Foliar penetration of silver nanoparticles in 3 tree species: role of macro and micro-structures of leaves in foliar penetration

GEO 511 Master's Thesis

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Abstract

Particles between 1 and 100 are called nanoparticles or ultrafine particles. Nanoparticle research is currently an area of intense scientific interest driven by the desire to fabricate materials with novel and improved properties due to a wide variety of potential applications in the areas of physical, chemical, biological, and health sciences and other interdisciplinary fields of science and engineering. The main characteristic of silver nanoparticles is the high surface area to volume ratio, allowing the nanostructure to be extensively exploited in several areas such as biotechnology, electronics, medicine, environmental remediation, biosensors, agriculture, and the food industry. The extensive use of silver nanoparticles is reflected in numbering of worldwide suppliers, and the global consumption of these nanoparticles in electrical, healthcare, food, and textile. Once released into the environment, the formation of surface water on the nanoparticle surface is considered one of the main points of entry, enhancing dispersion of the nanoparticles and establishing a link between silver nanoparticles and key environmental components, such as biota, sediment. Therefore, seedlings of beech, birch, and poplar were exposed to silver nanoparticles via leaf application. In birch and beech leaves there were significantly higher concentrations of Ag than in poplar.

Keywords: Silver nanoparticles (AgNPs), uptake via leaves, stomata

1. Introduction

Silver nanoparticles (AgNPs) are found to have antimicrobial properties and are therefore used in plant pest disease management. Although it kills approximately 650 types of pathogenic microbes such as bacteria, fungi, viruses, and yeasts and does not need much time to do so (Mishra and Singh, 2015) it can exert detrimental effects too. It is believed to be toxic against mammals, invertebrates, or micro-organisms, but the potential negative effects will depend on the sensitivity of each organism itself (Li et al., 2017; Tortella et al., 2020). Negative effects range from membrane damage, reactive oxygen species (ROS) denaturation, mitochondrial dysfunction, DNA damage, and inhibition of cell proliferation (G R Tortella et al., 2020). While the mechanisms of uptake via roots have undergone abundant studies the pollutant uptake via leaves is still under debate and since AgNPs enter the stem of trees faster via leaves rather than via roots (Cocozza et al., 2019) there is a need to further investigation in that field to uncover the underlying processes which are not understood in detail so far (Mishra and Singh, 2015). Plants are known to uptake particles and ions through cuticular pores and stomata in case of foliar exposure and since there is an increase of AgNPs emissions in the atmosphere adverse responses on plants and reduction of biomass and impacts on the photosynthesis system of plants are expected (Wu et al., 2020). Furthermore, the atmospheric deposition of metallic nanoparticles is a major contributor to the pollutant load of plants. This poses food safety at risk and as there is a potential transfer into the food chain human health and ecosystem quality are at risk (Kranjc et al., 2018; Vitali et al., 2019).

There is comprehensive knowledge on uptake via roots but scarce knowledge when it comes to leaf uptake of nanoparticles (NP). Moreover, different studies show controversial results about the leaf uptake because of the scarce knowledge. We know about humans` uptake but we do not know so much about trees. Additionally, NPs undergo an increased application and subsequent release into the environment, but their effect and presence in trees are largely unexplored (G. R. Tortella et al., 2020).

The aims of this study were to: a) investigate the fate of silver nanoparticles in trees after foliar application, b) investigate differences in silver uptake in three different tree species concerning: the macrostructure (size, shape, surface) and microstructure of the leaves (stomatal density, wax, trichomes, presences of hair), and c) determine the gas exchange rates (stomatal conductance, photosynthesis) and their influence on the Ag uptake by the leaves.

1.1 Research questions

- Are silver nanoparticles transported through the stomata inside the leaves?
- If yes, are there differences in the uptake about the leaf structure of different tree species?

1.2 Hypothesis

We hypothesize that nanoparticles will enter the leaves by their stomata which serve as a gas exchange from the leaves to the environment and vice versa (Halley and Meteorology, 1981). Nanoparticles are trapped by microstructures of the leaves that could facilitate their penetration through the stomata. Stomata are assumed to be open at high relative humidity; the higher the density of the stomata the higher maybe the uptake. Bigger stomata may allow more nanoparticle uptake than smaller ones.

Nanoparticles might be washed away by meteorological events or directly attached to the leaf because of the polarity of water and subsequent become transferred inside the leaf through the stomata.

