potential to interact with aquatic plants and may affect them. At present, however, little is known about the influence of Ag NPs on the photosynthetic performance of aquatic plants.

In the present study, the photosynthetic performance of *L. minor* was assessed following exposure to cAg NPs, $AgNO_3$, and mPEG at different concentrations (up to 100 mg/L). The results demonstrated that cAg NPs had no toxic impact on F_v/F_m and F_q'/F_m'. In agreement with these results, Wang *et al.* (2008) found no significant effect of TiO_2 NPs (up to 100 mg/L) on F_v/F_m and F_q'/F_m' of *C. reinhardtii* over a 24 h period. Even at higher concentrations up to 1000 mg/L, Juhel *et al.* (2011) demonstrated no effect of Al_2O_3 NPs (at 10 and 1000 mg/L) on F_v/F_m in *L. minor*.

In contrast to the results of the present study, several studies have shown inhibitory effects of ENPs on the photosynthetic performance of aquatic plants. In one study that investigated the impact of Ag NPs at 5 mg/L on *S. polyrhiza*, Jiang *et al.* (2012) observed a significant decrease in F_v/F_m. In comparative study, Kim *et al.* (2011) demonstrated that *Lemna paucicostata* was more sensitive towards Ag NPs (at concentrations $\geq$ 1 ppm) than TiO_2 NPs (at concentrations $\geq$ 250 ppm). In a recent study, Oukarroum *et al.* (2015) reported that NiO NPs especially at a high concentration of 1000 mg/L caused a significant decrease in the F_v/F_m of *L. gibba*. Also, Perreault *et al.* (2014) noticed that lower formation of ROS in *L. gibba* was induced when CuO NPs, Cu ions or, the capping agent (polymer emulsion) applied alone. However, when CS-CuO NPs applied (at

400 mg/L) the formation of ROS was increased, causing higher toxicity on F_q'/F_m'. The authors therefore concluded that surface properties may change the nature of ENPs toxicity. Thus, the contrasting results between studies could be attributed to variations in ENPs type and physicochemical properties. Such variations have been highlighted to cause different toxicities towards plants (Juhel *et al.*, 2011).

The lack of toxicity observed in the present study may be attributed to the possibility that once cAg NPs were added to the medium growth they undergo a sedimentation leading to a decrease in Ag dissolution and subsequently a reduction in cAg NPs toxicity. Sedimentation is a key factor which can control the fate of ENPs in aquatic environments (Markus *et al.*, 2015), as up to 75% of ENPs may sediment (Zhang *et al.*, 2012). Previous studies have shown that the dissolution of ions from ENPs is dependent on the form of ENPs in the aquatic suspension. For example, Oukarroum *et al.* (2013) demonstrated that when Ag NPs (up to 10 mg/L) were added to growth medium, rapid agglomeration occurred, resulting in a low amount of Ag ions being released. Similarly, Gubbins *et al.* (2011) observed a dramatic increase in agglomeration of two different sizes of Ag NPs (from 14 nm to 130 nm, and from 97 nm to 700 nm) after being interacted with the medium solution. Santos *et al.* (2013) also observed that the addition of C_{60} NPs in Hutner's medium enhanced their agglomeration and thereby promoted larger clusters. Thus, analysing the properties of ENPs during experimental exposure is of great importance in order to understand to what extend the prosperities of ENPs were changed.

In contrast to cAg NPs, $AgNO_3$ at 50 and 100 mg/L completely inhibited F_v/F_m and F_q'/F_m', indicating the fundamental role of dissolved ions in Ag NPs toxicity. Indeed, heavy metal ions have been shown to be toxic to plants (Boucher and Carpentier, 1999; Giardi *et al.*, 2001; Vassilev

et al., 2002; Han *et al.*, 2008; Parmar *et al.*, 2013). In support of this, Jiang *et al.* (2012) reported that $AgNO_3$ at a concentration of 5 mg/L significantly decreased F_v/F_m of *S. polyrhiza*. Whilst Lee and An (2013) observed an inhibitory effect when the green algae *Pseudokirchneriella subcapitata* exposed to ZnO NPs and it was entirely attributed to the dissolved Zn ions. The inhibitory function of metal ions lies in their ability to form ROS, which have been reported to cause oxidative stress and may destroy the integrity of plant cells (Oukarroum *et al.*, 2013).

Coating agents are widely applied in order to increase the stabilization of ENPs (Perreault *et al.*, 2014). However, such agents could make ENPs toxic substances which have, for example, been shown to have adverse effects on seed germination and plant growth (Stampoulis *et al.*, 2009). In the present study, when *L. minor* was exposed to mPEG alone, no toxic impacts were observed (Appendix D). Similarly, Beddow *et al.* (2014a) did not observe any detrimental effects on the growth of *E. coli* and *B. subtilis* exposed to mPEG alone, whilst growth was significantly negatively affected by the cAg NPs. In addition, the capping agent citrate (used to cap Ag NPs) had no effect on the growth of mung beans (Lee *et al.*, 2012).

In conclusion, the present study confirmed that the toxicity of cAg NPs towards *L. minor* can be mainly attributed to the dissolved ions, as neither cAg NPs nor the mPEG capping agent demonstrated any toxicity towards the photosynthetic performance of *L. minor* compared to $AgNO_3$, which was highly toxic. Additionally, this study provides evidence that Chlorophyll *a* fluorescence imaging is a reliable tool that may be used to assess the potential risk of toxicants to aquatic plants. Importantly, increasing amounts of ENPs are expected to enter the aquatic environment in the near future and thus information about the toxicity of higher concentrations in addition to several types of ENPs on aquatic plants is urgently needed.

Chapter 8. Conclusion and future work

Nanotechnology has grown rapidly in the last decade with a wide range of different applications and products being industrially developed and provided today (Petersen *et al.*, 2014). There is no doubt that nanotechnology has the potential to improve the environment, for example, through the removal of pollutants, in the design of cleaner industrial processes, and by the creation of environmentally responsible nanotechnological products (Brar *et al.*, 2010). Despite this, there is a risk to the environment as a consequence of the release of ENPs, from nanotechnological products, into the environment (Gottschalk and Nowack, 2011). Measurable concentrations of ENPs are expected to be found in terrestrial, atmospheric, and aquatic environments in the near future (Nowack and Bucheli, 2007; Wang *et al.*, 2008a). Consequently, ENPs are often viewed as novel toxicants that may impact living organisms, including plants. Due to their small size, ENPs have a large surface to volume ratio and thus behave differently to larger materials of the same type. Hence, they have a greater chance to interact and enter plants. ENPs have been reported to accumulate in plants and thus may modify food quality and quantity (Gardea-Torresdey *et al.*, 2014; Zhao *et al.*, 2015). Furthermore, the potential for plant-accumulated ENPs to be used for human consumption is not excluded, which could result in health risks (Larue *et al.*, 2014).

Whilst products containing Ag NPs represented a total of 313 items of all nanotechnological products recorded in 2011 (Larue *et al.*, 2014), by 2015 this had increased to 442 products, or 24% of all recorded products (Woodrow Wilson database, 2015), implying a preference for use of Ag NPs in commercial applications and products over other ENPs. Acknowledging the uncertainty of Ag NPs toxicity and the urgent need to assess their ecotoxicological impacts, the aim of this study was to investigate the impact of capped silver

nanoparticles (cAg NPs) on plants. Due to the important role of soil microbes in plant survival and growth, the influence on plant-associated soil microbial community structure was also analysed.