2. Material and methods

2.1 Silver nanoparticle application

Leaves of three different tree species were investigated concerning AgNPs uptake. Therefore, 10 two-year-old seedlings of beech (*Fagus sylvatica L.*), birch (*Betula pendula L.*), and, poplar (*Populus L.*) were in winter 2019 planted in 12 cm diameter pots with a mixed soil substrate (‘Containererde’, Ökohum GmbH, Switzerland) and kept under natural conditions (80 % RU, 25-30° C) in a greenhouse at the WSL (Birmensdorf, Switzerland) (47°21'16" N, 8°26'16" E; 518 m asl). Since the humidity during the application of Ag was kept between 90-100%, stomata were not put under dryness pressure. As a consequence, all stomata should be fully opened. Right from the beginning, 5 trees of each species were separated and so became the control group, while on the remaining five trees AgNPs were applied. On the abaxial side of 10 leaves per tree, AgNPs from the company Nanocomposix (San Diego) with the size of 40 nm ± 5 nm were applied. Applicated by a syringe, 20 drops of 1-2 µL ultrapure MilliΩ water and within mixed AgNPs (50 mg AgNPs /L ultrapure MilliΩ water) were given along the midrib and along the first lateral veins. Same procedure was done for birch (*Betula pendula L.*).

However, poplar was poor on leaves. Compensating that issue, only 5 leaves instead of 10 leaves per tree were treated but with the double amount of (40) drops. Simplifying the application, tree seedlings were put upside down. Covered previously by a Parafilm layer, contamination of leaves by eventually dropping soil was inhibited (Figure 1). Short after the application, the surfaces of the treated leaves were dry and the application spots were visible. Shortly thereafter, an additional application on the same visible spots without AgNPs but only ultrapure MilliΩ water was given. Thus, enhancing a possible further uptake of the previous applicated AgNPs. Once a day, this routine was carried out from 03.08.2020 to 13.08.2020 (ten times in total). Subsequently, the treated leaves were collected and oven-dried at 105°C for 24 h. Total dry biomass weight (DW, mg) of leaves was measured.

Figure 1: Sampling trees in the greenhouse. Silver nanoparticles were applied on the lower side of the leaves by holding the plants upside down. Therefore, pots were sealed by a Parafilm layer

2.2 Stomatal density

Since the focus lies on the stomata, their amount has to be evaluated. Taken 12 untreated control plants of beech, birch, and poplar, one leaf from each tree was harvested. Subsequently, nail varnish was applied on the lower and upper sides of the sampling leaves. Then the transparent adhesive tape was put on the parts, where the varnish was applied. By pulling away the adhesive tape from the leaf, an imprint was generated which subsequently was affixed to a microscope glass. One microscope glass contained both, the imprint of the lower and the upper side of one leaf (Figure 2).

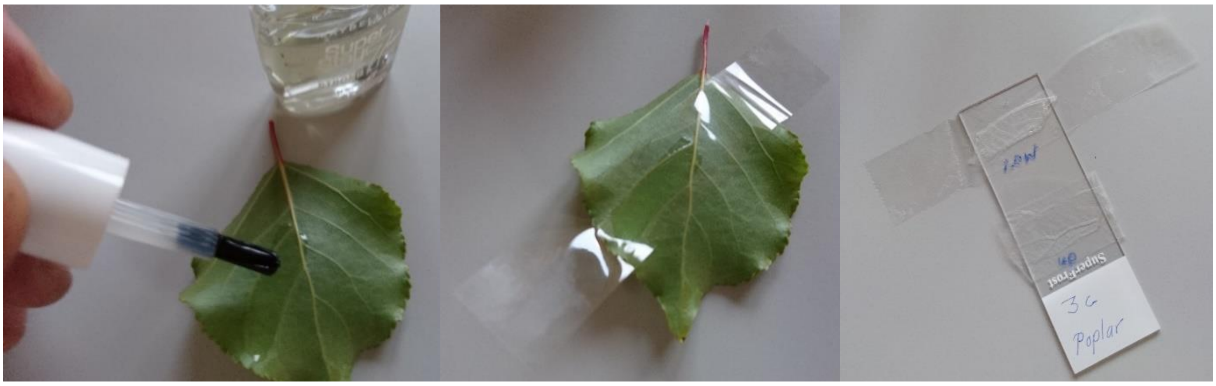

Figure 2:(left side): Application of nail varnish on the abaxial side of a poplar leaf. (middle part) As soon as the nail varnish was dry, an adhesive tape was pressed on the nail varnish and carefully removed. (right side) Fixed on one microscope glass, imprints of the lower and upper part of a poplar tree were ready for microscopical evaluation.