The importance of this study comes from several points. It is one of the first studies to consider the impact of one type of ENP (i.e. cAg NPs) on different plants, including both terrestrial and aquatic species, using multiple techniques. Moreover, it covers all possible interactions and entry pathways including seeds, roots, and leaves. Most previous studies have investigated the impact of ENPs on certain endpoints such as seed germination and root length (Lin and Xing 2007; Wang *et al.*, 2012a; Thuesombat *et al.*, 2014), whilst little attention has been given to the impact on photosynthetic performance. Furthermore, even when photosynthetic performance has been considered, it has been conducted in isolated chloroplasts (Lei *et al.*, 2007a; Lei *et al.*, 2007b; Giraldo *et al.*, 2014), whilst in the present study it was conducted on attached leaves. It could be argued that the process of chloroplast isolation may modify photosynthetic performance even before exposure to ENPs, whereas investigating photosynthesis with chlorophyll in its original state could provide more realistic information about the impact of ENPs on photosynthesis.

Where the aim of Chapter 3 was to evaluate the impact of cAg NPs on seed germination and assess any downstream effects on plant growth, results demonstrated that seed germination of *A. thaliana* significantly decreased following exposure to cAg NPs. This toxicity was shown to be concentration dependent, as toxicity was only observed above 12 mg/L. As $AgNO_3$ demonstrated greater toxicity compared to cAg NPs, the toxicity of cAg NPs was attributed to the released Ag ions. Whilst it is known that Ag ions acts as an ethylene inhibitor (Locke *et al.*, 2000; Turhan, 2004; Chaudhuri and Kar, 2008), ethylene is an important factor in promoting seed germination (Hermann *et al.*, 2007; Lin *et al.*, 2013). Additionally, the photosynthetic performance of *A.*

thaliana, germinated for three weeks after seeds exposure, showed no toxicity. These unsurprising results were related to the well-established root system, activation of protective mechanisms and/or cAg NPs entered seeds remained trapped in roots without any effect on the activity of the plants (Parsons *et al.*, 2010; Larue *et al.*, 2012; Thuesombat *et al.*, 2014).

The application of cAg NPs (up to 50 mg/kg) to soil was carried out, with the aim to elucidate soil microbial responses to cAg NPs and investigate any related effects on the photosynthesis of plants. Results demonstrated however, no impact on soil microbial community structure or the photosynthetic performance of *V. faba* (Chapter 4). Moreover, $AgNO_3$ at the same concentrations also had no effect. It is thought that humic acids and sulphur compounds found in soil may play a role in reducing the toxicity of cAg NPs towards the microbial community through increased aggregation of Ag NPs and/or interaction of Ag ions with chloride ions, forming less-toxic Ag complexes (Chio *et al.*, 2009; Liu and Hurt, 2010; Calder *et al.*, 2012; Beddow *et al.*, 2014b). Additionally, Ag-resistance genes exist in some environmental bacteria (Silver, 2003) and it is not excluded that such strains were present in the soil used in this study, which would also help to explain the lack of toxicity observed (Beddow *et al.*, 2014b). The plant root system represents a crucial point of interaction and entry for ENPs. However, a low bioavailability of ENPs in soil (Asli and Neumann, 2009) in addition to a weak water uptake capacity of plants (Schwade *et al.*, 2013; Zaho *et al.*, 2015) may explain why no impacts by cAg NPs were observed on photosynthetic performance herein. Importantly, in the present study single application of cAg NPs was performed. However, the addition of ENPs to agricultural soil may occur multiple times and so commutable effects on soil microbes and plants are possible. Therefore, the impact of repeated application of ENPs on soil microbes and plants needs further investigation.

Foliar interaction of ENPs with plants is inevitable and may lead to the penetration of ENPs into internal tissues. The impact of foliar exposure of cAg NPs by injection, spray, and deposition methods were applied, with the aim of assessing any impacts on the photosynthetic performance of *V. faba.* At the same time, the role of light in cAg NPs toxicity was investigated. Results from the injection study demonstrated that cAg NPs at 12 mg/L had no effect on *V. faba* (Chapter 5). However, at higher concentrations (50 and 100 mg/L), cAg NPs significantly decreased chlorophyll fluorescence and gas exchange parameters. This toxicity was thought to be related to the released Ag ions, which can cause the formation of ROS (Jiang *et al.*, 2014). Although obtaining a mechanistic understanding of the role of ROS generation in cAg NP toxicity on the photosynthetic performance of *V. faba* was one of the primary aims in this study, the AR probe was not sensitive enough, especially at higher cAg NPs concentrations, to clarify to what extent ROS formation played a role in cAg NPs toxicity. Therefore, further studies are needed to elucidate the exact roles of ROS in cAg NPs toxicity. Meanwhile, the use of an alternative probe such as DCFH-DA is suggested to be considered in future studies. Changes in the transport of electrons from chlorophyll to cAg NPs may occur (Falco *et al.*, 2015), which could also help to explain the observed decrease in photosynthetic performance. Interestingly, in some instances, plants showed some level of recovery, indicating that plants may be able to activate protection mechanisms in order to cope with cAg NPs. In addition to concentration and time, this study demonstrated the role of light in enhancing the toxicity of cAgNPs. When plants were grown in a high level of light, a faster decrease in photosynthetic performance by cAg NPs was observed compared to growth in a low level of light. Light has previously been suggested to play a role in ENPs toxicity (Ma *et al.*, 2014) by facilitating dissolution and the consequent formation of ROS (Santos *et al.*, 2013).

The main aim of Chapter 6 was to investigate the impact of foliar spray and deposition of cAg NPs on the photosynthetic performance of *A. thaliana* and *V. faba*. However, in contrast to the results of Chapter 5, when foliar exposure was applied by spray and deposition, the cAg NPs had no effect on photosynthetic performance in *A. thaliana* and *V. faba*. In support with this finding, Qi *et al.* (2013) reported no disruptions to photosynthetic performance in tomato leaves exposed aerially to TiO_2 NPs, at concentrations of 0.05 and 0.1 g/L. In general, the toxicity of foliar-applied, by means of spray and deposition, cAg NPs may be influenced by several factors including leaf morphology, the amount of wax and exudates present on leaves, the density and morphology of trichomes, any microorganisms present on leaf surface, the physicochemical properties of the ENPs themselves (Larue *et al.*, 2014), stomatal aperture density (Eichert *et al.*, 2008), and working environment (Wang *et al.*, 2013b). Additionally, repeated exposure to ENPs, relevant to the environment, is also an important factor which can affect plant activity. Thus, application method needs to be considered when interpreting the toxicity of ENPs.

Aquatic plants are also at risk of interaction with ENPs (Oukarroum *et al.*, 2013), thus the aim of Chapter 7 was to investigate the impact of cAg NPs on *L. minor* by monitoring changes in photosynthetic parameters during cAg NPs exposure. Results demonstrated that exposure of *L. minor* to cAg NPs at concentrations of 12, 50, and 100 mg/L did not cause any significant impact on photosynthetic performance. This study confirmed that the toxicity of cAg NPs can be mainly attributed to the dissolved ions, as $AgNO_3$ at high concentrations (up to 100 mg/L) was highly toxic and completely inhibited plants. Nevertheless, it is recommended that ENPs toxicity towards aquatic plants requires further investigation, including exposure to different concentrations in addition to several types of ENPs.

The production of ENPs is predicted to increase (Navaaro *et al.*, 2008) and so it is possible that ENPs will be intentionally and/or unintentionally released into the environment in high concentrations especially in the event of a spillage. Additionally, new nanotechnological products with novel release properties that are not yet available, could lead to significantly greater exposure (Gottschalk *et al.*, 2009). Thus, further ecotoxicological studies of ENPs on plants, with a focus on crop plants, are urgently needed. These studies should acknowledge new techniques which cover both exposure and assessment methods. For example, aerosolization of ENPs, thermal imaging, and stomatal behaviour, in addition to those applied in the present study should be considered in future studies. Furthermore, the impact of cAg NPs on plant hormones and genes that participate in photosynthesis could be included.