Using a light microscope Olympus BX 51, stomata on the adhesive tapes could be made visible by utilizing enlargement factor 20x and 40x. Since imprints of the stomata do not also have extension in length and width, but also in height, larger imprints of stomata in the observed area were clear in shape, whereas smaller stomata were blurred or could not even be depicted as stomata. After taking several pictures of the same section with different sharpness adjustments, hard copy prints of the several sections were printed out on A4 sheets separately. Using a computer program for counting the stomata failed due to the similarity of the between the stomata lying cells to the stomata, which together with the blurring stomata inhibited an exact evaluation. After dividing the A4 sheets into smaller sections (Figure 3), exact amounts of stomata were evaluated. There were no stomata found on the upper side of beech and birch. However, there were found stomata on both sides of poplar leaves (Figure 4). Poplar is not the only plant species that have stomata on the upper part of the leaves. Under strong light irradiation, tomatoes can also built-up stomata on both, the upper and lower side of a leaf. Hence, the stomatal density in the upper part (30 stomata/mm^2) of the leaf remains considerably lower than in the lower part of the tomato leaf (100 stomata/mm^2) (Schopfer and Brennicke, 2010). In case the stomata were only partly visible due to the edge of the paper it had been counted too. In case of sharpness issues with different stomata sizes or tape adhesive tape bend, all A4 sheets of the one section were printed out and because all stomata at least in one of the printed A4 were sharp, the total stomata of the section with blurring stomata could be evaluated too. Using a calibrating plate, the stomatal lengths of beech, birch, and poplar were determined.

Figure 3: Divided into several sectors, partial amounts of stomata from the abaxial side of a poplar leaf were evaluated and subsequently summed.

Figure 4: (Left side) Divided into several sectors, the lower part of a poplar leaf with stomata, (right side) Same poplar leaf exhibiting stomata on the upper leaf side.

2.3 Licor measurements

Evaluating the physiological leaf characteristics of the 3 tree species next to the greenhouse, a LI-COR 6800 device depicted all relevant data, like transpiration rate, assimilation rate, and stomatal conductance. A leaf still attached to the tree was clamped into the device and three measurements were taken and subsequently recorded into the LI-COR 6800 device (Figure 5). From every tree, a leaf was chosen. Although all measurements took place on a sunny and warm day, the device offset all environmental influences by cooling or heating the area where the leaf was clamped. Due to that, all measurements were taken under the same conditions previously set. Subsequently, combining the stored data with the evaluated stomatal amounts of beech, birch, and poplar respectively, several indexes could be calculated.

Figure 5: Clamped by the LICOR device, a birch leaf is under investigation to evaluate several physiological leaf properties (stomatal conductance, assimilation rate, transpiration rate)

2.4 Sample preparation for ICP-MS analysis

2.4.1 Harvest of trees

Leaves were harvested 10 days after the Ag foliar treatment. Before the leaves were dried and subsequently evaluated in the ICP-MS, all leaves were washed with ultra-pure water. Samples were oven-dried at 105°C for 24 h and the total dry biomass weight (DW, mg) of leaves was measured.

2.4.2 Sample preparation for ICP-MS analysis

Digestion was performed with a microwave digester (TurboWAVE, Terminal 640, MLS GmbH) in the laboratory of the Department of Environmental Systems Science, ETH (Zurich, Switzerland), led by Prof. Dr. Denise Mitrano. The digester operated at a pressure of 60 bar and a temperature of 220 °C. Full crumbled leaves were digested in Teflon tubes and caps. A digestion solution (1 mL 65% HNO_3 – Sigma Aldrich, Switzerland, and 1 mL 30% H_2O_2 – VWR Chemicals, VWR International) was added to the leaf samples. After the acid microwave digestion, the samples were transferred into Falcon tubes (Figure 6). Each digestion tube was rinsed with ultrapure water and the sample in the Falcon tube was filled up to 10 mL (final dilution prior to measurement was 1:50). Samples were centrifuged (Allegra X-30R Centrifuge, Beckman Coulter, IN, USA) for 5 min to eventually allow complete deposition of particles in suspension.

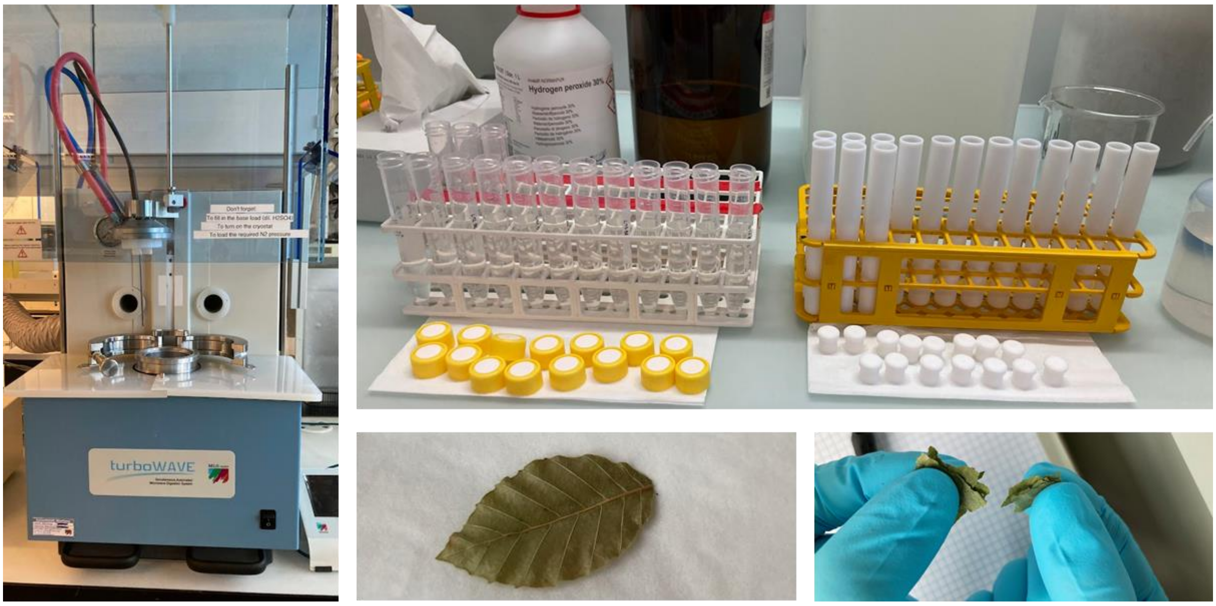

Figure 6: Crumbled leaves were digested in Teflon tubes using a microwave digester (left photo) and then were transferred into Falcon tubes.

Samples were measured by an inductively coupled plasma-mass spectrometer (Figure 7, Agilent 7900 ICP-MS, Agilent Technologies, Inc., USA) along with blanks and reference materials, against an external calibration series of 0.1, 0.5, 1, 2.5, 5, 12.5, 25 ug L^{-1} of a multielement standard solution 5 for ICP containing silver (TraceCERT, Sigma Aldrich, Switzerland), prepared in 1% HNO_3 and 1% Internal Standard on the same day as samples were digested. All samples and controls were adjusted during the measurement against Rhodium and Yttrium as internal standards. As suitable reference materials for measuring Au-NPs in plant tissue are not available yet, leaves and wood powder of *F. sylvatica* were spiked with Ag-NP suspensions to reach a target concentration of 10 ppm Ag. This material was then used for quality control.

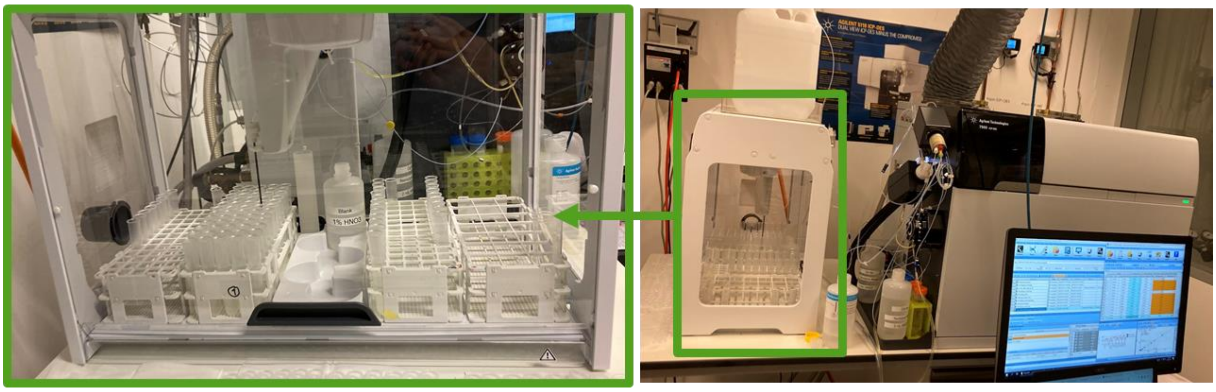

Figure 7: Inductively coupled plasma-mass spectrometer.