In summary, laboratory controlled-studies are essential in order to assess the potential impacts of ENPs on plants (Bour *et al.*, 2015). In this study, multiple techniques were applied and different end points were considered in order to obtain detailed information about the responses of plants to cAg NPs. The results of this study show that concentration, exposure method, released ions, plant species, light intensity, and growth mediums are key factors that influence the toxicity of cAg NPs. Nevertheless, the behaviour, location, and state of cAg NPs during interaction with biological matter represent areas that require further investigation. The results of this study could help to establish a threshold for the toxic concentration of cAg NPs to plants. Additionally, they can be used to inform the direction in future studies. The injection method applied herein may be used to understand to what extent plants can tolerate ENPs once inside plants. However, great care should be taken with this method as the technique itself can lead to disruption in plant performance. This study also demonstrates that chlorophyll fluorescence imaging and gas exchange are very sensitive and useful tools that can be applied in nanotoxicological studies. Finally, there is

no uniform conclusion at the present time about the extent of Ag NPs toxicity to plants, with many contrasting results observed in the literature. Thus, it is suggested that caution must be taken in regard to the use and production of Ag NPs in industrial sectors.

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Appendices

APPENDIX A: Assessment of cAg NPs effect on intercellular carbon dioxide concentration (C_i) in *V. faba*

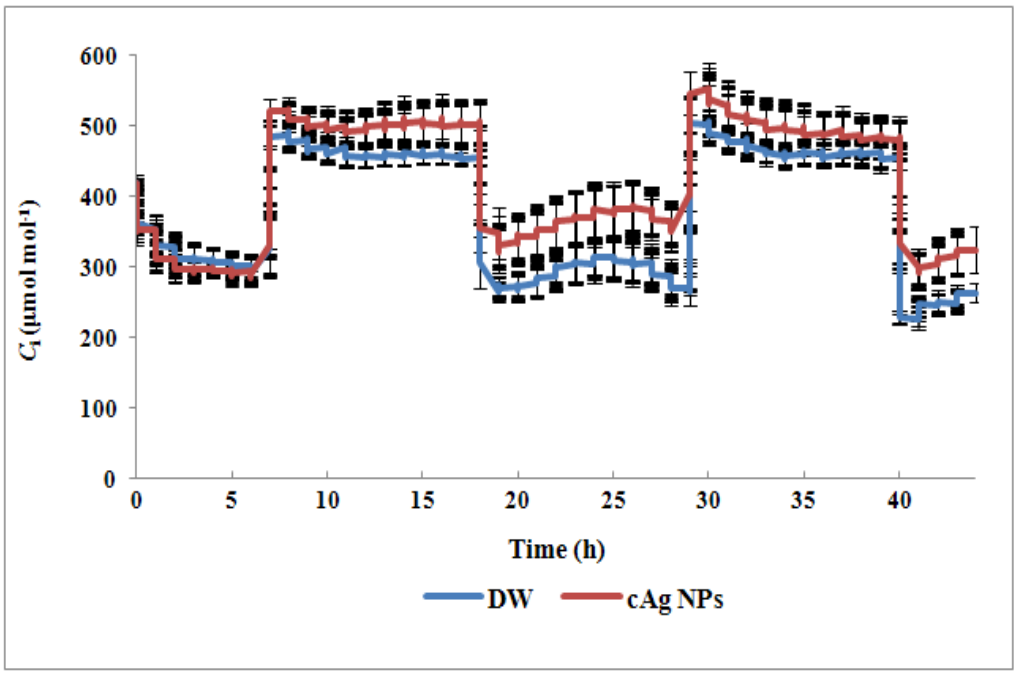

Fig. A1. Response of C_i to injected cAg NPs at 100 mg/L.

APPENDIX B: Impact of the injected-capping agent (mPEG) on the photosynthetic performance in *V. faba*

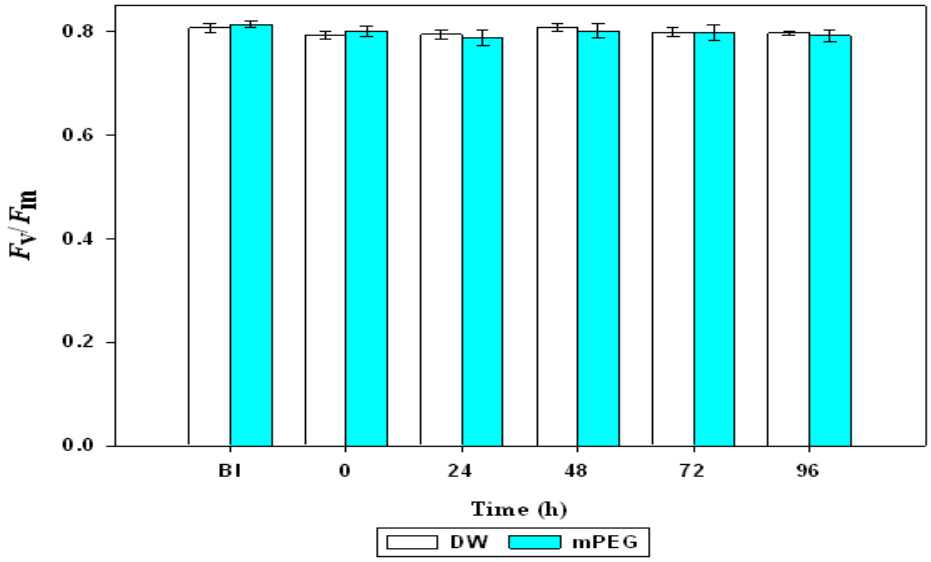

Fig. A2. Impact of injected mPEG at 12 mg/L on the chlorophyll fluorescence parameter F_v/F_m.

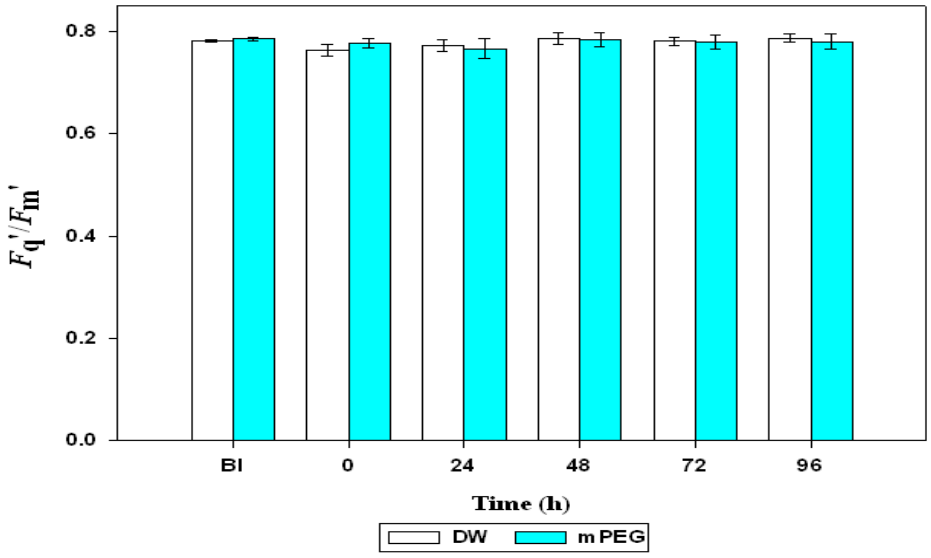

Fig. A3. Impact of injected mPEG at 12 mg/L on on the chlorophyll fluorescence parameter F_q'/F_m' at 100 µmol m^{-2} s^{-1} PPFD.

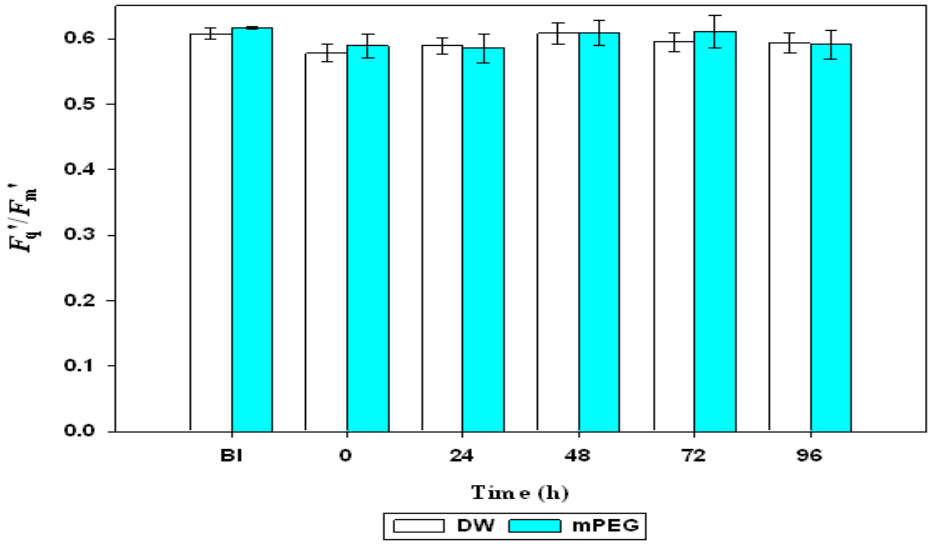

Fig. A4. Impact of injected mPEG at 12 mg/L on the chlorophyll fluorescence parameter F_q'/F_m' at 500 µmol m^{-2} s^{-1} PPFD.

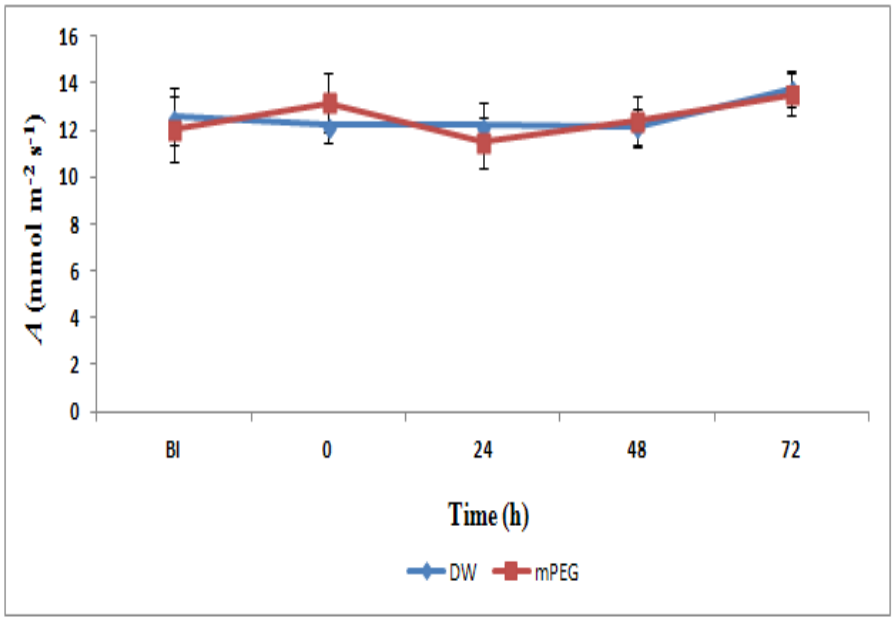

Fig. A5. Response of *A* to injected mPEG at 12 mg/L.

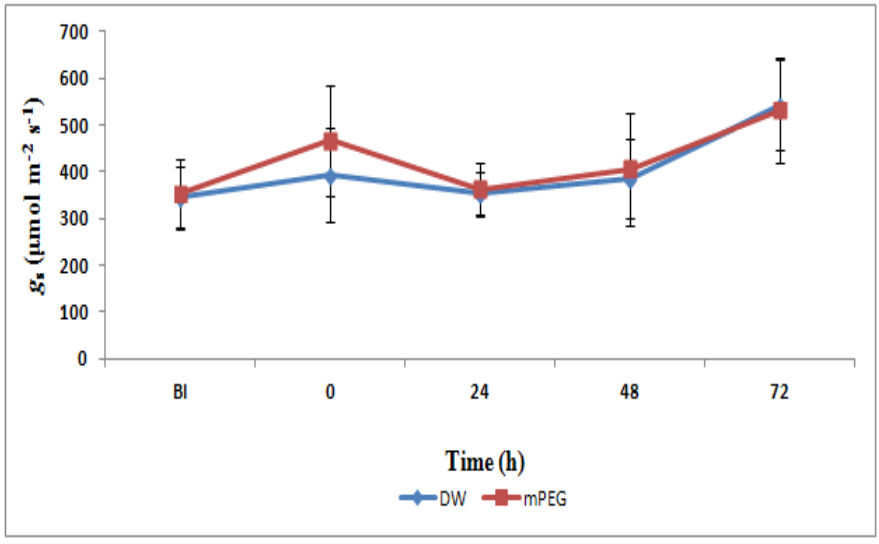

Fig. A6. Changes in g_s induced by injected mPEG at 12 mg/L.

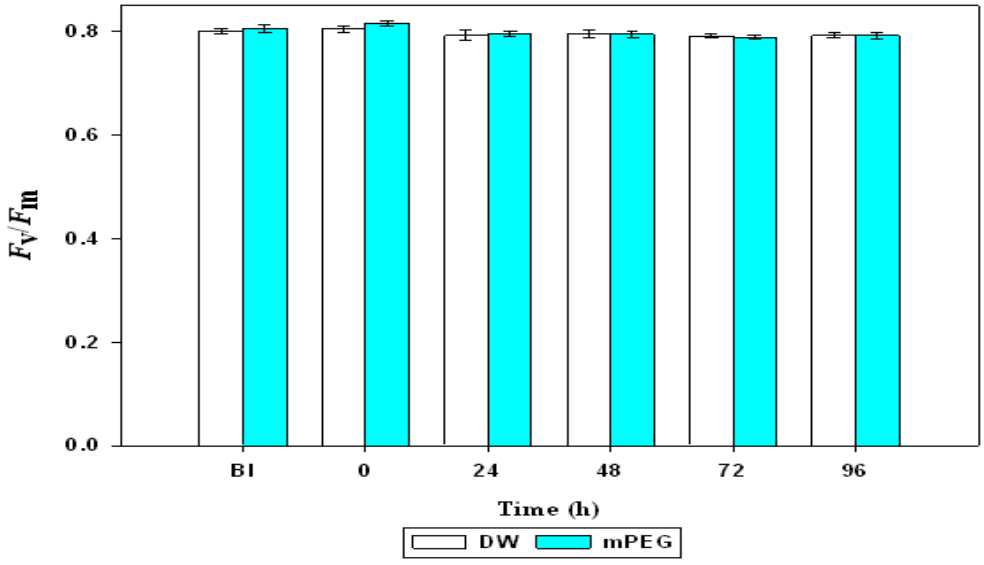

Fig. A7. Effect of injected mPEG at 100 mg/L on the chlorophyll fluorescence parameter F_v/F_m.