2.5 Statistical Analysis

ANOVA one-way was performed to determine differences among species regarding the gas exchange parameters, the stomatal length, stomatal density, and Ag concentration.

3. Results

Transpiration of 0.0020 mol $m^{-2}s^{-1}$ from poplar is almost double as high as the value of birch which rated 0.0011 mol $m^{-2}s^{-1}$. Beech with 0.0084 mol $m^{-2}s^{-1}$ had the lowest value. However; poplar had significantly higher values than both, birch and beech (Figure 8). Stomatal conductance of Poplar was 0.098 mol m^{-2} s^{-1} the highest of the 3 species. Birch with 0.067 mol m^{-2} s^{-1} ranges in the middle with approx. 1/3 less of the poplar value, whereas beech, reveals with 0.049 mol m^{-2} s^{-1} the lowest value. There is a significant difference between beech and poplar in stomatal conductance (Figure 9). Birch (n=88) and poplar (n=108) ranked approximately at the same low level of stomatal density. Outnumbering with the factor 3 for birch and more than 2 for poplar respectively, beech revealed the significantly highest stomatal density (Figure 10). Ranking the highest, poplar had stomata that were longer than 37 µm. In the range of poplar but slightly smaller, birch had stomata over 32 µm. Meanwhile, with less than 20 µm, beech had the significantly lowest stomatal length of all of the tree species (Figure 11). With Ag less than 7 mg/kg, poplar revealed the lowest value. Showing values above 10 mg/kg Ag, beech and birch had significantly higher values than poplar (Figure 12). Poplar showed with 6.74 mg/kg of Ag the lowest value, meanwhile, beech and birch had 15.8 mg/kg and 16.1 mg/kg of Ag almost the same high values, respectively. Overall, beech and poplar showed significantly higher values than poplar.

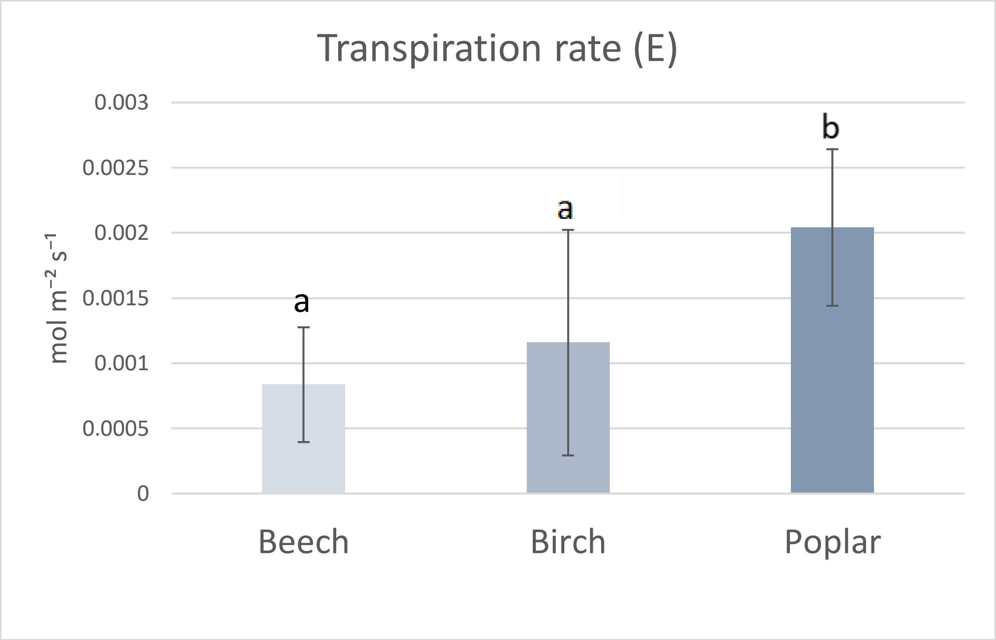

Figure 8: Transpiration rate in beech, poplar, and birch. Data are the means ± standard deviation. Lowercase letters represent significant differences among tree species at P<0.05.