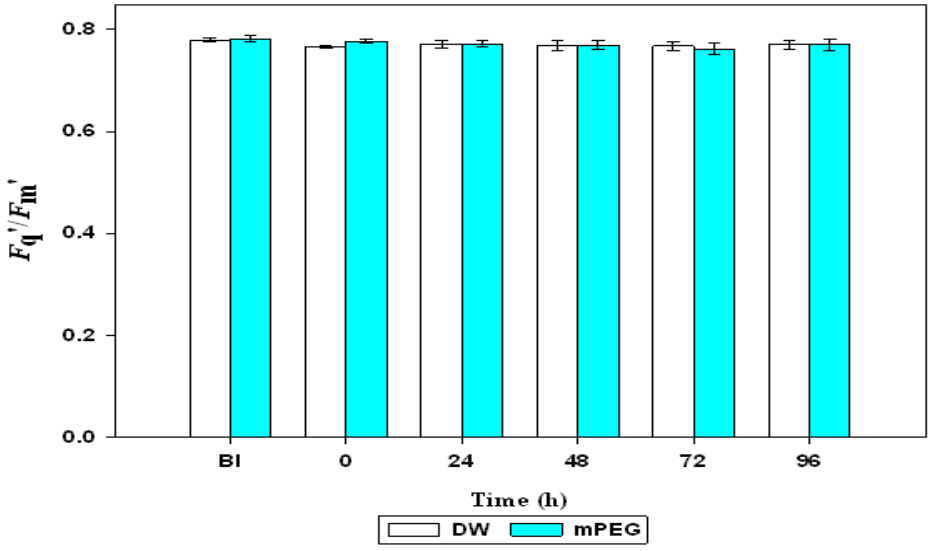

Fig. A8. Impact of injected mPEG at 100 mg/L on the chlorophyll fluorescence parameter F_q'/F_m' at 100 µmol m^{-2} s^{-1} PPFD.

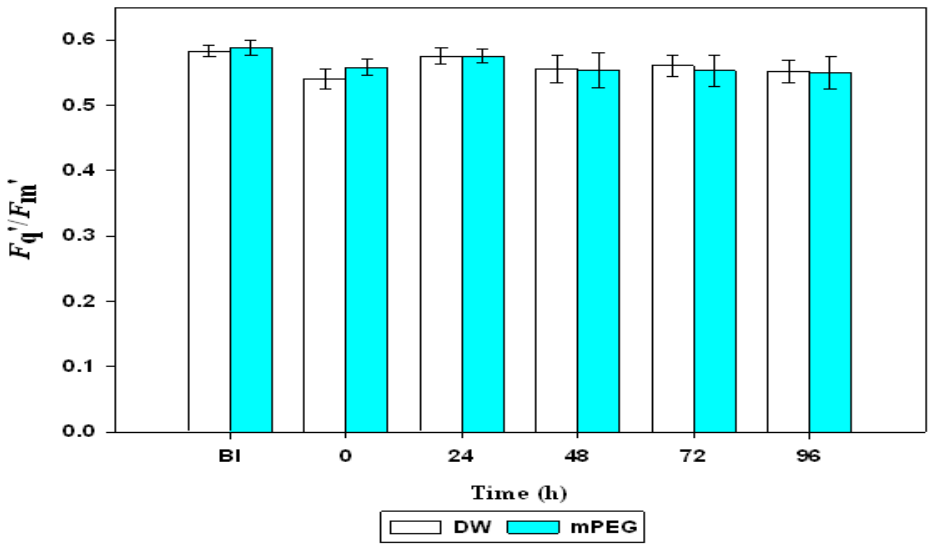

Fig. A9. Influence of injected mPEG at 100 mg/L on the chlorophyll fluorescence parameter F_q'/F_m' at 500 µmol m^{-2} s^{-1} PPFD.

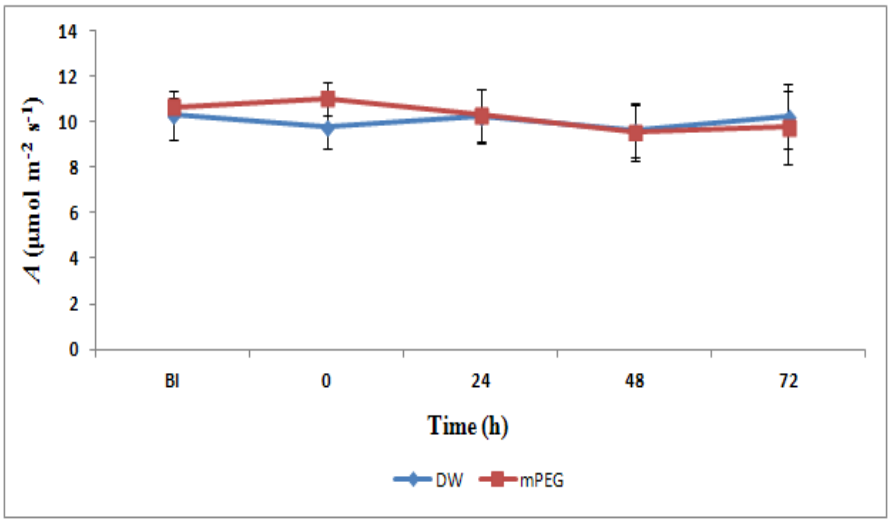

Fig. A10. Changes in *A* induced by injected mPEG at 100 mg/L.

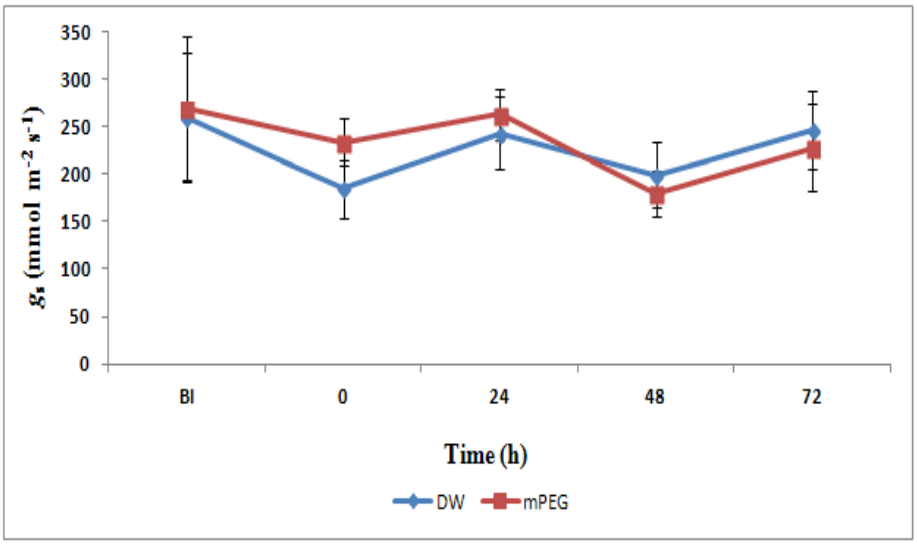

Fig. A11. Response of g_s to the injected mPEG at 100 mg/L.

APPENDIX C: Influence of the deposition and spray of cAg NPs on the photosynthetic performance in *V. faba*

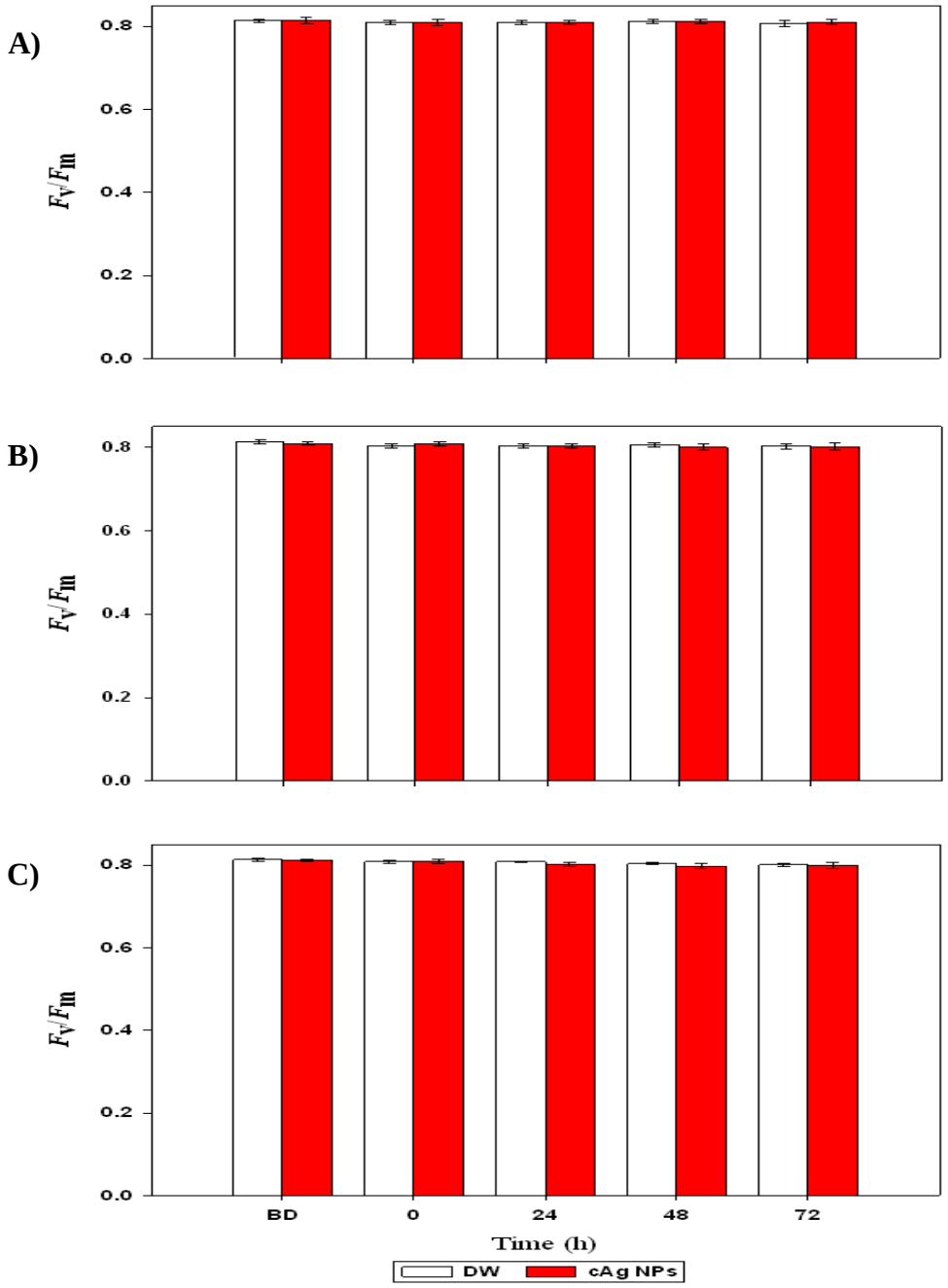

Fig. A12. Effect of deposited cAg NPs at 12 (A), 50 (B), 100 (C) mg/L on the chlorophyll fluorescence parameter F_v/F_m.

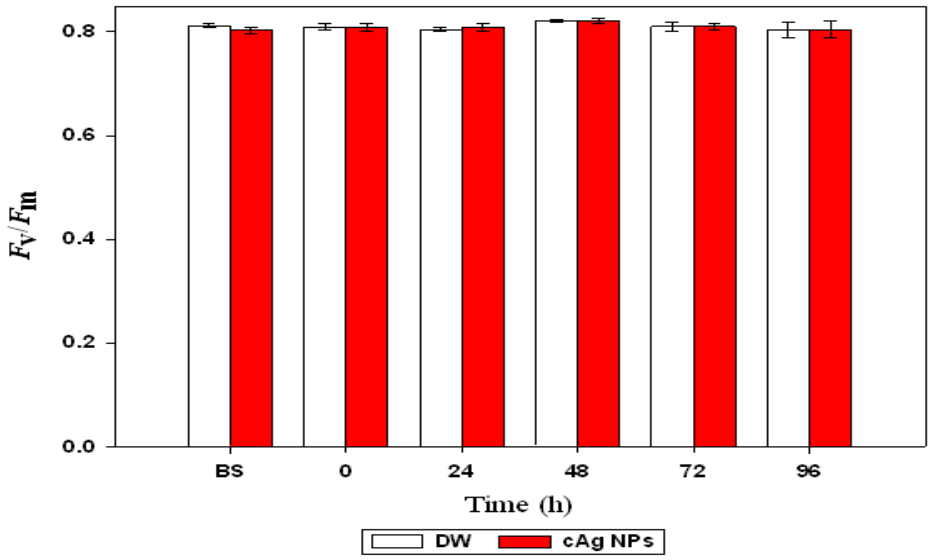

Fig. A13. Effect of sprayed cAg NPs at 100 mg/L on the chlorophyll fluorescence parameter F_v/F_m.