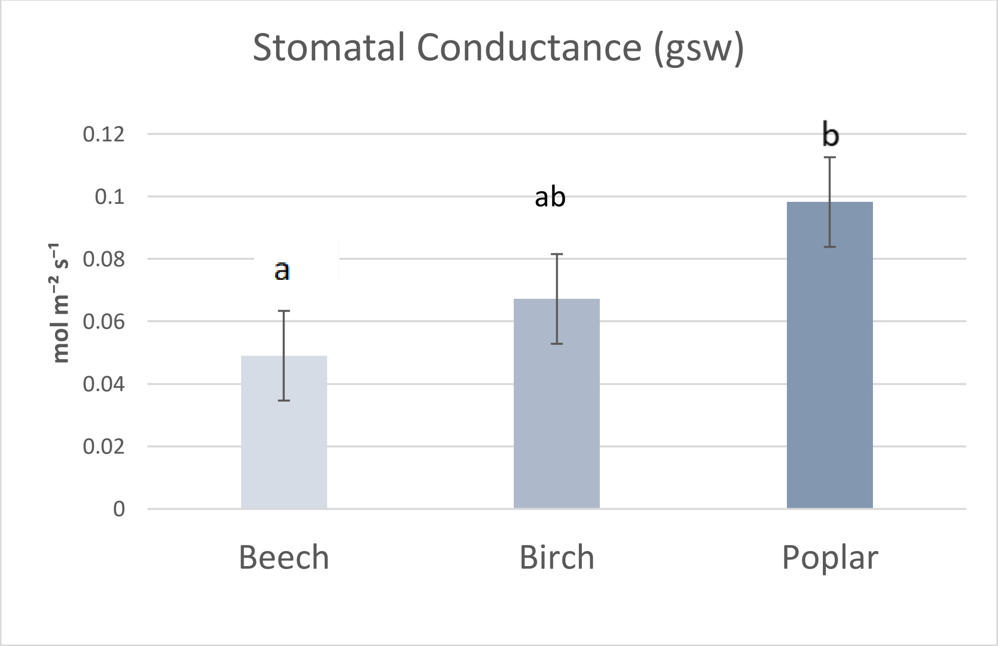

Figure 9: Stomatal conductance of beech, birch, and poplar. Data are the means ± standard deviation. Lowercase letters represent significant differences among tree species at P<0.05.

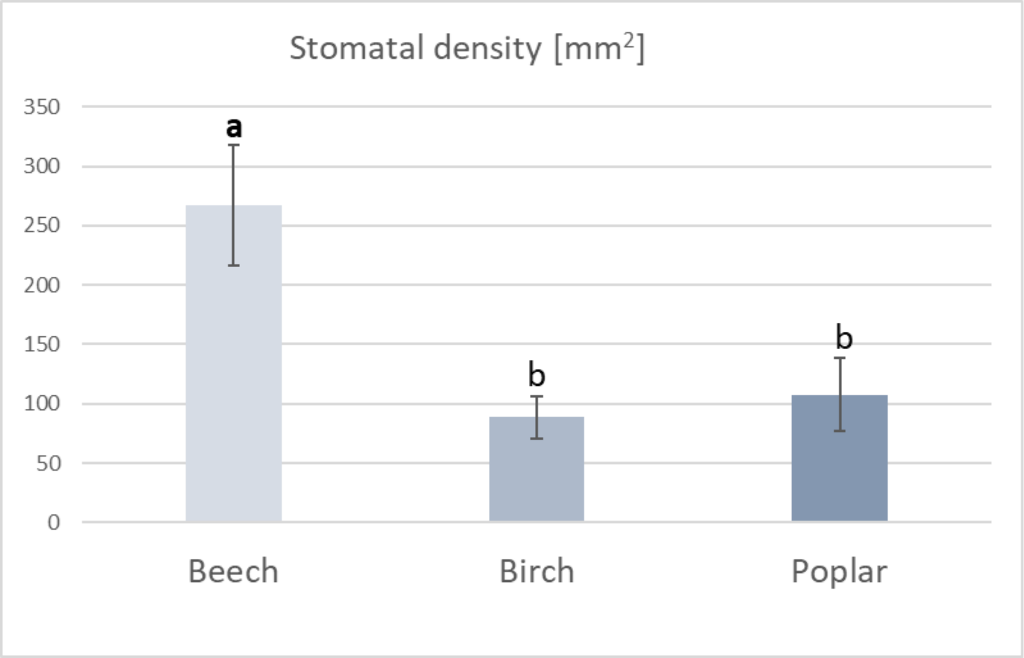

Figure 10: Stomatal density of beech, birch, and poplar. Data are the means ± standard deviation. Lowercase letters represent significant differences among tree species at P<0.05.