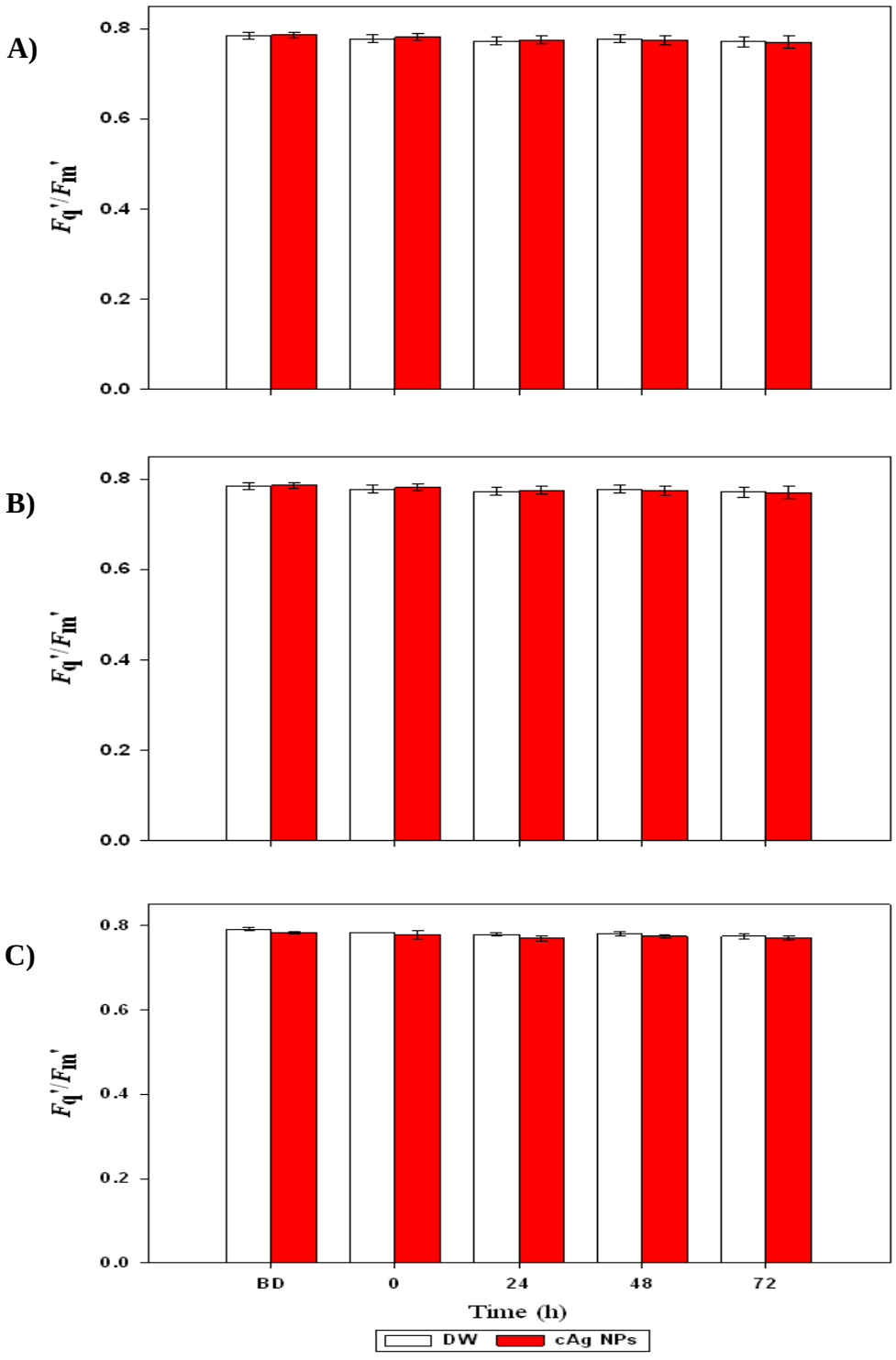

Fig. A14. Impact of deposited cAg NPs at 12 (A), 50 (B), and 100 (C) on the chlorophyll fluorescence parameter F_q'/F_m' at 100 µmol m^{-2} s^{-1} PPFD.

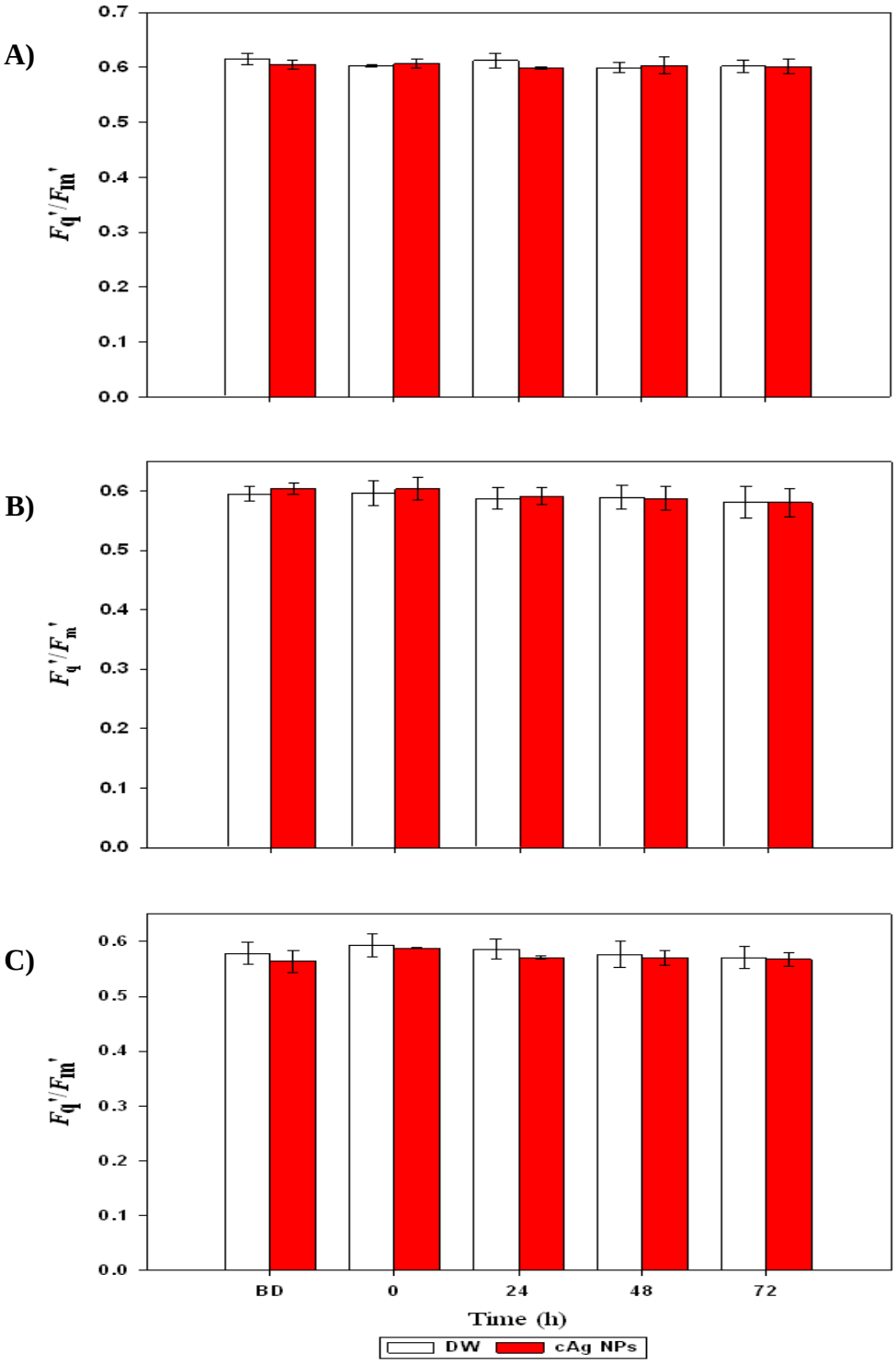

Fig. A15. Effect of deposited cAg NPs at 12 (A), 50 (B), and 100 (C) on the chlorophyll fluorescence parameter F_q'/F_m' at 500 µmol m^{-2} s^{-1} PPFD.