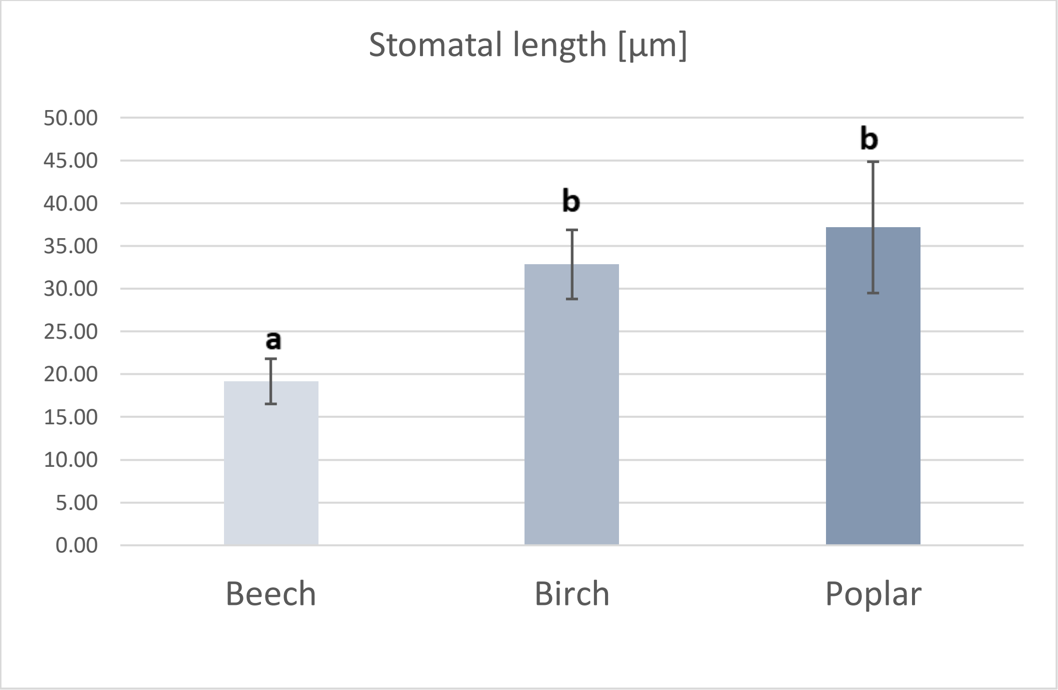

Figure 11: Average stomatal length of beech, birch, and poplar. Data are the means ± standard deviation. Lowercase letters represent significant differences among tree species at P<0.05.

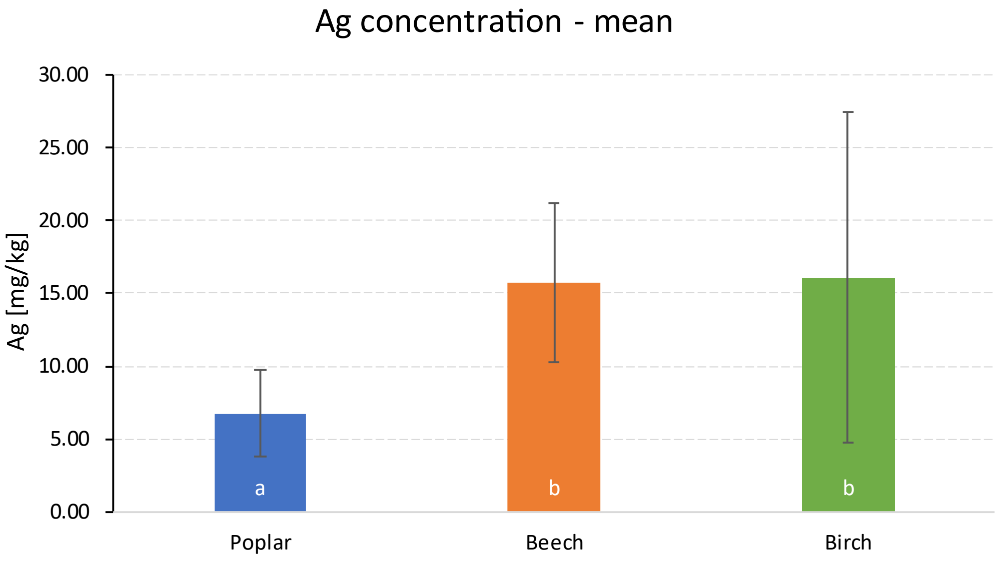

Figure 12: Silver concentration in leaves of poplar, beech, and birch. Data are the means ± standard deviation (n=25 poplar; n=50 beech and birch). Lowercase letters represent significant differences among tree species at P<0.05.

4. Discussion

Assuming silver nanoparticles enter the leaf by passing the stomata (Husen and Iqbal, 2019), poplar should have the most silver content assimilated due to its significantly highest transpiration rate (Figure 8). Additionally, the stomatal conductance of poplar is higher of beech and birch. In comparison with beech, poplar has even significantly higher stomatal conductance. Poplar having a significantly higher stomatal length, poplar should have an advantage against beech in incorporating silver nanoparticles. However, poplar shows the patchier stomatal distribution meanwhile the one of beech is more evenly distributed. Maybe the even stomatal distribution of beech outweighs the higher stomatal conductance of the poplar or poplar incorporates more silver nanoparticles than both, beech and birch but due to its higher metabolism (Al Afas et al., 2006) will have transported it to the branch or stem already.

Since plant diseases often come along with humidity on plant tissue, trichomes play a major role in repelling water from the leaf surface. Under wet conditions, more trichomes were built up. Since trichomes are hydrophobic, water is urged to build droplets that fall off the leaf (Lihavainen et al., 2017). Birch having trichomes solely, AgNP application in birch may be enhanced due to leaves being headfirst during the application. As a consequence, during the drying process water with its soluble AgNPs will be pushed away from the trichomes towards the surrounding lying stomata (Figure 13) which may uptake these additional AgNPs beside the particles which were put on the stomata directly. Although birch had significantly lower stomatal density than the beech has, it may be assumed that the significantly bigger stomata of the birch and the assumed pushing effect of the trichomes of the birch will end in almost the same Ag concentration in both, beech and birch. Assuming the hydrophobic effect of the birch trichomes, AgNPs uptake under real environmental conditions in a rain event may be even reduced. Birch leaf not turned headfirst, due to both, the hydrophobic effect of the trichomes and gravitation enhanced drop falling will occur. As a consequence, previously attached nanoparticles will be rather washed away and fall off the leaf instead of pressed through the stomata. After the rain event, most or all of the AgNPs would be washed away from the leaf. During the subsequent drying process, there are no AgNPs to be pushed by the trichomes to the stomata. Done the AgNPs application in 2020, the subsequent evaluation of physiological properties of the leaves was taken in the summer of 2021. However, the trees were the same but the leaves were not. Maybe there was some adaption of the leaves or other factors which could have altered the new leaves and their properties in the year 2021 in some way. Known to build up different leaves when cut down, leaves of all poplar sample plants were attached to a new driving out. There had been a cutting down of all poplar sampling trees before summer 2021. In general, leaves from a new driving out are growing faster (Al Afas et al., 2006) as they can rely on a settled root system and this allows quick and strong regrow. If there is also a change in the physiological properties it also has to be considered.

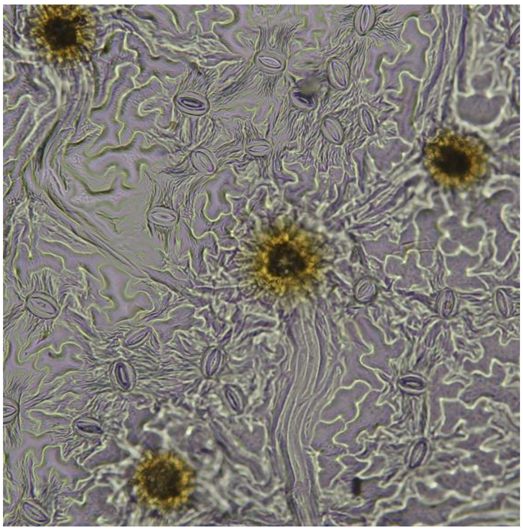

Figure 13: Trichomes (yellow) surrounded by stomata on the abaxial side of a birch leaf (microscopic view 20x).

5. Conclusion

This study shows that trees are able to take up silver nanoparticles through their leaves. The uptake is species-specific, with beech and birch taking the most and poplar the least. It seems that the stomatal density and stomatal size play an important role in the uptake of nanoparticles. The presence of trichomes in birch could be responsible for the adhesion on the surface or uptake rate of silver nanoparticles.

Although there are still many open questions that need additional work for a complete understanding, this study shows that the choice of tree species is important and contributes to a better understanding of the interaction of NPs with different tree species.

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List of figures

Declaration of Authorship

I hereby declare that I wrote this thesis on my own, that I did not use this thesis or parts of it for other qualifying thesis and that I declared parts or Ideas I took from other sources.

Kurt Weber Zürich, 30.06.2022