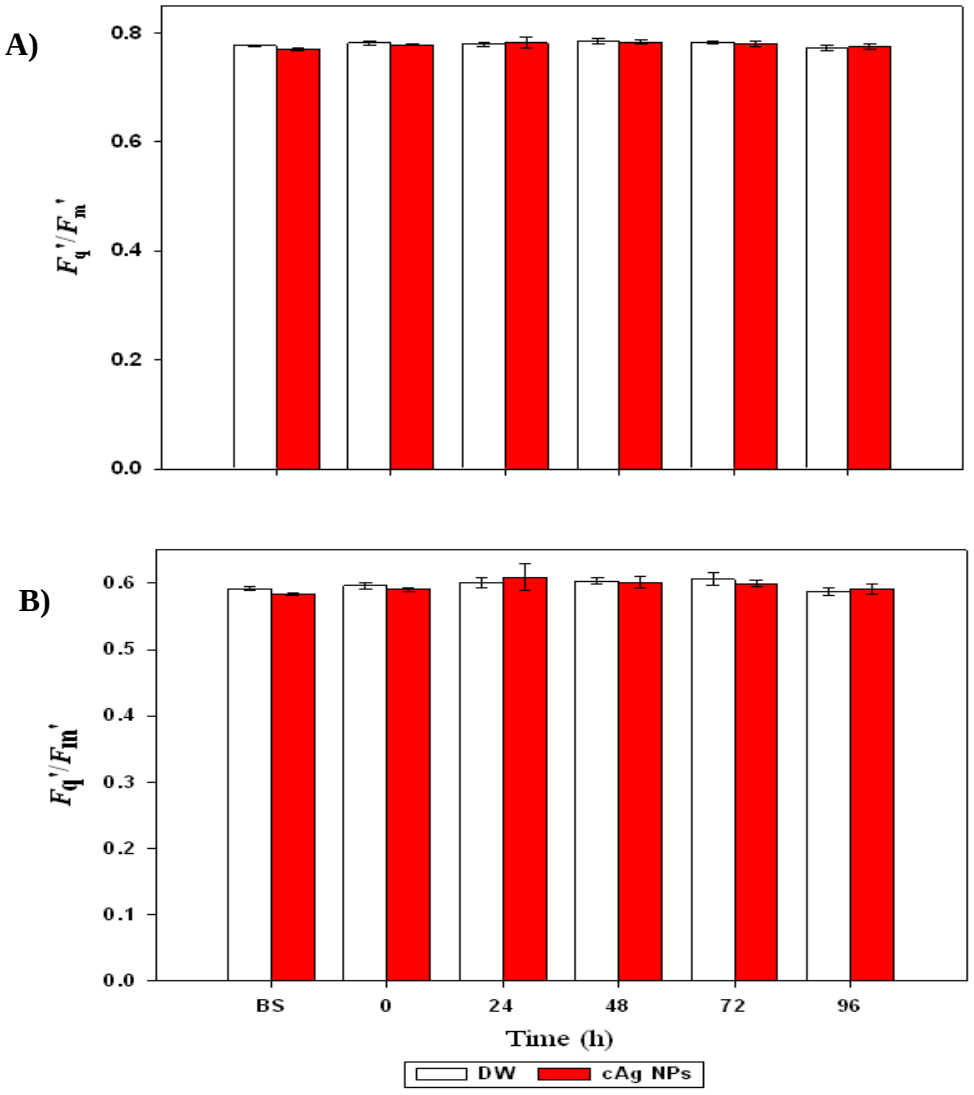

Fig. A16. Effect of sprayed cAg NPs at 100 on the chlorophyll fluorescence parameter F_q'/F_m' at 100 (A) and 500 (B) µmol m^{-2} s^{-1} PPFD.

APPENDIX D: Effect of the capping agent on the photosynthetic performance in *L. minor*

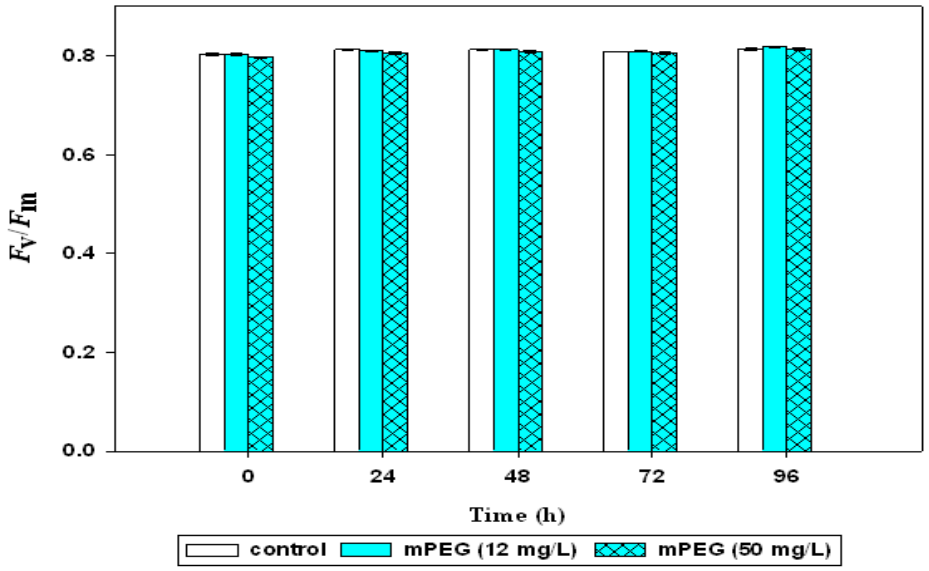

Fig. A17. Impact of mPEG on the chlorophyll fluorescence parameter F_v/F_m.

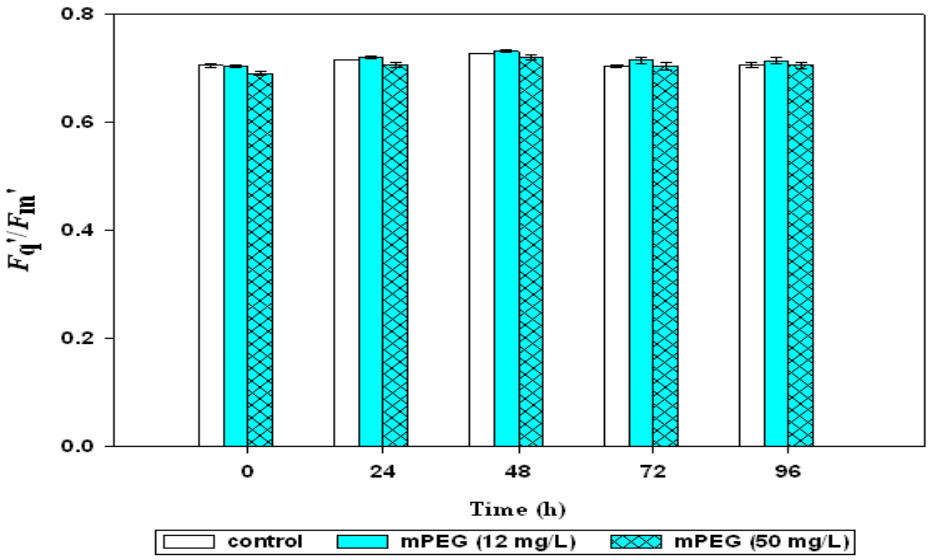

Fig. A18. Influence of mPEG on the chlorophyll fluorescence parameter F_q'/F_m' at 100 µmol m^{-2} s^{-1} PPFD.

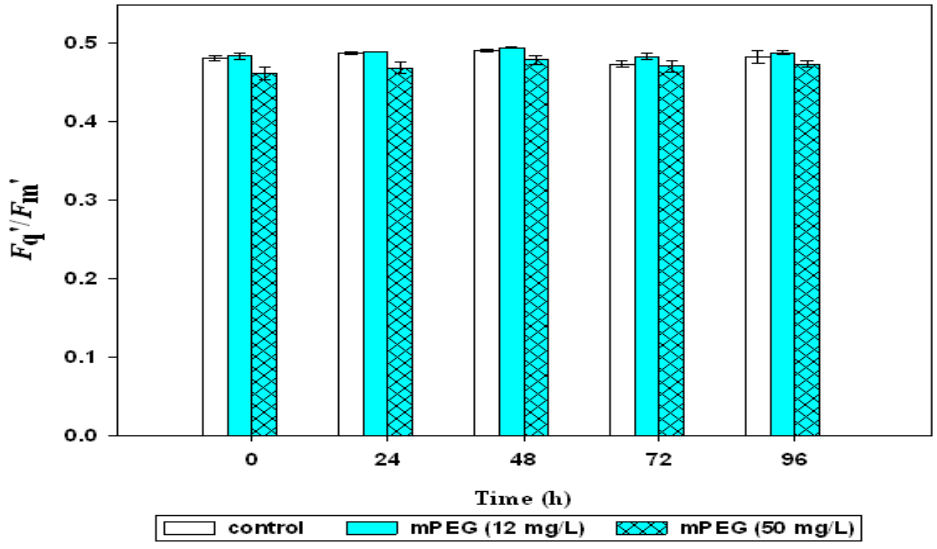

Fig. A19. Impact of mPEG on the chlorophyll fluorescence parameter F_q'/F_m' at 500 µmol m^{-2} s^{-1} PPFD.